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Cyclin Y Is a Novel Conserved Cyclin Essential for Development in Drosophila

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ABSTRACT

The Drosophila gene *CG14939* encodes a member of a highly conserved family of cyclins, the Y-type cyclins, which have not been functionally characterized in any organism. Here we report the generation and phenotypic characterization of a null mutant of *CG14939*, which we rename *Cyclin Y* (*CycY*). We show that the null mutant, $CycY^{E8}$, is homozygous lethal with most mutant animals arresting during pupal development. The mutant exhibits delayed larval growth and major developmental defects during metamorphosis, including impaired gas bubble translocation, head eversion, leg elongation, and adult tissue growth. Heat-shock-induced expression of *CycY* at different times during development resulted in variable levels of rescue, the timing of which suggests a key function for zygotic *CycY* during the transition from third instar larvae to prepupae. *CycY* also plays an essential role during embryogenesis since zygotic null embryos from null mothers fail to hatch into first instar larvae. We provide evidence that the *CycY* protein (CycY) interacts with Eip63E, a cyclin-dependent kinase (Cdk) for which no cyclin partner had previously been identified. Like *CycY*, the *Eip63E* gene has essential functions during embryogenesis, larval development, and metamorphosis. Our data suggest that CycY/Eip63E form a cyclin/Cdk complex that is essential for several developmental processes.

CYCLINS are a family of highly conserved proteins that activate cyclin-dependent kinases (Cdks) to regulate the cell cycle, transcription, and other cellular processes. The founding members of the family, cyclins A and B, were first discovered as proteins that oscillated throughout the cell cycle, peaking in late G2 and M phases (Evans *et al.* 1983). These proteins were later shown to be required to activate the serine/threonine protein kinase, Cdc2 (also known as Cdk1), which is required for entry into M phase in most eukaryotes (Morgan 1997). Other cyclins with sequence similarity to cyclins A and B were subsequently identified and shown to be required at other points during the cell cycle (Murray 2004). The best characterized of these in metazoans include D-type cyclins, which partner with Cdk4 to control G1 phase events, and E-type cyclins, which partner with Cdk2 to control the transition from G1 to S phase. Several other members of the cyclin family do not show cell-cycle-dependent degradation or synthesis and some have been shown to play roles in cellular processes that are not directly related to cell cycle regulation. One group of cyclins, for example, regulates transcription by activating Cdks that can phosphorylate the carboxy-terminal tail of the large subunit of RNA polymerase II (Loyer *et al.* 2005). Several additional members of the cyclin family remain uncharacterized. Here we describe the initial characterization of one such novel cyclin encoded by the Drosophila gene *CG14939*, which we renamed *Cyclin Y* (*CycY*). Although this cyclin is highly conserved through evolution, no member of its family has been functionally characterized in any organism.

The defining feature of the cyclin family is a homologous region called the cyclin domain (Hadwiger *et al.* 1989; Hunt 1991), which includes the region responsible for interaction with a Cdk. Detailed studies on specific examples of Cdk/cyclin complexes have shown that the cyclin domain is essential and sufficient for interaction with and activation of the Cdk partner (Jeffrey *et al.* 1995; Morgan 1996). Thus, while specific Cdk partners have not been identified for every cyclin, all are thought to play the role of activating one or more Cdks. In addition to activating kinase activity, the cyclin may influence the substrate specificity or determine the subcellular localization of the active complex (Miller and Cross 2001).

Here we present data suggesting that one Cdk partner for Drosophila Cyclin Y is Ecdysone-induced protein 63E (Eip63E). The *Eip63E* gene encodes five highly related and apparently functionally redundant protein isoforms, all of which have homology to cyclin-dependent kinases (Sauer *et al.* 1996; Stowers *et al.* 2000). The

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proteins are most similar to the poorly characterized mammalian Cdks called PFTAIRE, so named because of the amino acid sequence in the conserved helix that binds to cyclins. Although a cyclin partner for Eip63E has not been identified, rescue experiments using mutant variants of the protein have suggested that its activity depends on cyclin binding (Stowers *et al.* 2000). In those experiments, mutation of a conserved glycine adjacent to the PFTAIRE (G243), which in other Cdks is required for cyclin binding, abolished the ability of an *Eip63E* transgene to rescue null Drosophila embryos to adulthood. Similarly, mutation of a conserved isoleucine (I249), which is also required for cyclin binding in other Cdks, diminished the ability of Eip63E to promote development. A directed yeast two-hybrid screen by Rascle *et al.* (2003) identified two potential regulators of Eip63E, Pif-1 and Pif-2, but neither of these proteins has any similarity to cyclins. In a high throughput yeast two-hybrid screen, however, one Eip63E-interacting protein that was identified was the *CG14939* protein (Stanyon *et al.* 2004).

The name of Eip63E derives from the fact that one of the three transcription units of the *Eip63E* gene is induced in response to pulses of the steroid hormone ecdysone, which triggers crucial developmental transitions including metamorphosis. Phenotypic characterization of *Eip63E* loss-of-function mutants has shown that it has essential roles in several developmental processes (Stowers *et al.* 2000). The majority of zygotic null mutants die during larval development, while only a small percentage survive to pupation. The mutants that survive take 2–3 days longer to pupariate than their heterozygous siblings and are generally smaller than wild-type pupae. These phenotypes point to a role for Eip63E in larval development and metamorphosis and further suggest that this Cdk may be involved in growth control. Mutant eye clones, however, show no morphological or cell cycle defects, leading Stowers *et al.* (2000) to conclude that Eip63E does not regulate the cell cycle. *Eip63E* proteins have also been shown to be important for embryogenesis since zygotic null embryos from null mothers fail to hatch into first instar larvae. Interestingly, this maternal effect can be complemented by zygotic expression (Stowers *et al.* 2000). Thus, while it is clear that this ecdysone-inducible gene is important for metamorphosis and other developmental events, the molecular partners for Eip63E and the pathways in which it may function have yet to be discovered.

Here we report the generation of a null mutant allele of *CycY* and show that its phenotype is similar to that of *Eip63E* mutants. We show that *CycY* plays major essential roles during metamorphosis, especially during pupariation. We also show that maternally expressed *CycY* is essential for embryogenesis and that this requirement could be partially rescued by zygotic expression. Finally, we confirm that CycY and Eip63E specifically interact in Drosophila cells and show that the interaction depends on a conserved phosphorylation target on CycY, Ser389.

MATERIALS AND METHODS

Fly stocks: The stocks carrying *crol*^{04418} (D'Avino and Thummel 1998), *Eip63E*^{81} (Stowers *et al.* 2000), *Df(3L)GN50*, also known as *Eip63E*^{GN50} (Stowers *et al.* 2000), P{*Δ2-3*} (Robertson *et al.* 1988), *ovo*^{D1} *neoFRT40A* (Chou and Perrimon 1996), *Tubulin-Gal4*, and *Df(2L)Exel6030* were obtained from the Bloomington Stock Center (stock nos. 11374, 4513, 3687, 3664, 2121, 5138, and 7513, respectively). The XP insertion line d03228 (Thibault *et al.* 2004) was obtained from the Exelixis mutant collection at Harvard Medical School.

Plasmid cloning for *P*-element transformation: pAS1 (A. Soans and R. L. Finley, unpublished results) is a modified pUAST (Brand and Perrimon 1993) vector encoding a myc-tag followed by 5′ and 3′ recombination tags (RTs) to facilitate cloning of open reading frames containing the same RTs from yeast two-hybrid vectors (Giot *et al.* 2003; Stanyon *et al.* 2004). A map of pAS1 is available at http://www.proteome.wayne.edu. pAS1–CycY was constructed by subcloning a fragment of the *CycY* cDNA beginning with the ATG and ending with the stop codon. The fragment was generated by PCR from the yeast two-hybrid clone using oligonucleotides (forward: 5′ TTG ACTGTATCGCCGGAATTC; reverse: 5′ CCGGAATTAGCTT GGCTGCAG), which provided the 5′ and 3′ RTs at either end, respectively. The fragment was subcloned by gap repair in *Escherichia coli* (Parrish *et al.* 2004). The P{*CycY*} genomic clone was constructed by sequentially subcloning a 3.6-kb *Bam*HI/*Not*I fragment and then a 4.0-kb *Avr*II/*Eco*RI fragment, each from BACR05B13 (BACPAC resources center), into pCaSpeR2 (Thummel *et al.* 1988). The whole insert is 7.3 kb, which includes the entire *CycY* gene and sequences 4032 bp upstream of the *CycY* start codon, and 1970 bp downstream of the stop codon, and includes none of the coding regions of *crol* or *Pde1c*. A *P*-element carrying the *CycY* cDNA expressed from a heat-shock promoter, pCaSpeR-hs-CycY, was constructed by subcloning the 1.5-kb *Hpa*I/*Stu*I fragment encoding myc–CycY from pAS1–CycY into the *Hpa*I/*Stu*I sites of pCaSpeR-hs (Thummel *et al.* 1988). *P*-element-mediated transformation was performed as previously described (Rubin and Spradling 1982).

Generation and molecular characterization of a *CycY* mutant allele: We used *P*-element imprecise excision to generate *CycY* mutant alleles. The starting *P*-element in d03228 is inserted 1958 bp downstream of the *CycY* stop codon and 5723 bp upstream of the *crol* start codon (Figure 1A). d03228 virgin females were mated with *w**; *CyO/Sp*; *Δ2-3*, *Sb/TM6* males, which provided P-transposase. F_1 females (w*; d03228/*CyO*; *Δ2-3*, *Sb*/+) were then mated to *w**; *CyO/Sp*; TM3, *Ser/Sb* males, and F_2 progeny were screened for *P*-element excision by the reversion of eye color to white (Carney *et al.* 2004). One hundred white-eyed flies were collected and further balanced individually to make stocks. For each stock, genomic DNA was extracted from single flies and analyzed by PCR for the presence of *CycY* gene regions close to the d03228 insertion site. The excision that removes all *CycY* coding sequences was named E8 and was shown here to be a null allele of *CycY* (*CycY*^{E8}). To make sure there is not a second site mutation on the same chromosome as *CycY*^{E8}, the *CycY*^{E8} chromosome was cleaned up by homologous recombination with d03228 for seven generations. To determine the precise end point of the E8 deletion, genomic DNA was extracted from heterozygous *CycY*^{E8} adults and the region was amplified by PCR using primers 5′-GGGCCAAGCACAAATA CAAACG-3′ and 5′-TGGTGAACGGCGAACAGAGC-3′. The

PCR product, which is about 1 kb, was gel purified and sequenced from both ends. The deletion end points were determined by sequence alignment with wild-type genomic sequence. $CycY^{E8}$ is missing 6119 bp of DNA from 734 bp downstream of the *Pde1c* stop codon to 1411 bp downstream of the *CycY* stop codon, and a second small region of 988 bp from 1955 bp downstream of the *CycY* stop codon to the first noncoding exon of *crol* transcripts RA-RC or the first intron of *crol* transcript RD. This removes the entire *CycY* transcript along with noncoding portions of the last exon of *Pde1c* and the first noncoding exon of *crol* (Figure 1A).

Lethal phase analysis: Eggs were collected from *w*; $CycY^{E8}$/CyO, Act5C-GFP* flies for 12 hr on apple juice plates with fresh yeast paste. After another 30 hr, the numbers of unhatched embryos and hatched first instar larvae were counted. Since homozygous *CyO* balancer is lethal during embryogenesis, an ~75% hatching rate suggests no embryonic lethality. One hundred eighty *w*; $CycY^{E8}$/CyO, Act5C-GFP* and 180 *w*; $CycY^{E8}$* (lacking *GFP*) first instar larvae were picked under a fluorescence dissection microscope and transferred into regular vials. The numbers of wandering third instar larvae, pupae, and adults were counted once a day for 15 days to score for a delay in puparium formation, progression through metamorphosis, and adult eclosion. To estimate the delay of puparium formation more accurately, we followed 180 first instar larvae for each genotype and calculated the average time to form pupa by counting the number of pupa newly formed after each 24-hr period and averaging over all individuals and days. Similarly, we analyzed the following animals: (1) *w*; $CycY^{E8}$/CyO, Act5C-GFP; P{CycY}*, (2) *w*; $CycY^{E8}$; P{CycY}*, (3) *w*; $CycY^{E8}$/CyO, Act5C-GFP* and *Df(2L)Exel6030/CyO, Act5C-GFP*, (4) *w*; $CycY^{E8}$/Df(2L)Exel6030*, (5) *w*; $CycY^{E8}$/CyO, Act5C-GFP; P{CycY}* and *w*; Df(2L)Exel6030/CyO, Act5C-GFP; P{CycY}*, (6) *w*; $CycY^{E8}$/Df(2L)Exel6030; P{CycY}*, (7) *w*; $Eip63E^{S1}$/TM3, Ser Act5C-GFP* and *w*; $Eip63E^{GN50}$/TM3, Ser Act5C-GFP*, and (8) *w*; $Eip63E^{S1}$/$Eip63E^{GN50}$*. All flies were incubated at 25° except where noted.

Phenotypic characterization: Two hundred *w*; $CycY^{E8}$/CyO, Act5C-GFP* and 200 *w*; $CycY^{E8}$* first instar larvae were collected (see above), transferred into individual fresh vials, and allowed to develop for 9 days. For each genotype, pupae at all developmental stages were collected, weighed, and imaged. The relative pupal length was measured on the basis of the image size. The average pupa weight and length were then calculated. To document the pupal phenotype, pupae at all developmental stages were carefully removed from the wall. For pharate adults, the pupal case was gently dissected. Images were taken with the Leica MZ 16FA stereomicroscope and Leica DFC 490 camera (kindly provided by Markus Friedrich). Similarly, we analyzed the following animals: (1) *w*; $CycY^{E8}$/CyO, Act5C-GFP; P{CycY}*, (2) *w*; $CycY^{E8}$; P{CycY}*, (3) *w*; $CycY^{E8}$/CyO, Act5C-GFP* and *Df(2L)Exel6030/CyO, Act5C-GFP*, (4) *w*; $CycY^{E8}$/Df(2L)Exel6030*, (5) *w*; $CycY^{E8}$/CyO, Act5C-GFP; P{CycY}* and *w*; Df(2L)Exel6030/CyO, Act5C-GFP; P{CycY}*, (6) *w*; $CycY^{E8}$/Df(2L)Exel6030; P{CycY}*, (7) *w*; $Eip63E^{S1}$/TM3, Ser Act5C-GFP* and *w*; $Eip63E^{GN50}$/TM3, Ser Act5C-GFP*, and (8) *w*; $Eip63E^{S1}$/$Eip63E^{GN50}$*.

Heat-shock induction and rescue efficiency: Eggs from a self cross of *$CycY^{E8}$/CyO; hs-CycY/TM3, Ser* flies were collected in glass vials with standard cornmeal Drosophila media for 24 hr and then heat shocked on each day as indicated in Figure 4. For each heat-shock induction, glass vials were incubated in a 37° water bath for 1 hr. Vials were kept at 25° otherwise. The numbers of flies with or without curly wings were counted separately. If *$CycY^{E8}$/CyO* and *$CycY^{E8}$* flies have equal viability (full rescue), the number of *$CycY^{E8}$* flies should be half of that of *$CycY^{E8}$/CyO* flies. The rescue efficiency was then determined by the number of *$CycY^{E8}$* adult flies divided by half of the number of *$CycY^{E8}$/CyO* adult flies. The genotype of representative flies was confirmed by single-fly PCR.

Generation of mosaic germline clones with homozygous $CycY^{E8}$: *$CycY^{E8}$* was recombined with FRT40A as previously described (Xu and Rubin 1993). Germline clones with homozygous *$CycY^{E8}$* were generated on the basis of the well-established approach of Chou and Perrimon (1996) with minor modification. *w*; $CycY^{E8}$ FRT40A/CyO* females were crossed with *hs-FLP/Y; ovo^{D1} FRT40A/CyO* males for 3 days. Eggs were collected for 3 days and aged for 2 more days. Larvae, which were at either second or third instar stages, were heat shocked at 37° in a water bath for 2 hr. Females with straight wings (*hs-FLP/w*; $CycY^{E8}$ FRT40A/ovo^{D1} FRT40A*) from the above cross were then mated to *$CycY^{E8}$/CyO, Act5C-GFP* males to test for a maternal requirement for *CycY*. *GFP* positive (*$CycY^{E8}$ FRT40A/CyO, Act5C-GFP)* and *GFP* negative (*$CycY^{E8}$ FRT40A/$CycY^{E8}$*) first instar larvae were picked and development was followed as described above.

Gene expression: Gene expression was assayed by reverse-transcription PCR (Figure 1B) or quantitative real-time PCR (qPCR) (supporting information, Figure S5). Flies at the indicated developmental stages were collected and total RNA was extracted using the RiboPure kit (Ambion). The RNA samples were then treated with DNase from a DNA-free kit (Ambion) to remove contaminating genomic DNA. cDNAs were synthesized with a Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer's protocol. qPCR reactions were performed using Brilliant SYBR Green QPCR Master Mix (Stratagene) in a 96-well plate. qPCR reactions were carried out in triplicate for each RNA sample. The primers used in this work are listed in Table S5. *rp49* was used as the internal control and the RNA level of *CycY* was normalized to *rp49* levels.

Co-affinity purification (co-AP) assays: Co-AP assays were conducted by expressing pairs of N-terminally myc-tagged and NTAP-tagged proteins in Drosophila S2R+ cells, purification of the NTAP-tagged protein, and detection of associated myc-tagged proteins by immunoblotting. Myc-tagged proteins were expressed from pAS1. NTAP-tagged proteins were expressed from pDL4, a derivative of pUAST–NTAP (Veraksa *et al.* 2005) containing the NTAP tag followed by the same 5′ and 3′ RTs found in pAS1. Coding regions from the ATG to the stop codon of various Cdks or cyclins were amplified from yeast two-hybrid clones (Stanyon *et al.* 2004) with primers that added the 5′ and 3′ RTs and the products were subcloned into pAS1 or pDL4 by gap repair in *E. coli*. S2R+ cells were cotransfected with pairs of myc-tagged and NTAP-tagged plasmids along with pMT–Gal4 (Klueg *et al.* 2002), a Gal4 expression vector induced by $CuSO_4$. Cells were grown in Schneider's Drosophila media (Invitrogen) supplemented with 10% FBS and 0.1 mg/ml gentamicin and were induced with 1 mm $CuSO_4$. Cells were harvested 2 days after induction and lysed with 50 mm Tris-HCl pH 7.4, 180 mm NaCl, 5 mm EDTA, 1% NP-40, 10% glycerol, 50 mm NaF, 1 mm Na_3VO_4, 1× protease inhibitor cocktail (Roche). Clarified cell lysate was then incubated with rabbit IgG-conjugated agarose beads (Sigma) at 4° for 2 hr with shaking. After five washes with lysis buffer, co-purified proteins were eluted with 1× LDS sample loading buffer (Invitrogen), resolved by SDS–PAGE and detected by Western blotting using mouse anti-myc primary antibody (Santa Cruz) and goat anti-mouse HRP conjugated secondary antibody (BioRad) or goat anti-protein A peroxidase-conjugated antibody (Rockland Immunochemicals).

RESULTS AND DISCUSSION

CycY is a conserved uncharacterized cyclin: Drosophila *CG14939* has a single predicted transcript that

encodes a protein with 406 residues (Figure 1A, Figure S1, B). Between amino acids 205 and 328 lies a cyclin domain, a conserved region that defines the cyclin family of proteins. The closest human homolog of *CG14939* is a poorly characterized gene called *Cyclin Y* (*CCNY*). Genes in a number of other species have also been named *Cyclin Y* on the basis of their sequence similarity to human *CCNY*. *CG14939* is more similar to the Y cyclins from other species than it is to any other *Drosophila melanogaster* gene (Figure S1 and Table S1), indicating that it belongs to this orthologous family of proteins. We therefore renamed *CG14939 Cyclin Y* (*CycY*). Outside of the cyclin domain the protein has virtually no sequence similarity to other cyclins. However, *CycY* has been highly conserved through evolution. Clear *CycY* orthologs are found in all metazoans with fully sequenced genomes, including bilaterians (*e.g.*, insects, nematodes, vertebrates), cnidarians (*e.g.*, the sea anenome, *Nematostella vectensis*), and the placozoan, *Trichoplax adhaerens*. Cyclin Y is also found in the choanoflagellate, *Monosiga brevicollis*, the closest known unicellular relative of metazoans, suggesting that the Y-type cyclins originated prior to the first multicellular species. Cyclin Y proteins from all of these species share substantial sequence similarity over most of their length, including regions outside of the cyclin domain (Figure S1, B). In contrast, plants, fungi, and other nonmetazoan species do not have proteins with extensive sequence similarity to CycY, though they do contain the CycY-specific cyclin domain; this cyclin domain is distinct from other cyclin domains and appears to be conserved throughout the eukaryotic kingdom (Figure S2). In metazoan species the level of CycY conservation is particularly high. For example, the Drosophila protein shares 52% identity with the human *CCNY* protein. This level of conservation is much higher than that observed for the cell cycle cyclins (*e.g.*, cyclins A, B, D, and E), which share between 20 and 41% identity between human and Drosophila (Finley *et al.* 1996). This suggests that CycY has an important and potentially conserved function. Surprisingly, the function of Cyclin Y has not been studied and *CycY* mutants have not been reported for any model organism.

Generation of a *CycY* mutant: To determine the function of Drosophila *CycY*, we set out to generate a loss-of-function mutant allele. We took advantage of the availability of a strain, d03228, bearing a *P*-element inserted 1958 bp downstream of the *CycY* stop codon and 5723 bp upstream of the start codon of the neighboring gene, *crol* (Figure 1A). This insertion itself has no visible effect on the function of any genes in this region since the homozygous d03228 adults are completely viable and normal. We used imprecise excision to generate a small deletion around the original *P*-element. The deletion, E8, completely removed the *CycY* coding region while leaving the coding regions of the neighboring genes intact (Figure 1A), as determined by

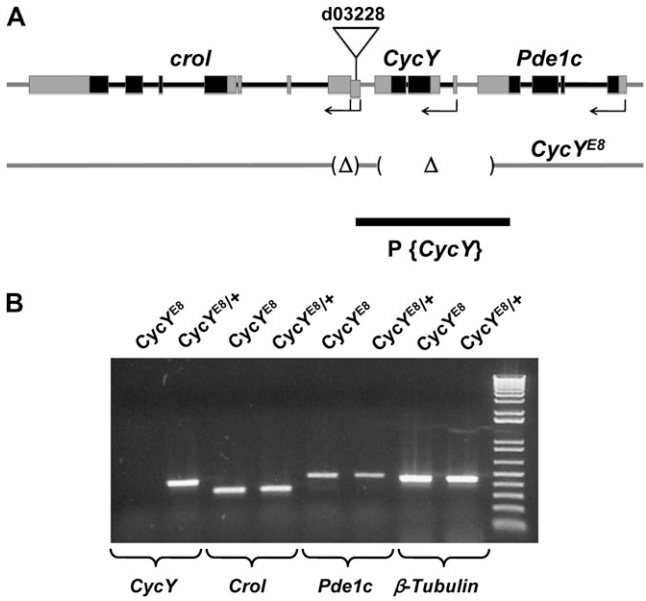

Figure 1.—(A) The genomic region of *CycY* and the flanking genes *crol* and *Pde1c*. Exons of the *CycY*, *crol*, and *Pde1c* transcripts are indicated by boxes. Solid boxes represent coding regions and shaded boxes represent untranslated regions. Direction of transcription and approximate start sites are shown with arrows; *Pde1c* has five predicted transcripts that all start from the same position, *CycY* has one predicted transcript, and *crol* has three predicted transcripts (RA, RB, and RC) that start from the same position and one (RD) that starts further upstream as shown. The *P*-element in strain d03228 is inserted just upstream of exon 1 of *crol* transcripts RA-RC, and within exon 1 (offset box) of *crol* transcript RD. The two deleted regions in the $CycY^{E8}$ allele, which was isolated by imprecise excision of the *P*-element in strain d03228, are indicated as Δ. The deletion removes the entire *CycY* gene, the first noncoding exon of *crol*, and a noncoding portion of the last exon of *Pde1c*. The genomic fragment used to create a transgene P{*CycY*} that complements $CycY^{E8}$ is also depicted (solid bar). (B) Reverse-transcription PCR detecting expression of *CycY*, *crol*, *Pde1c*, or β-tubulin in homozygous $CycY^{E8}$ or heterozygous $CycY^{E8}$ ($CycY^{E8}$/+) second instar larvae; the "+" chromosome is a *CyO* balancer with *Act5C–GFP*. A band of the expected size is detected for all genes in both genotypes, except for *CycY* in the homozygous $CycY^{E8}$ larvae.

PCR and sequencing (materials and methods). Expression of the neighboring genes, *crol* and *Pde1c*, was confirmed using RNA extracted from homozygous and heterozygous E8 second instar larvae (Figure 1B). In contrast, *CycY* transcription was undetectable in homozygous E8 larvae. Hereafter we refer to the E8 deletion as $CycY^{E8}$.

Two additional lines of evidence indicate that *CycY* is the only gene affected in strain $CycY^{E8}$. First, $CycY^{E8}$ fully complemented $crol^{04418}$, a lethal null allele of the neighboring gene (D'Avino and Thummel 1998); $crol^{04418}$ also complemented the mutant phenotype of $CycY^{E8}$ (see below). Thus, although $CycY^{E8}$ lacks the first noncoding exon of *crol*, a *crol* transcript is expressed and appears to be fully functional. Second, as described in detail below, all of the abnormalities that we observed in homozygous

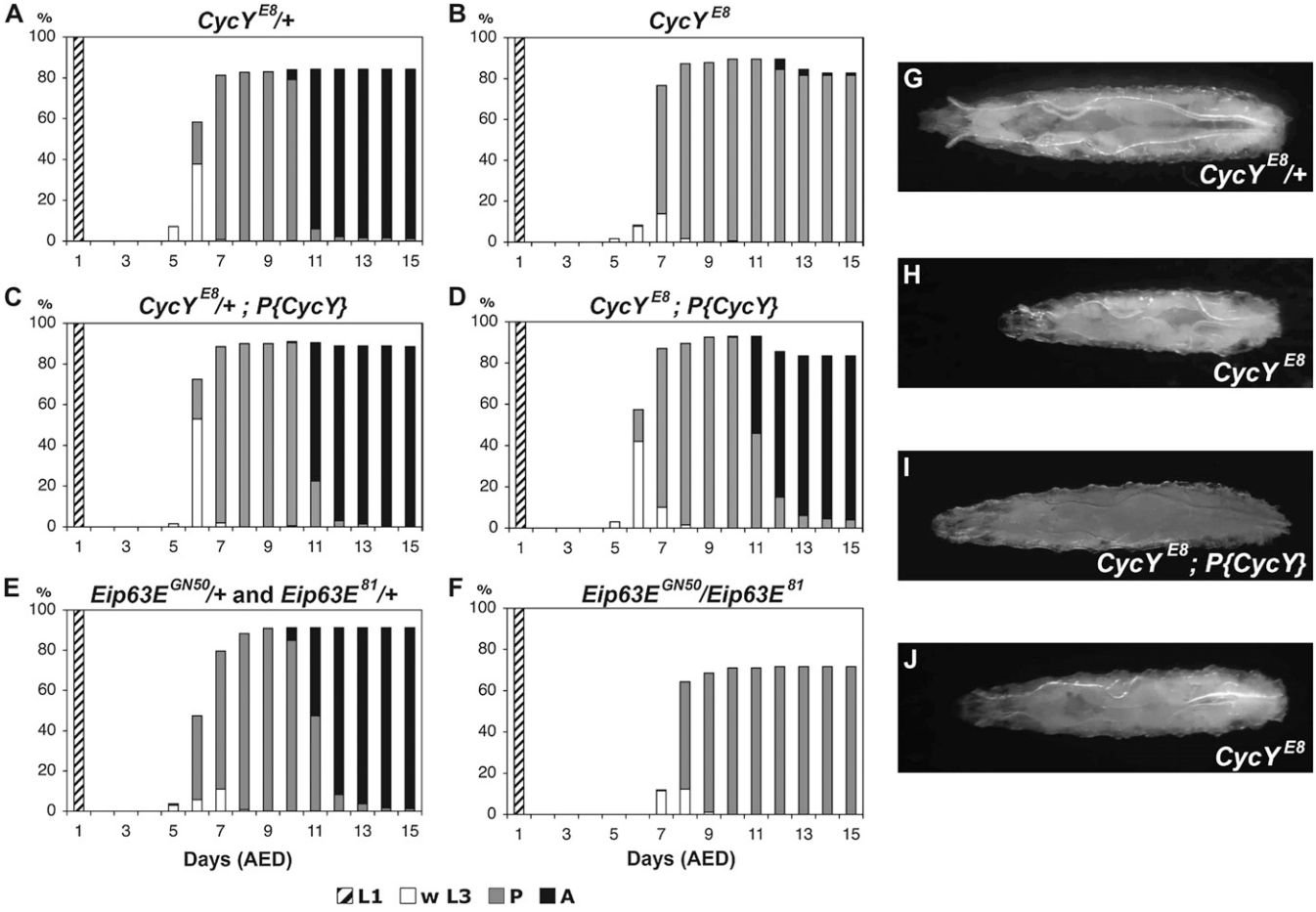

FIGURE 2.—Developmental timing of *CycY* and *Eip63E* mutants. (A–F) The development of 180 first instar larvae (L1) of each genotype was followed for 15 days. Genotypes include heterozygous $CycY^{E8}$ (A and C) or homozygous $CycY^{E8}$ (B and D). Larvae in C and D harbored a genomic *CycY* transgene on the third chromosome (*P{CycY}*). Larvae heterozygous for the *Eip63E* mutants, $Eip63E^{GN50}$ or $Eip63E^{81}$ (E), or transheterozygous $Eip63E^{GN50}/Eip63E^{81}$ (F) were also analyzed. The percentage of first instar larvae that developed into wandering third instar larvae (w L3), pupae (P), and adults (A) on each day after egg deposition (AED) is shown. (G–J) Typical third instar larvae of $CycY^{E8}/+$ (G), $CycY^{E8}$ (H), $CycY^{E8}$*; P{CycY}* (I) at the same time point and $CycY^{E8}$ after an additional day (J). In A, C, and G, the plus symbol (+) stands for an *Act5C–GFP*-marked *CyO* balancer chromosome; in E, + stands for an *Act5C–GFP*-marked *TM3, Ser* balancer chromosome.

$CycY^{E8}$ mutants can be rescued either by a *CycY* genomic transgene (Figure 1A) or by ubiquitous expression of a *CycY* cDNA using heat-shock induction (see below). Combined these results indicate that the $CycY^{E8}$ mutant strain is a null mutant for *CycY*.

***CycY* null mutants show delayed entry into pupariation and are pupal lethal:** Homozygous $CycY^{E8}$ mutants or $CycY^{E8}$ over a deficiency that removes *CycY* (*Df(2L)Exel6030*) produce no viable adults, indicating that *CycY* is an essential gene. To analyze the lethal phase, eggs from a self-cross of $CycY^{E8}/CyO$ flies were collected for 12 hr and aged for another 30 hr. Of 366 embryos examined, 89 (24.3%) remained unhatched while 277 (75.7%) hatched to first instar larvae. Since ~25% of the embryos from this cross should be homozygous *CyO*, which is lethal during embryogenesis, one-third of the embryos that hatched should be homozygous $CycY^{E8}$, indicating that zygotic expression of *CycY* is not essential for embryogenesis.

To evaluate whether *CycY* is required during larval and pupal development, we picked 180–200 first instar larvae of *CycY* null mutants (homozygous $CycY^{E8}$ or $CycY^{E8}/Df(2L)Exel6030$) or their siblings and followed their morphology and development for 15 days, after which no additional adults eclosed. *CycY* null mutants did not show obvious larval lethality since the majority (90 or 93%) of first instar larvae developed into pupae, which is a rate similar to their heterozygous siblings (84 or 94%, respectively) (Table S2). However, we did observe delayed growth during larval development. By the time third instar larvae in the heterozygous group started to wander, *CycY* null mutant larvae were still at the feeding stage and exhibited dramatically smaller body sizes (Figure 2, A, B, G, and H; Figure S3, A and B). The $CycY^{E8}$ homozygotes eventually grew to sizes that were 80–90% of the heterozygotes before pupariation (Figure 2, G and J). The delay in larval growth could be rescued with a genomic *CycY* transgene (Figure 2, C, D,

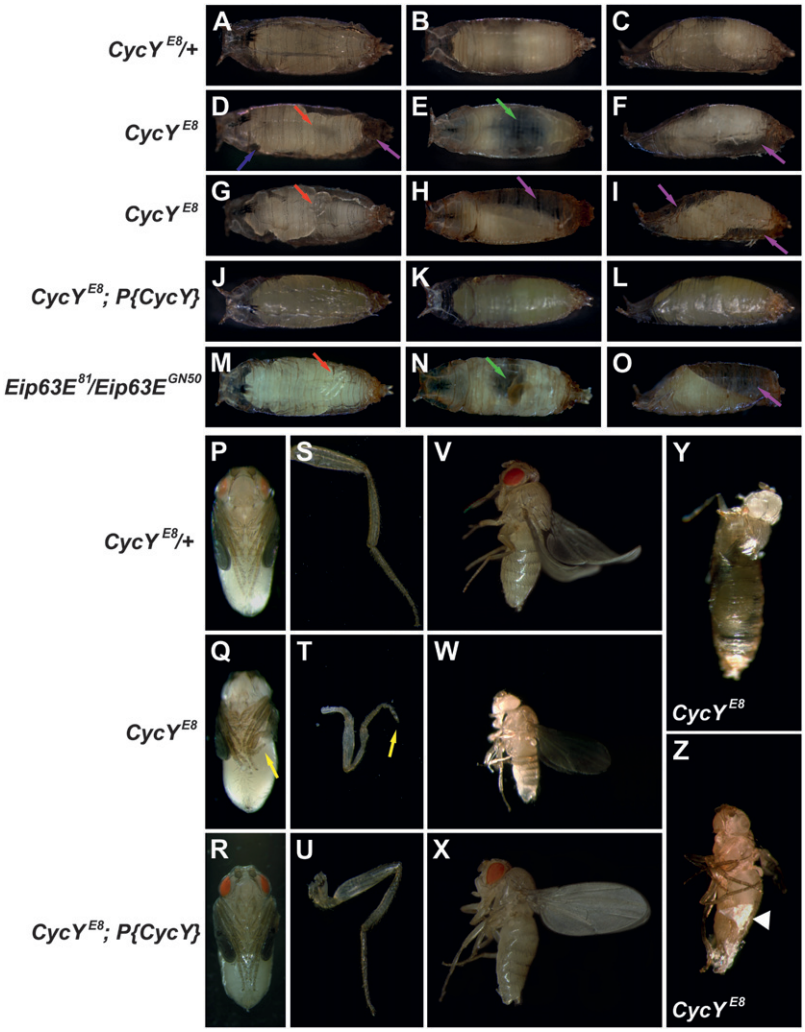

Figure 3.—Metamorphosis defects in *CycY* and *Eip63E* mutants. (A–O) Representative early pupae from ventral, dorsal, and lateral views (left, middle and right columns, respectively). Genotypes include $CycY^{E8}$/+ (A–C), homozygous $CycY^{E8}$ (D–I), homozygous $CycY^{E8}$ with the *P{CycY}* transgene (J–L), and $Eip63E^{81}/Eip63E^{GN50}$ (M–O). Defects are indicated by colored arrows. The $CycY^{E8}$ homozygous mutant early pupae (second and third rows) and *Eip63E* null mutant early pupae (fifth row) show defects of leg elongation (red), head eversion (blue), gas bubble translocation (green), and adult tissue growth (purple). Early pupae of $CycY^{E8}$ homozygotes with a genomic *CycY* transgene have no defects (fourth row). (P–R) Representative pharate adults of $CycY^{E8}$/+ (P), homozygous $CycY^{E8}$ (Q), and homozygous $CycY^{E8}$ with the *P{CycY}* transgene (R). Homozygous $CycY^{E8}$ mutant pharate adults have an obvious bent leg phenotype (yellow arrow in Q. $CycY^{E8}$ homozygous mutant adult escapers either die soon after eclosion or survive for <2 days and have a much smaller body size (W) than heterozygous control adults (V) or $CycY^{E8}$ mutants complemented with the *P{CycY}* transgene (X). Many of the adult escapers had malformed legs (T, yellow arrow), whereas legs were normal in heterozygous control adults (S), or $CycY^{E8}$ mutants complemented with the *P{CycY}* transgene (U). In $CycY^{E8}$ mutants arrested during eclosion (Y and Z), when the pupal case was manually removed (Z) a layer of white tissue (arrowhead) was evident. "+" stands for a *CyO* balancer chromosome with *Act5C–GFP*.

and I; Figure S3, C and D). The delay was also evident in the timing of pupariation. As shown in Figure 2, A and B, the first pupa of $CycY^{E8}$ heterozygotes was observed at 6 days after egg deposition (AED), while the first pupa of $CycY^{E8}$ homozygotes was observed at 7 days AED. On the basis of the number of pupae that formed each day in the two strains, we estimated that puparium formation of $CycY^{E8}$ homozygous mutants was delayed for ~13 hr relative to that of the heterozygous controls (materials and methods). The genomic *CycY* transgene shortened this delay to ~5 hr. Similar results were obtained with the $CycY^{E8}/Df(2L)Exel6030$ mutant (Figure S3).

CycY null mutants were arrested predominately during pupal stages, but with variable expressivity. We scored the final developmental stages of animals from each genotype on the basis of the presence of defined morphological markers (Bainbridge and Bownes 1981). Two major lethal phases were observed. The early lethal phase was between pupal stages P3 and P5; for example, all 162 $CycY^{E8}$ mutants that pupated developed to stage P3 but only 61% reached stage P5 (Table S2). In contrast, all of the 152 heterozygotes that pupated reached stage P5, and all but two eventually emerged as adults. The *CycY* null pupae that were arrested at stage P3 or P4 showed a variety of developmental defects, including defects in gas bubble translocation, head eversion, leg elongation, and adult tissue growth (Figure 3 and Table S3). Many mutant individuals stopped further development with the newly formed gas bubble still in the middle of the abdomen (Figure 3E). In others the gas bubble translocated to the posterior portion of the puparium as in wild type, but then failed to completely relocate to the anterior (Figure 3H), which may hinder head eversion (Chadfield and Sparrow 1985). Many of the mutant pupae showed different amounts of empty space inside the pupal case (Figure 3, D–I), which was probably due either to the failure of gas bubble translocation or to insufficient adult tissue growth. A defect in leg elongation was also prevalent. Some mutant individuals had partially elongated legs that were either shorter than normal and did not reach the bottom of the abdomen or were bent (*e.g.*, Figure 3G). More severe cases showed no sign of leg elongation (Figure 3D). Wings also did not achieve full extension. The $CycY^{E8}/Df(2L)Exel6030$

TABLE 1

***CycY* and *Eip63E* mutant pupae are smaller than wild-type pupae**

Genotype[c]	Average weight[a] mg (%)	*n*	Average length[b] % ± SD	*n*
$CycY^{E8}$/+	1.12 (100)	161	100 ± 4.0	31
$CycY^{E8}$/+ and *Df(2L)Exel6030*/+	1.18 (100)	239	100 ± 4.8	44
$CycY^{E8}$	0.65 (58)	137	90 ± 8.6	36
$CycY^{E8}$/*Df(2L)Exel6030*	0.75 (64)	230	92 ± 6.4	44
$CycY^{E8}$/+; *P{CycY}*	1.19 (100)	265	100 ± 4.9	72
$CycY^{E8}$; *P{CycY}*	1.13 (95)	239	102 ± 5.0	72
$CycY^{E8}$/+; *P{CycY}* and *Df(2L)Exel6030*/+; *P{CycY}*	1.21 (100)	246	100 ± 4.4	47
$CycY^{E8}$/*Df(2L)Exel6030; P{CycY}*	1.14 (94)	279	101 ± 4.2	48
$Eip63E^{GN50}$/+ and $Eip63E^{81}$/+	1.25 (100)	104	100 ± 3.7	30
$Eip63E^{GN50}$/$Eip63E^{81}$	0.75 (60)	142	90 ± 5.3	40

[a] Percentage of average weight is calculated relative to heterozygous siblings (100%).
[b] Percentage of average length is calculated relative to heterozygous siblings (100%).
[c] The plus symbol (+) stands for an *Act5C-GFP*-marked balancer chromosome; either *CyO* with $CycY^{E8}$ and *Df(2L)Exel6030* or *TM3, Ser* with $Eip63E^{GN50}$ and $Eip63E^{81}$.

mutant had the same range of phenotypes as homozygous $CycY^{E8}$ (Table S3 and Figure S4).

The late lethal phase of the *CycY* null was between stages P14 and P15, almost at the end of pupal development. For example, while 41% of the $CycY^{E8}$ homozygous pupae reached stage P14, only 13% reached stage P15 (Table S2). The P14-arrested mutants exhibited the prominent malformed leg phenotype that was also observed during earlier pupal stages (Figure 3Q). In addition to the morphological defects, *CycY* null pupae were generally shorter and much lighter than wild-type pupae (Table 1).

Among the small fraction of $CycY^{E8}$ pupae that reached stage P15, 8 out of 23 (35%) arrested during the process of eclosion. The remainder eclosed into adults, but the majority (13 out of 15) died very quickly with their wings still folded. Most of these adults displayed short bent legs (Figure 3T). Only two animals successfully eclosed into adults that looked normal, though they were smaller than newly emerged heterozygous control adults (Figure 3, V and W) and they survived for <2 days. When the mutants that were arrested during eclosion were manually dissected from the pupal case, a layer of white tissue could be seen, which seemed to adhere adult structures to the inside wall of the pupal case (Figure 3, Y and Z). All of the *CycY* null mutant defects described above could be rescued by introduction of a *CycY* genomic transgene (Figure 3, Table 1, Table S2, Table S3, and Figure S4).

The expression of CycY is essential during the transition from third instar larvae to prepupae: The null mutant phenotype of *CycY* suggested an important function during metamorphosis. To determine the developmental time point at which *CycY* expression is required, we generated transgenic flies that expressed myc-tagged CycY from a heat-shock promoter. A series of different heat-shock regimes was performed to compare their ability to rescue the lethality of homozygous $CycY^{E8}$ (Figure 4). Heat shock on the first 3 days after egg laying failed to rescue the viability of homozygous $CycY^{E8}$ mutants. However, when heat shock was extended for 1 or 2 more days, which included late third instar larvae, the rescue ability was dramatically increased to 30–35%. If *CycY* was also provided during early pupal stages, the rescue ability increased further to 50–60%. If *CycY* expression was withheld until 4 days after egg laying, a 50% rescue rate could still be achieved. However, if heat shock was delayed for 1 more day, the rescue ability decreased to only 13% (Figure 4). Combined, these data suggest that the most important period for zygotic *CycY* expression is from the late larvae to the early stages of pupal development, consistent with the first major lethal phase of the $CycY^{E8}$ mutant.

To see whether *CycY* is expressed at the developmental times when it appears to be needed, we used quantitative real-time PCR to determine the *CycY* mRNA levels. We found that the relative abundance of *CycY* mRNA fluctuated over a narrow range during development (Figure S5). The highest mRNA level was observed in 0- to 1-hr embryos, most likely due to maternal deposition. *CycY* message levels then decreased from later embryogenesis through the first and second instar larval stages but increased again in third instar larvae and peaked at pupal stages. The transcription variation of *CycY* is thus consistent with its essential requirement for pupariation.

***CycY* shows a maternal effect that can be partially rescued by zygotic expression:** The mutant phenotypes described above were based on zygotic null mutants, which showed normal embryogenesis and slow but otherwise normal larval development. To test whether maternally expressed *CycY* contributes to early development, we generated maternal null mutants using the ovo^{D1} dominant female sterile technique (Chou *et al.*

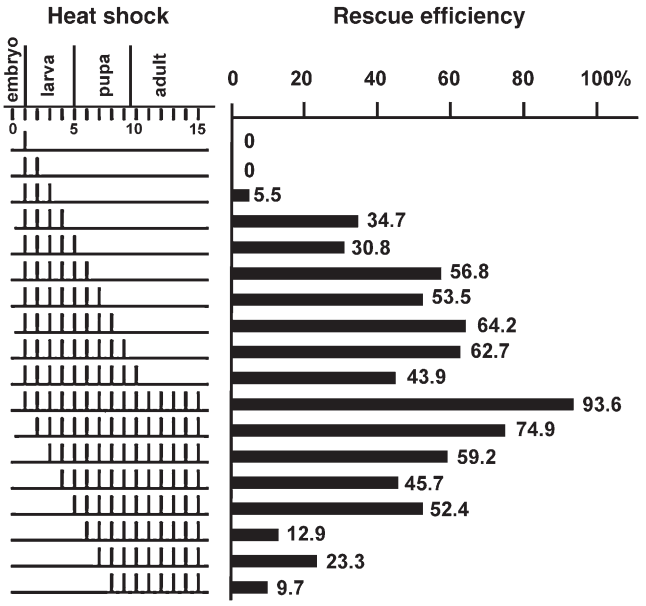

Figure 4.—Temporal requirements for the expression of *CycY*. Embryos from a *CycY*^{E8}*/CyO; hs-CycY/TM3, Ser* self-cross were collected for 24 hr and heat shocked for different regimes. Each row indicates a different heat-shock schedule. On the left side, each bar represents a single 1-hr heat shock at 37° on that particular day. The efficiencies of rescue to adulthood are shown on the right. The genotype of each adult was determined by the presence or absence of *CyO* and *Ser* balancer chromosomes (materials and methods). Representative adult genotypes were confirmed by single-fly PCR. For each condition, the total number of adults analyzed was between 200 and 300.

1993). *Hs-FLP/w*; CycY*^{E8} *FRT40A/ovo*^{D1} *FRT40A* females were heat shocked for 2 hr during larval development to express FLP recombinase and promote homologous recombination between the *CycY*^{E8} *FRT40A* and *ovo*^{D1} *FRT40A* chromosomes. Since *ovo*^{D1} is dominant female sterile, mothers will lay eggs only if homozygous *CycY*^{E8} *FRT40A* germline cells are generated and *CycY* is not essential for oogenesis. Mothers that received heat-shock treatment during larval development were crossed with *w*^{1118} males and the number and development of the eggs laid were monitored. We observed that heat-shock treated *CycY*^{E8} *FRT40A/ovo*^{D1} *FRT40A* females could lay similar numbers of eggs as heat-shock treated *FRT40A/ovo*^{D1} *FRT40A* females, indicating that *CycY* is not essential for at least some of the major processes of oogenesis. However, ~40% of the eggs from *CycY*^{E8} mothers had fused dorsal appendages (data not shown), suggesting that *CycY* may play a role in axis specification.

To test for a maternal contribution to embryogenesis, females with homozygous *CycY*^{E8} germline cells were generated using the *ovo*^{D1} dominant female sterile technique (Chou *et al.* 1993), and were crossed with *CycY*^{E8}*/CyO, Act5C-GFP* males. Zygotic null progeny were identified by absence of the *GFP* balancer. Interestingly, the majority (99.6%) of zygotic null embryos from null mothers failed to hatch, suggesting that maternal expression of *CycY* is essential for embryogenesis. Surprisingly, when females with homozygous *CycY*^{E8} germline cells were crossed with *w*^{1118} males, 7.3% of the embryos hatched into first instar larvae and 73% of these larvae developed into normal adults. Taken together, these data suggest that maternally provided *CycY* plays an important role during embryogenesis, but that this role can be accomplished at least to a limited extent by zygotic expression.

Eip63E is a potential binding partner of CycY: Cyclin proteins generally serve as regulatory subunits for Cdks. In a previous high throughput yeast two-hybrid screen (Stanyon *et al.* 2004) we identified an interaction between CycY and Eip63E, a Cdk with no known cyclin partner (Rascle *et al.* 2003; Stowers *et al.* 2000). To test specificity, we conducted additional two-hybrid assays using additional Cdks and cyclins (File S1 and Table S4). We found that CycY interacted only weakly or not at all with other Cdks, including Cdk1, Cdk2, Cdk4, Cdk5, Cdk7, Cdc2rk, and CG7597. Likewise, Eip63E interacted with CycY and CycC, a protein known to be promiscuous in two-hybrid assays, but only weakly or not at all with CycA, CycB, CycB3, CycD, CycE, CycG, CycH, CycJ, CycK, CycT, Koko, and CG16903. To further confirm and test the specificity of the Eip63E–CycY interaction, we expressed tagged versions of Cdks and cyclins in cultured Drosophila cells and tested interaction by co-AP followed by immunoblotting (materials and methods). In the co-AP assay, CycY interacted strongly with Eip63E but only weakly or not at all with Cdk2, Cdk4, or Cdc2rk (Figure 5, A and B). Eip63E, on the other hand, interacted much more strongly with CycY than with other cyclins tested, including CycK, CycD, and CG31232 (Koko) (Figure 5C). As expected, Glycine 243 (G243) of Eip63E, which is essential for its function *in vivo* (Stowers *et al.* 2000), is required for binding to CycY (Figure 5D). In further support of the interaction between these proteins, a recent study demonstrated an interaction between the human homolog of Eip63E, PFTK1, and human CycY using yeast two-hybrid and co-AP assays from human cells (Jiang *et al.* 2009). Taken together, our data and the studies with the human orthologs support the notion that CycY and Eip63E constitute a conserved cyclin–Cdk pair.

A recent large-scale phosphoproteome study in Drosophila embryos identified several phosphorylated peptides from the CycY protein (Zhai *et al.* 2008). A number of the phosphorylation sites are in highly conserved serine residues, suggesting that they may affect CycY function (Figure S1, B). One of these residues, S389, has also been found to be phosphorylated in human CycY, both in nuclear and cytoplasmic fractions (Beausoleil *et al.* 2004; Olsen *et al.* 2006). Position Ser389 in the Drosophila protein is conserved in every species that we examined (Figure S1, B). Moreover in one of the two preceding positions of every CycY there is another serine (S388 in Drosophila), which was also identified

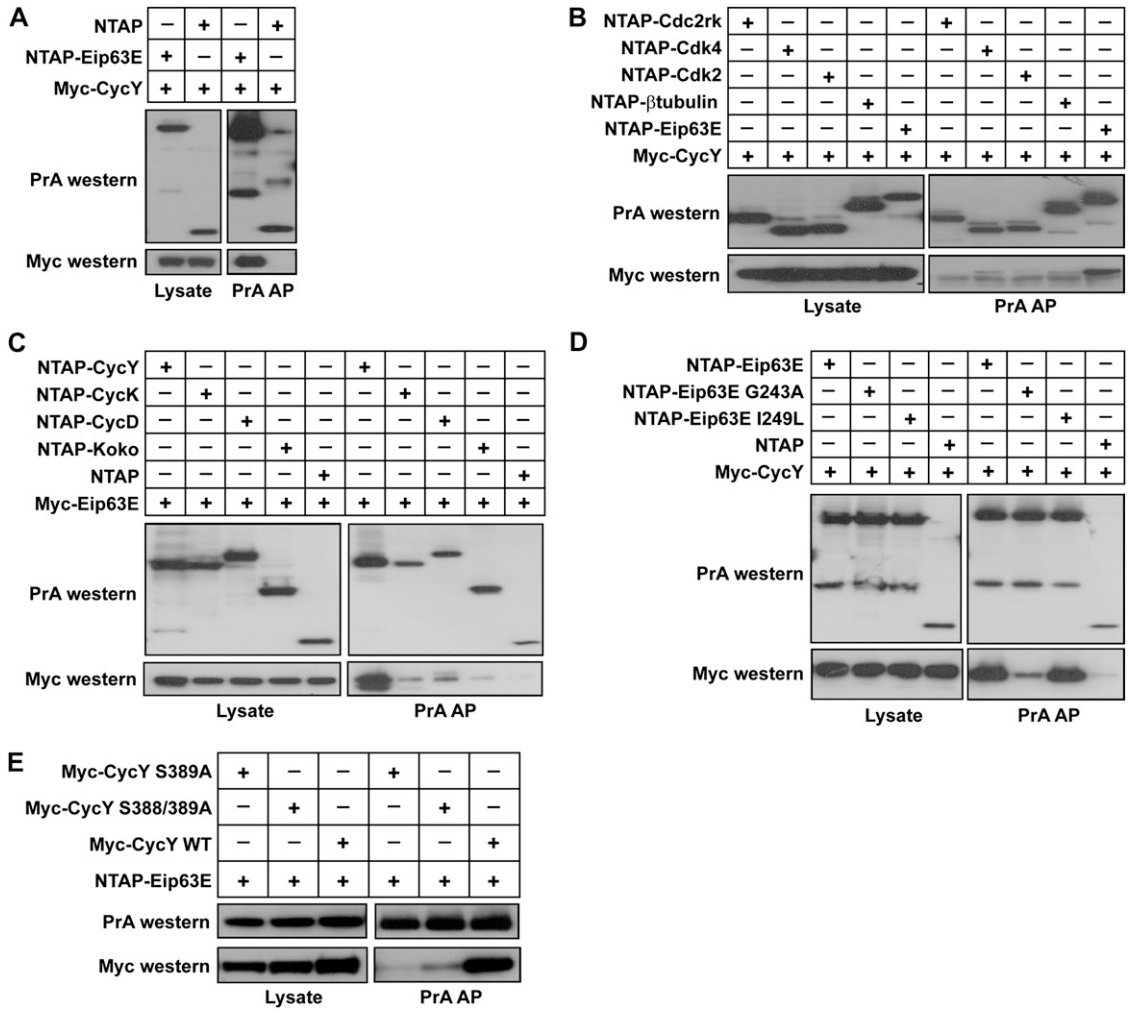

FIGURE 5.—CycY preferentially interacts with Eip63E in Drosophila cells. Cells were cotransfected with the indicated constructs and lysed for co-affinity purification (co-AP) using IgG beads. Co-purified proteins were further detected by Western blot using anti-Myc or anti-protein A (PrA) antibody. (A) CycY interacts with Eip63E. (B) CycY interacts much more strongly with Eip63E than with Cdk2, Cdk4, or Cdc2rk. (C) Eip63E interacts much more strongly with CycY than with CycD, CycK, or CG31232 (Koko). (D) Eip63E G243A mutant interacts poorly with CycY. (E) CycY S389A mutants display decreased affinity for Eip63E.

as a phosphorylated residue in the human protein. As a first test of the potential importance of these residues we generated a Drosophila CycY S389A mutant and S388A/S389A double mutant and tested their Cdk-binding ability. The Ser389A mutant had a dramatically decreased ability to bind Eip63E (Figure 5E). The double mutant did not further diminish Cdk binding, indicating that S388 does not contribute to the interaction. While these results point to a role for S389 in Cdk interaction, we were unable to show that phosphorylation is important, since a S389E mutant also failed to interact with the Cdk (data not shown).

***CycY* and *Eip63E* have similar mutant phenotypes:** If Eip63E and CycY form a functional Cdk/cyclin complex *in vivo*, we might expect their mutant phenotypes to be similar. Previous studies have shown that *Eip63E* is important for embryogenesis, larval development, and morphogenesis (STOWERS *et al.* 2000). Those studies demonstrated that the majority of *Eip63E* null mutants die during larval development, while a small percentage survive to pupal stages with an occasional adult escaper. STOWERS *et al.* (2000) also showed that puparium formation in *Eip63E* mutants is delayed by 2–3 days, pupae are small, and the rare adult escapers have a bent-leg phenotype and short life spans. All of these phenotypes are similar to those we observed for $CycY^{E8}$. To further compare the *Eip63E* and *CycY* loss-of-function phenotypes, we performed a detailed side-by-side phenotypic characterization. We used a transheterozygous null mutant, $Eip63E^{81}/Eip63E^{GN50}$ (STOWERS *et al.* 2000) and compared its phenotype with that of $CycY^{E8}$. We found that *CycY* and *Eip63E* null mutants showed similar developmental defects, though the *Eip63E* null mutant phenotype was generally more severe. Both mutants displayed a major lethal phase during metamorphosis (Figure 2, A, B, E, and F). While *CycY* mutants showed

lethality during early or late pupal stages, the majority of *Eip63E* mutants died at earlier pupal stages (Table S2). Both mutants also showed similar metamorphosis defects, including gas bubble translocation defects, failed head eversion, and leg elongation defects (Figure 3; Table S3). In addition, pupae of both mutants were similarly small in weight and length (Table 1). Finally, both mutants exhibited delayed puparium formation, for 13 hr in the case of *CycY*, and 37 hr for *Eip63E* (Figure 2). We also note that Stowers *et al.* (2000) showed that Eip63E has a zygotically rescuable maternal contribution to embryogenesis, similar to our observation for *CycY*. The striking similarity between the mutant phenotypes of *Eip63E* and *CycY*, combined with the specific physical interaction between the proteins in yeast two-hybrid and co-AP assays, supports the idea that CycY and Eip63E may function together *in vivo*. We cannot exclude the possibility, however, that one or both proteins have additional partners. For example, one potential explanation for the earlier lethality and more severe phenotype of *Eip63E* mutants relative to the *CycY* null is that Eip63E may have functions independent of CycY and these may involve other cyclin partners. Alternatively, the subtle differences in *CycY* and *Eip63E* mutant phenotypes may be due to differences in the levels of perdurance of their maternal components. Further *in vivo* analysis of the interaction will be needed to distinguish these possibilities.

Conclusions: Cyclin Y is a highly conserved protein that has not been characterized in any model organism. Only minimal information is available for the human ortholog, *CCNY*. The gene is broadly expressed in human tissues, with particularly high levels in testis (Jiang *et al.* 2009; Li *et al.* 2009). Localization studies with *GFP* fusions in cell lines have shown that one isoform of human CycY, which has also been called Cyclin X, is nuclear while another isoform may be anchored to the cell membrane via a conserved myristoylation signal (Jiang *et al.* 2009). Recently, *CCNY* was identified as a potential susceptibility factor for inflammatory bowel disease (IBD), a complicated genetic disorder affecting the intestinal mucosa. A single nucleotide polymorphism (SNP) located in an intron of *CCNY* was found to be strongly associated with the two IBD subphenotypes, Crohn's disease and ulcerative colitis (Franke *et al.* 2008; Weersma *et al.* 2009), though it is not yet clear whether *CCNY* plays a direct role in these diseases. Another study found that human CycY is among a number of proteins that are significantly upregulated in metastatic colorectal cancer cells (Ying-Tao *et al.* 2005), though again it is not clear whether this cyclin contributes to the phenotype of these cells. The establishment of a *CycY*-deficient animal model could provide a system for studying conserved functions of Cyclin Y and for understanding its potential role in human diseases.

Here we described the first mutant allele for a Y-type cyclin, a null for Drosophila *CycY*. We showed that *CycY* is an essential gene that is required for a broad range of developmental processes, including normal oogenesis, embryogenesis, larval and pupal development. The most obvious defects in the null were visualized during pupal development, and included defects in gas bubble translocation, head eversion, leg elongation, and adult tissue growth. Similar phenotypes have been described for a number of genes involved in the response to the steroid hormone ecdysone, including *E74*, *EcR*, *BR-C*, and *crol*, (Kiss *et al.* 1988; Fletcher and Thummel 1995; Bender *et al.* 1997; D'Avino and Thummel 1998). CycY may also be involved in the ecdysone response. Consistent with this possibility, we provide several lines of evidence, suggesting that at least one of the Cdk partners for CycY is the ecdysone-inducible protein, Eip63E. CycY and Eip63E preferentially interact in yeast two-hybrid assays and in co-AP assays from cultured Drosophila cells. The human orthologs of these proteins have also been shown to interact and to colocalize in human cell lines (Jiang *et al.* 2009). Finally, the mutations in Eip63E and CycY show a similar range of phenotypes. Our findings in Drosophila should provide a model system for further biochemical and genetic studies on the function of this conserved Cdk/cyclin pair.

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Cyclin Y Is a Novel Conserved Cyclin Essential for Development in Drosophila

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FILE S1

Supporting Materials and Methods

Sequence alignments: We determined the reciprocal best-match BLAST hits between *Drosophila* and human cyclins (Table S1). BLAST searches were conducted with each of the *Drosophila* cyclins listed below to identify the top matching human cyclins. In cases where a gene had multiple protein isoforms, the longest isoform that had a cyclin domain was used. The top matching human proteins were then used in BLAST searches against the *Drosophila melanogaster* annotated proteins and the top matching protein was identified. Reciprocal best-match BLAST hits are listed in Table S1. An example of how reciprocal best-match hits are interpreted is as follows: Human CCND1, CCND2, and CCND3 are the human proteins most similar to *Drosophila* CycD, and *Drosophila* CycD is the *Drosophila* protein most similar to human CCND1, CCND2, or CCND3, according to BLAST. Multiple sequence alignment was performed using ClustalX version 2 (LARKIN *et al.* 2007; THOMPSON *et al.* 2002). Pair-wise percent identity was determined by dividing the number of identical sites in the alignment by the length of the alignment, including gaps and unaligned ends. The dendrogram shown in Figure S1A was constructed using ClustalX with the neighbor-joining algorithm. For Figure S1B, the reciprocal best-match BLAST hits between *Drosophila* CycY and proteins from several divergent species were aligned using ClustalW followed by manual corrections to improve identities. Only the top matching CycY-like protein from each species is shown. The proteins aligned were as follows, where Genbank accession numbers are in parentheses: *Aedes aegypti* hypothetical protein AaeL_AAEL010543 (XP_001660900.1); *Caenorhabditis elegans* hypothetical protein ZK353.1a (NP_498858.2); *Danio rerio* hypothetical protein LOC767752 (NP_001070188.1); *Drosophila melanogaster* CG14939-PA (NP_609519.1); *Gallus gallus* CCNYL1 cyclin Y-like 1 (XP_425973.2); *Homo sapiens* cyclin fold protein 1 variant b (AAL78999.1); *Mus musculus* cyclin fold protein 1 (NP_080760.2); *Xenopus laevis* hypothetical protein LOC431857 (NP_001084816.1); *Nematostella vectensis* predicted protein (XP_001641126); *Trichoplax adhaerens* hypothetical protein (XP_002116466); *Monosiga brevicollis* hypothetical protein (XP_001750168).

To identify proteins with similarity to CycY in more distant species, reciprocal best-match BLAST hits between *Drosophila* CycY and proteins in the species listed below were determined. The identified proteins were also determined to be reciprocal best-match BLAST hits with the human *CCNY* protein. For all of the identified proteins the sequence similarity with the human or *Drosophila CycY* proteins was restricted to the annotated cyclin domain (MARCHLER-BAUER *et al.* 2009) and immediate flanking regions, referred to as the "cyclin+" region in Figure S2. The cyclin+ regions were aligned using ClustalW and a consensus sequence was determined by identifying residues that were found in >50% of the sequences (Figure S2A). The dendrogram shown in Figure S2B was obtained by aligning the cyclin+ region of the proteins most similar to CycY, and the annotated cyclin domains of reciprocal best-match hits of *Drosophila* CycA and CycB for the species shown. Only the top matching CycY-like protein from each species is shown; gene or genome duplications in some lineages have resulted in several

parologous CycY-like proteins (not shown). The following proteins from non-metazoan species were reciprocal best-match hits of *Drosophila* CycY or the human *CCNY* protein, where Genbank accession numbers are in parentheses: *Arabidopsis thaliana* CYCP4;3 (NP_196362.1); *Coprinopsis cinerea* predicted protein (XP_001832875); *Cryptococcus neoformans* cyclin (XP_566770); *Dictyostelium discoideum* cyclin domain-containing protein (XP_642568); *Giardia intestinalis* Cyclin fold protein 1 (EET00183.1); *Laccaria bicolor* predicted protein (XP_001886042); *Medicago truncatula* unknown (ACJ84314); *Paramecium tetraurelia* hypothetical protein (XP_001460214); *Perkinsus marinus* hypothetical protein (EER16009); *Phaeodactylum tricornutum* CYCP1 (XP_002182703.1); *Phytophthora infestans* cyclin-Y-like (EEY67633.1); *Populus trichocarpa* predicted protein (XP_002302113); *Ricinus communis* cyclin (XP_002520742.1); *Saccharomyces cerevisiae* PCL1 (NP_014110.1); *Tetrahymena thermophila* Cyclin, N-terminal domain containing protein (EAS05969); *Toxoplasma gondii* cyclin, N-terminal domain-containing protein (EEE19730); *Trypanosoma cruzi* cyclin 6 (AAG44389.1); *Yarrowia lipolytica* hypothetical protein (XP_505742).

Yeast two-hybrid assays: Yeast two-hybrid assays (FIELDS and SONG 1989) were performed using the LexA system (GYURIS *et al.* 1993) and interaction mating assays (FINLEY and BRENT 1994). Yeast strains and vectors, the protocol for one-on-one mating assays, and the reporter scoring methods were previously described (ZHONG *et al.* 2003). All of the cyclins tested were expressed as activation domain (AD) fusions, whereas all of the Cdks were expressed as DNA-binding domain (BD) fusions. AD and BD strains were obtained from the arrays of LexA-based yeast two-hybrid clones previously described (STANYON *et al.* 2004).

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TABLE S1

***Drosophila*-Human reciprocal best-match proteins**

Drosophila protein	Genbank ID	Human Gene	Human protein	Genbank ID
CG14939-PA (CycY)	NP_609519	*CCNY*	Cyclin Y	NP_659449.3
CG14939-PA (CycY)	NP_609519	*CCNYL1*	Cyclin Y-like 1	NP_689736.1
CycA-PA	NP_524030	*CCNA1*	Cyclin A1	NP_001104516
CycA-PA	NP_524030	*CCNA2*	Cyclin A2	NP_001228.1
CycB-PB	NP_726246	*CCNB1*	Cyclin B1	NP_114172.1
CycB-PB	NP_726246	*CCNB1*	Cyclin B2	NP_004692.1
CycB3-PA	NP_651303	*CCNB3*	Cyclin B3	NP_149020.2
CycC-PA	NP_476848	*CCNC*	Cyclin C	NP_005181.2
CycD-PF (PC)	NP_727913.1	*CCND2*	Cyclin D2	NP_001750.1
CycD-PF (PC)	NP_727913.1	*CCND3*	Cyclin D3	NP_001751.1
CycD-PF (PC)	NP_727913.1	*CCND1*	Cyclin D1	NP_444284.1
CycE-PD	NP_723925	*CCNE1*	Cyclin E1	NP_001229.1
CycE-PD	NP_723925	*CCNE2*	Cyclin E2	NP_477097.1
CycG-PC	AAF57169.2	*CCNG1*	Cyclin G1	NP_004051.1
CycG-PC	AAF57169.2	*CCNG2*	Cyclin G2	NP_004345.1
CycH-PA	NP_524207	*CCNH*	Cyclin H	NP_001230.1
CycJ-PA	NP_523903	*CCNJ*	Cyclin J	NP_001127847.1
CG16903-PA	NP_569980	*CCNL2*	Cyclin L2	NP_112199.2
CG16903-PB	NP_569980	*CCNL1*	Cyclin L1	NP_064703.1
CycK-PB	NP_788083	*CCNK*	Cyclin K	NP_001092872.1
CycT-PB	NP_524127	*CCNT2*	Cyclin T2	NP_001232.1
CycT-PB	NP_524127	*CCNT1*	Cyclin T1	NP_001231.2
Koko-PA	NP_650721	*FAM58A*	Family 58A	NP_689487.2
Koko-PA	NP_650721	*FAM58B*	Family 58B	NP_001098987.1

TABLE S2

***CycY* and *Eip63E* mutants display variable expressivity[a]**

Genotype[b]		L1	w. L3	P1	P3	P4	P5	P14	P15	A
CycY^{E8}/+	n	180	152	152	152	152	152	151	150	150
	%	100	84	84	84	84	84	84	83	83
CycY^{E8}	n	180	162	162	162	158	110	74	23	15[c]
	%	100	90	90	90	88	61	41	13	8
CycY^{E8}/+; *P{CycY}*	n	200	180	180	180	180	180	180	180	180[d]
	%	100	90	90	90	90	90	90	90	90
CycY^{E8}; P{CycY}	n	200	185	185	185	185	185	185	177	177[e]
	%	100	93	93	93	93	93	93	89	89
CycY^{E8}/+ and	n	200	187	187	187	187	187	187	174	174[f]
Df(2L)Exel6030/+	%	100	94	94	94	94	94	94	87	87
CycY^{E8}/Df(2L)Exel6030	n	200	186	186	186	182	144	88	31	19[g]
	%	100	93	93	93	91	72	44	16	10
CycY^{E8}/+; *P{CycY}* and	n	200	178	178	178	178	178	178	177	177[h]
Df(2L)Exel6030/+; *P{CycY}*	%	100	89	89	89	89	89	89	89	89
CycY^{E8}/Df(2L)Exel6030	n	200	179	179	179	179	179	179	176	176[i]
; *P{CycY}*	%	100	90	90	90	90	90	90	88	88
Eip63E^{GN50}/+ and	n	180	164	164	164	164	164	164	162	162
Eip63E^{81}/+	%	100	91	91	91	91	91	91	90	90
Eip63E^{GN50}/Eip63E^{81}	n	180	135	129	129	76	59	0	0	0
	%	100	75	72	72	42	33	0	0	0

[a] 180 or 200 newly eclosed first instar larvae (L1) from each genotype were followed and the number that reached each stage, including wandering third instar larvae (w. L3), pupal stages (P1-P5, P14, and P15), and adults (A), was recorded.

[b] *P{CycY}* represents a genomic *CycY* transgene (Figure 1). In *CycY^{E8}*/+ and *Df(2L)Exel6030*/+, "+" stands for an *Act5C-GFP*-marked *CyO* balancer chromosome presumed to be wild type for *CycY*. In *Eip63E^{81}*/+ and *Eip63E^{GN50}*/+, "+" stands for an *Act5C-GFP*-marked *TM3, Ser* balancer chromosome presumed to be wild type for *Eip63E*.

[c] 13 out of the 15 *CycY^{E8}* adults that eclosed had leg and wing defects and died quickly, while the remaining two were much smaller than their heterozygous siblings and died within two days.

[d] 3 out of the 180 *CycY^{E8}*/+; *P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

[e] 18 out of the 177 *CycY^{E8}; P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

[f] One out of the 174 *CycY^{E8}*/+ and *Df(2L)Exel6030*/+ adults was found dead on the food surface with the wing still folded and without other obvious morphological defects.

[g] All of the 19 *CycY^{E8}/Df(2L)Exel6030* adults that eclosed had leg and wing defects and died quickly.

[h] 6 out of the 177 *CycY^{E8}*/+; *P{CycY}* and *Df(2L)Exel6030*/+; *P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

[i] 13 out of the 176 *CycY^{E8}/Df(2L)Exel6030; P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

TABLE S3

Metamorphosis defects in *CycY* and *Eip63E* mutants[a]

Genotypes	Total pupae	Eclosed (%)	Arrested between P1 and P14 (%)				
			Defects	-	+	++	+++
CycY^{E8}	162	14	Leg elongation	18	9	22	37
			Empty space inside pupal case	17	16	40	13
			Head eversion	44	18	10	14
CycY^{E8}; P{CycY}	185	96	Leg elongation	4	0	0	0
			Empty space inside pupal case	4	0	0	0
			Head eversion	4	0	0	0
CycY^{E8}/Df(2L)Exel6030	186	17	Leg elongation	20	24	16	23
			Empty space inside pupal case	19	24	28	12
			Head eversion	27	35	11	10
CycY^{E8}/Df(2L)Exel6030 ;P{CycY}	179	98	Leg elongation	2	0	0	0
			Empty space inside pupal case	2	0	0	0
			Head eversion	2	0	0	0
Eip63E^{GN50}/Eip63E^{81}	129	0	Leg elongation	2	17	17	64
			Empty space inside pupal case	32	26	23	20
			Head eversion	33	23	4	40

[a] Mutants terminally arrested between pupal stages P1 and P14 (Table S2) were scored for metamorphosis defects (leg elongation, head eversion, or empty space inside the pupal case). - no defect; + mild defect; ++ moderate defect; +++ severe defect.

TABLE S4

CycY/Eip63E interaction specificity by yeast two-hybrid assay

	CycY	
	Leu2	LacZ
Eip63E	3	1
Cdk1	1	0
Cdk2	1	0
Cdk4	0.5	0
Cdk5	0.5	0
Cdk7	0	0
Cdc2rk	0.5	0
CG7597	0	0

	Eip63E	
	Leu2	LacZ
CycY	3	1
CycA	0	0
CycB	0	0
CycB3	0	1
CycC	3	2
CycD	0	0
CycE	0	0
CycG	0	0
CycH	0	0
CycJ	0	0
CycK	0	0
CycT	0	0
Koko	0	0
CG16903	0	0

Interactions between activation domain (AD) -tagged CycY and LexA DNA-binding domain (BD)-tagged Cdks (top), or BD-tagged Eip63E and AD-tagged cyclins (bottom) were tested by yeast two-hybrid mating assays. Activity for the two reporter genes, *LEU2* and *lacZ*, was scored by the growth on plates lacking leucine (scale 0-3, where 0=no growth, 3=heavy growth) and blue color on X-gal plates (scale 0-5, where 0=white, 5=dark blue).

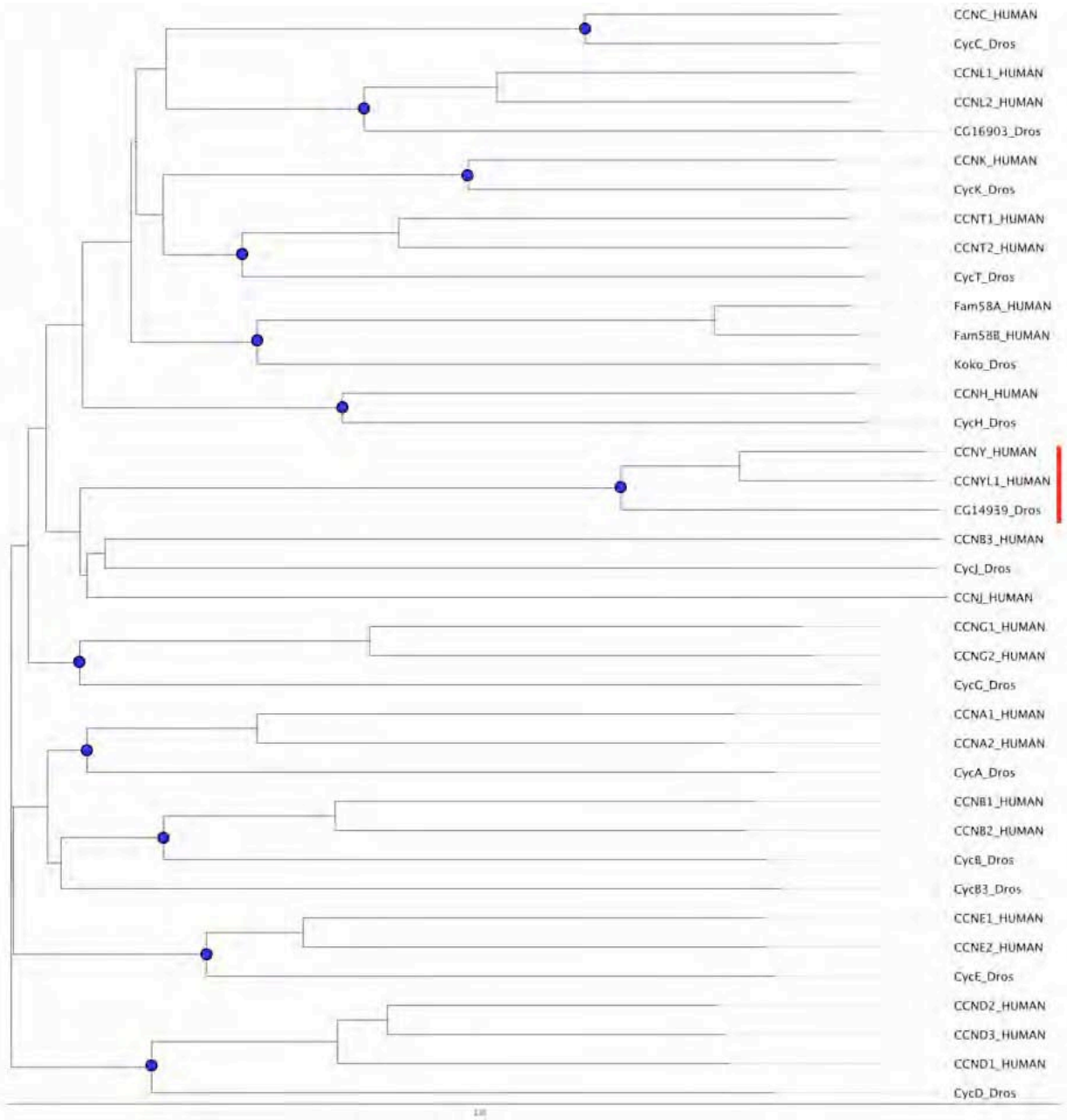
TABLE S5

Primers used for RT-PCR and qPCR

Gene	Primers	Sequence	Position[a]	Product length (bp)
CycY	Forward	5'-AGGAGAATGGCACCCAAC	765-782	414
	Reverse	5'-TACTCCCGGTGGCAATAG	1161-1178	
crol	Forward	5'-AGCTCGGTGCCATCAGTAG	1440-1458	332
	Reverse	5'-GCGGCATTATTCGTGGACG	1753-1771	
Pde1c	Forward	5'-GTGTGATCGCAACAATACGC	1622-1641	465
	Reverse	5'-TTGCTTTCCTCCGCTTCCCAG	2066-2086	
β-Tubulin	Forward	5'-GACCATGTCCGGCGTAAC	881-898	438
	Reverse	5'-AGCTCCTGGATGGCAGTG	1301-1318	
rp49	Forward	5'-GATATGCTAAGCTGTCGCACAAATGGC	95-121	118
	Reverse	5'-GTGCGCTTGTTCGATCCGTAACCG	189-212	

[a] Inclusive nucleotide positions in predicted transcript RA for each gene.

FIGURE S1.—*CG14939* encodes a highly conserved Y-type cyclin. (A) Dendrogram showing sequence similarity among all *Drosophila* and human cyclins.



(B) Alignment of Y cyclins from several species

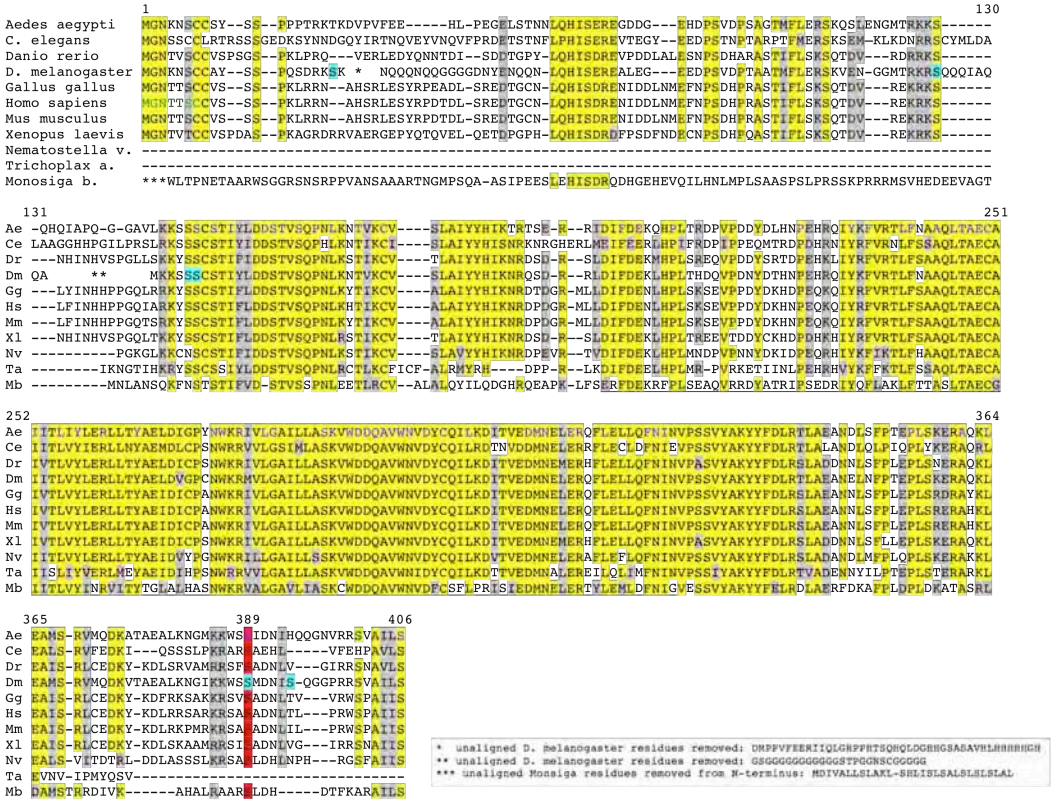


FIGURE S1.—*CG14939* encodes a highly conserved Y-type cyclin. (A) Phylogenetic tree resulting from alignment of all *Drosophila* cyclins and cyclin-like proteins and their corresponding human orthologs. Blue dots at branch points indicate that the attached nodes (proteins) are reciprocal best-match BLAST hits between *Drosophila* and human (see File S1). Lengths of the horizontal lines between nodes and branch points indicate relative sequence similarity; e.g., the human and *Drosophila* Y-type cyclins (red line) are more similar to each other than are any other human and *Drosophila* cyclins except for CycC. (B) Alignment of *Drosophila* CycY and the most similar proteins from several other species. The sequences available for *Nematostella* and *Trichoplax* may be truncated because the genome sequences were still in draft form. Yellow or grey shaded amino acids are identical or similar, respectively, in at least 8 out of the 11 species shown, or 7 of 9 species where the N- and C-terminal sequences of *Nematostella* and *Trichoplax* appear to be missing. Blue-shaded amino acids in the *D. melanogaster* sequence are known to be phosphorylated in embryos (ZHAI *et al.* 2008). Red-shaded serines, corresponding to S389 in *D. melanogaster*, are highly conserved and phosphorylated in both *Drosophila* and human CycY (BEAUSOLEIL *et al.* 2004; OLSEN *et al.* 2006; ZHAI *et al.* 2008). The N-terminal region of *H. sapiens* CycY contains a putative myristoylation signal (green lettering), previously noted by Jiang et al., (JIANG *et al.* 2009), which appears to be conserved in many other species. All of the sequences contain the conserved cyclin domain (underlined), corresponding to amino acids 205 to 328 of *Drosophila* CycY; this domain is annotated in these sequences by the Conserved Domain Database (MARCHLER-BAUER *et al.* 2009) and corresponds to pfam (FINN *et al.* 2009), domain pfam:00134, "Cyclin_N", the N-terminal cyclin fold found in the cyclin superfamily. Dashes indicate gaps in the alignment. Asterisks in the *D. melanogaster* sequence indicate unaligned residues that were removed and are shown below the alignment; one sequence is histidine-rich and the other is glycine-rich, and neither appears to be conserved. The unaligned N-terminal region of the *Monsiga brevicollis* sequence is also shown below. Numbers above the lines indicate residue numbers for the *Drosophila* protein. Gene names are listed in File S1.

A. Alignment of the cyclin domains from human, *Drosophila*, and *Monosiga* cyclin Y and the most related protein from many distantly related species.

B. The Y-type cyclin domain is only distantly related to other cyclin domains.

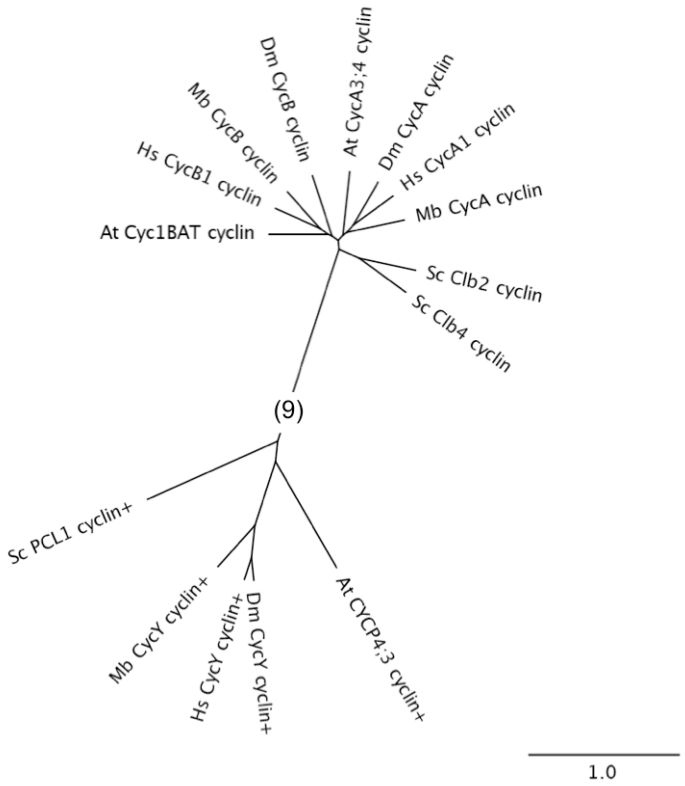


FIGURE S2.–The cyclin domain of Y-type cyclins is novel and conserved throughout the eukaryotic kingdom. (A) Alignment of the cyclin domains from the proteins that are reciprocal best-match BLAST hits of *Drosophila* CycY in many non-metazoan species. These proteins are also reciprocal best-match BLAST hits of human CycY. Alignments include the cyclin domains (arrows) as annotated by the Conserved Domain Database (MARCHLER-BAUER *et al.* 2009) along with the indicated flanking region of each protein. A consensus sequence was obtained as 31 residues that are identical in at least 50% of the proteins (colored); the *Drosophila* and human proteins each share 27 of these consensus residues. Only the top related protein from each species is shown. (B) Dendrogram showing sequence similarity among the cyclin domains from several distant species. Cyclin domains from *Drosophila melanogaster* (Dm) CycA, CycB, and CycY and their reciprocal best-match BLAST hits in human (Hs), *Monosiga brevicollis* (Mb), *Arabidopsis thaliana* (At), and *Saccharomyces cerevisiae* (Sc) were aligned. The cyclin domains from the Y-type cyclins included the annotated cyclin domain and small flanking regions as shown in Figure S2A (cyclin+). Only the top related protein from each species is shown. The length of the lines is proportional to sequence similarity. The lower cluster of Y-type cyclin domains and the upper cluster of A and B-type cyclin domains are separated by a relative distance of 9 (see scale bar for relative distances).

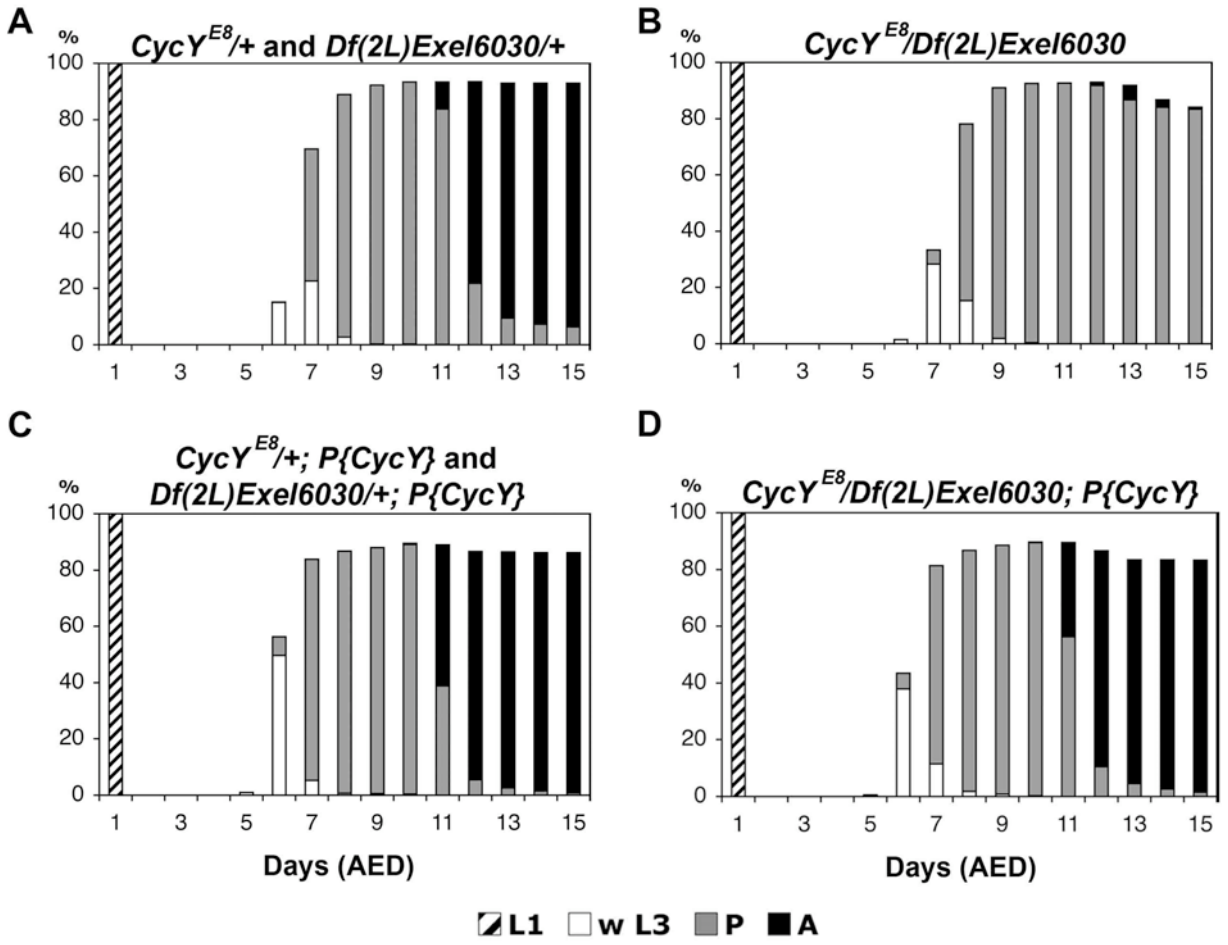


FIGURE S3.–Developmental timing of *CycY* null mutants with and without a *CycY* genomic transgene. The development of 200 first instar larvae of each genotype was followed for 15 days. Genotypes shown include *CycY*^{E8}/+ and *Df(2L)Exel6030*/+ combined (A), *CycY*^{E8}/*Df(2L)Exel6030* (B), *CycY*^{E8}/+; *P{CycY}* and *Df(2L)Exel6030*/+; *P{CycY}* combined (C), *CycY*^{E8}/*Df(2L)Exel6030*; *P{CycY}* (D). The percentage of first instar larvae (L1) that developed into wandering third instar larvae (w L3), pupae (P), and adults (A) on each day after egg deposition (AED) is shown. "+" stands for an *Act5C-GFP*-marked *CyO* balancer chromosome presumed to be wild type for *CycY*.

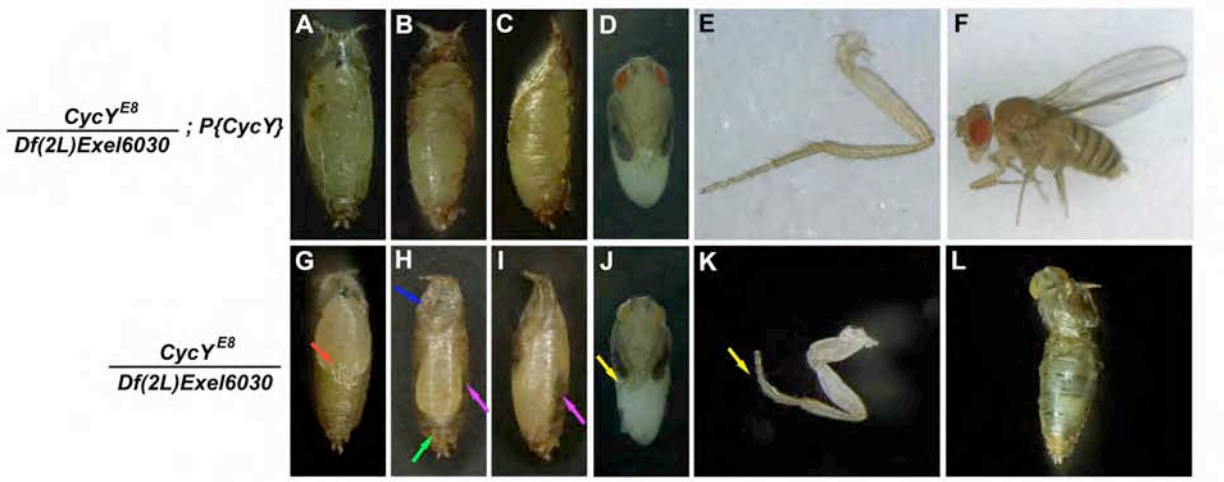


FIGURE S4.–Metamorphosis defects in *CycY* null mutants. Genotypes shown include *CycY^{E8}/Df(2L)Exel6030*; *P{CycY}* (A-F) and *CycY^{E8}/Df(2L)Exel6030* (G-L). Representative early pupae (A-C, G-I), pharate adults (D, J), dissected legs (E, K), or adults (F, L) are shown. For early pupae, the first, second, and third columns present the ventral, dorsal, and lateral views, respectively. Defects are indicated by colored arrows. The *CycY^{E8}/Df(2L)Exel6030* transheterozygous mutant early pupae (G-I) show defects of leg elongation (red), head eversion (blue), gas bubble translocation (green), and adult tissue growth (purple). *CycY^{E8}/Df(2L)Exel6030* transheterozygous mutant pharate adults have an obvious bent leg phenotype (J, yellow arrow), but the dorsal view is indistinguishable from the control (data not shown). *CycY^{E8}/Df(2L)Exel6030* transheterozygous mutant adult escapers die soon after eclosion, some of which also have malformed legs (K, yellow arrow). Some were arrested during eclosion (L).

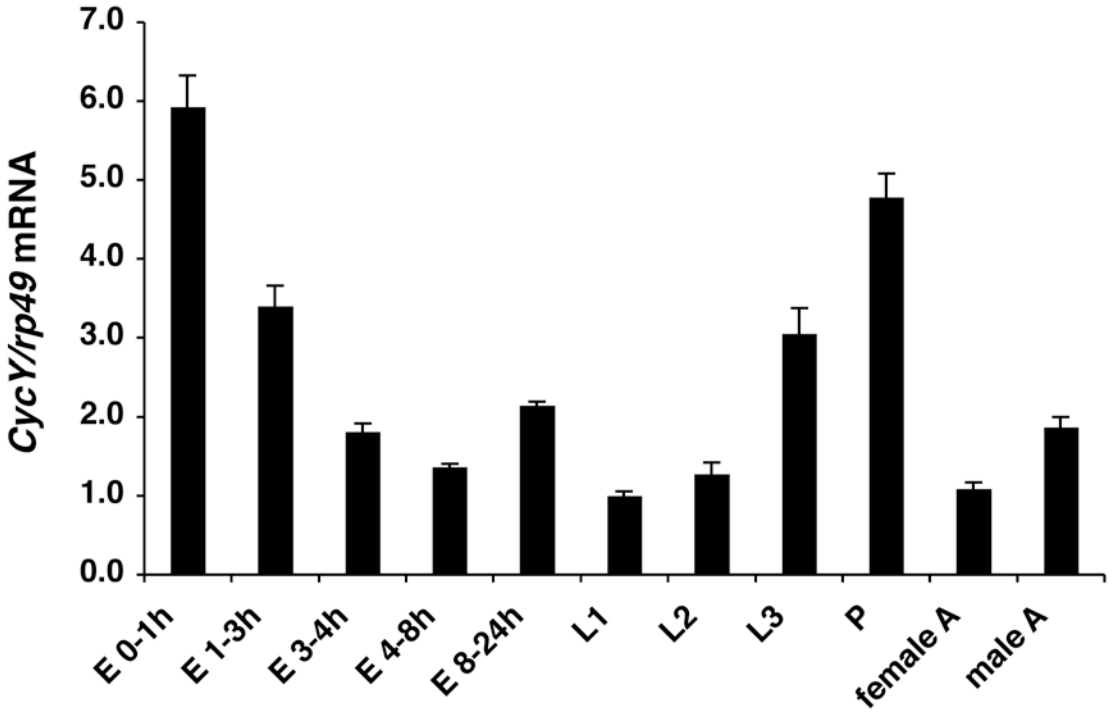


FIGURE S5.—Developmental expression pattern of *CycY*. Total RNA was extracted from *Drosophila* tissues at the indicated developmental time points and mRNA levels of *CycY* were determined by quantitative real-time PCR (qPCR) as described in Materials and Methods. Expression was normalized to the mRNA levels of the internal control *rp49*.