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Supporting Information
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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).

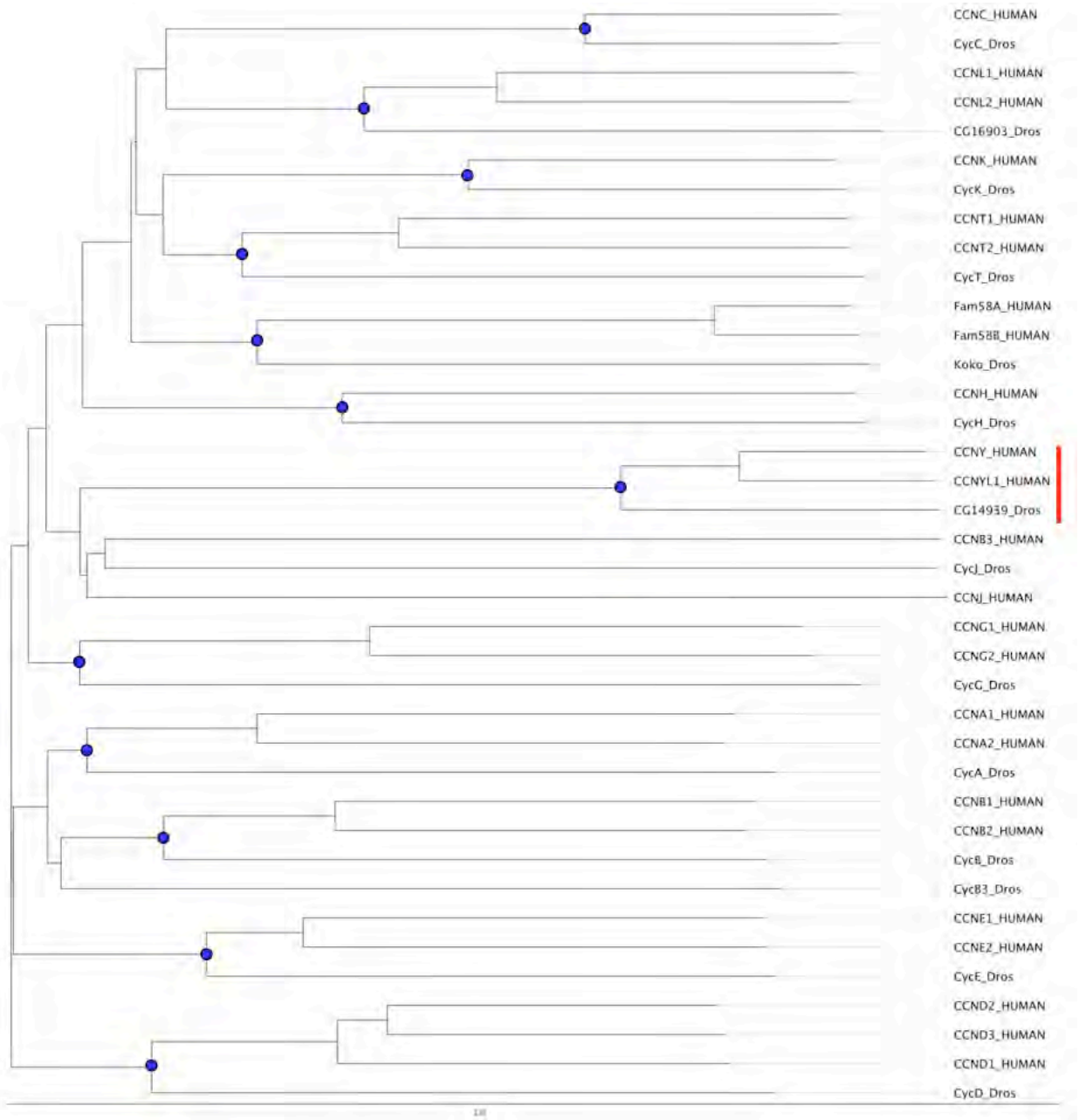
TABLE S5

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

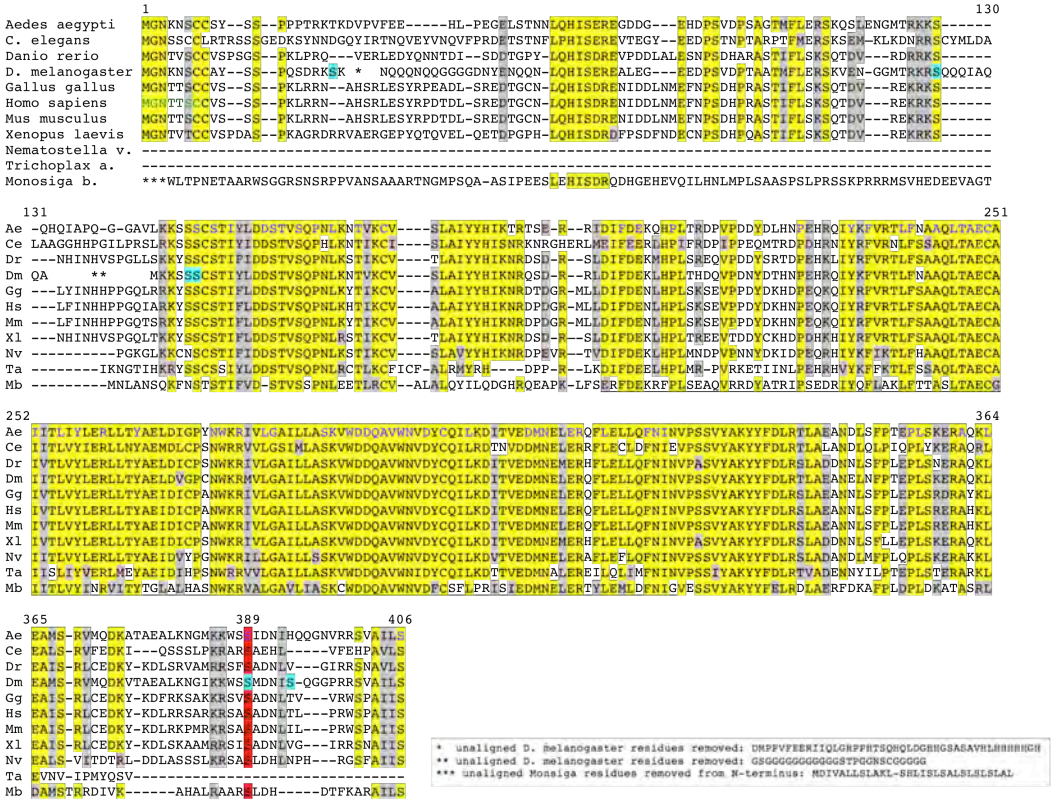


FIGURE S1.—*CG14939* encodes a highly conserved Y-type cyclin. (A) Phylogenetic tree resulting from alignment of all *Drosophila* cyclins and cyclin-like proteins and their corresponding human orthologs. Blue dots at branch points indicate that the attached nodes (proteins) are reciprocal best-match 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.

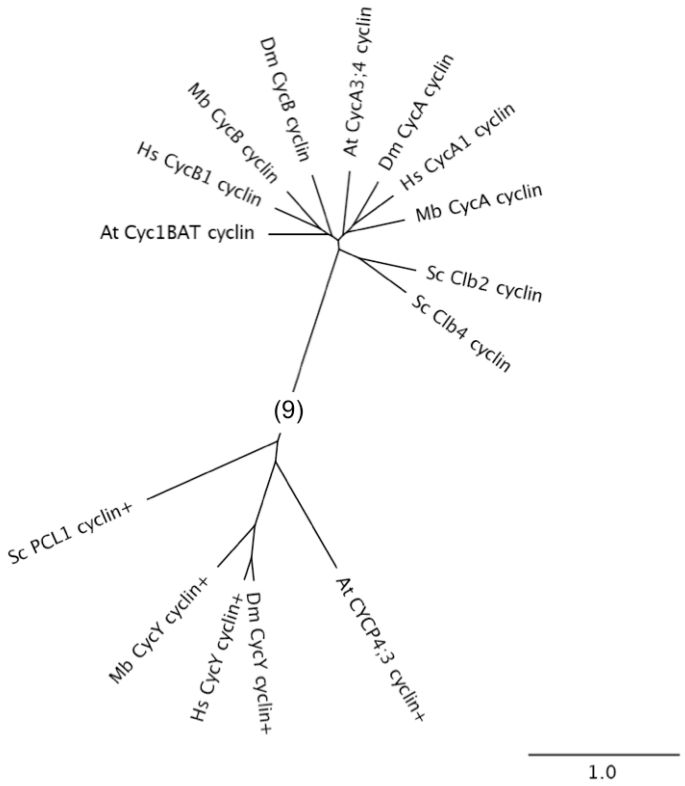


FIGURE S2.–The cyclin domain of Y-type cyclins is novel and conserved throughout the eukaryotic kingdom. (A) Alignment of the cyclin domains from the proteins that are reciprocal best-match BLAST hits of *Drosophila* CycY in many non-metazoan species. These proteins are also reciprocal best-match BLAST 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).

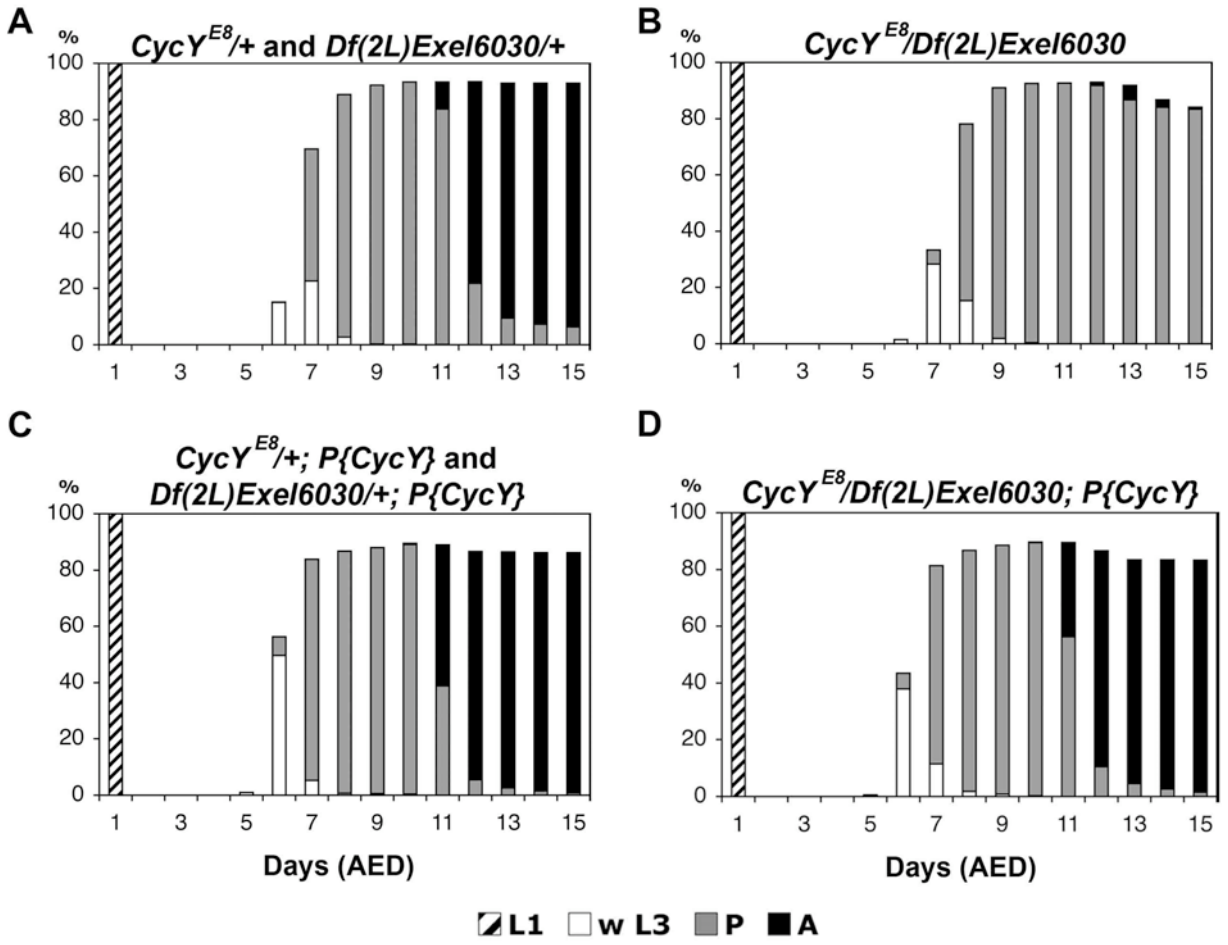


FIGURE S3.–Developmental timing of *CycY* null mutants with and without a *CycY* genomic transgene. The development of 200 first instar larvae of each genotype was followed for 15 days. Genotypes shown include *CycY*^{E8}/+ and *Df(2L)Exel6030*/+ combined (A), *CycY*^{E8}/*Df(2L)Exel6030* (B), *CycY*^{E8}/+; *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*.

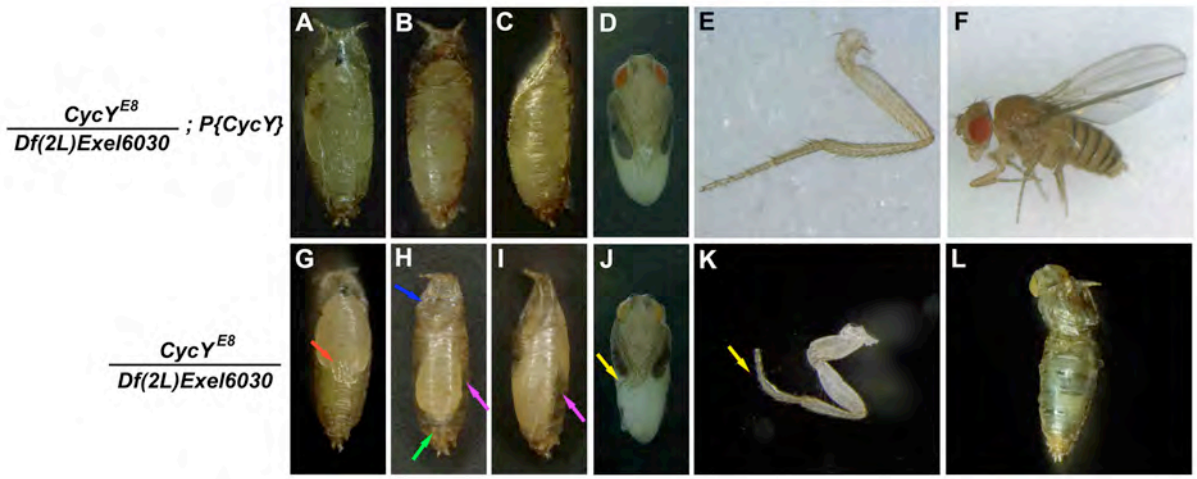


FIGURE S4.–Metamorphosis defects in *CycY* null mutants. Genotypes shown include *CycY^{E8}/Df(2L)Exel6030*; *P{CycY}* (A-F) and *CycY^{E8}/Df(2L)Exel6030* (G-L). Representative early pupae (A-C, G-I), pharate adults (D, J), dissected legs (E, K), or adults (F, L) are shown. For early pupae, the first, 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).

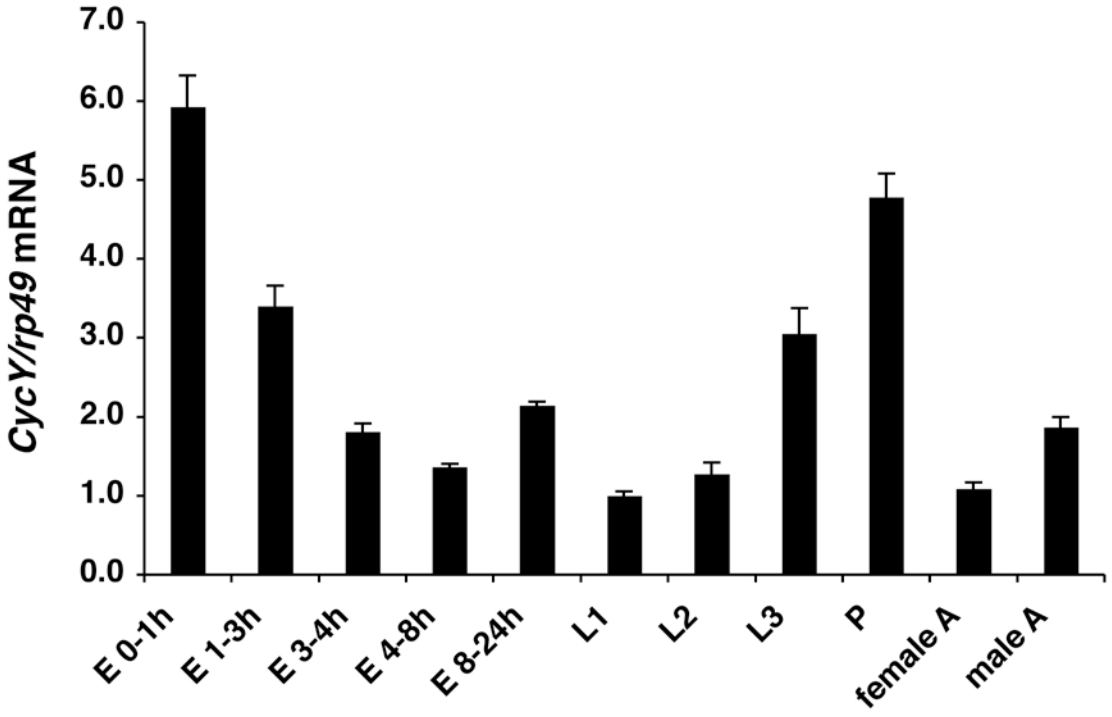


FIGURE S5.—Developmental expression pattern of *CycY*. Total RNA was extracted from *Drosophila* tissues at the indicated developmental time points and mRNA levels of *CycY* were determined by quantitative real-time PCR (qPCR) as described in Materials and Methods. Expression was normalized to the mRNA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