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Drosophila Myt1 Is the Major Cdk1 Inhibitory Kinase for Wing Imaginal Disc Development

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ABSTRACT

Mitosis is triggered by activation of Cdk1, a cyclin-dependent kinase. Conserved checkpoint mechanisms normally inhibit Cdk1 by inhibitory phosphorylation during interphase, ensuring that DNA replication and repair is completed before cells begin mitosis. In metazoans, this regulatory mechanism is also used to coordinate cell division with critical developmental processes, such as cell invagination. Two types of Cdk1 inhibitory kinases have been found in metazoans. They differ in subcellular localization and Cdk1 target-site specificity: one (Wee1) being nuclear and the other (Myt1), membrane-associated and cytoplasmic. Drosophila has one representative of each: dMyt1 and dWee1. Although dWee1 and dMyt1 are not essential for zygotic viability, loss of both resulted in synthetic lethality, indicating that they are partially functionally redundant. Bristle defects in *myt1* mutant adult flies prompted a phenotypic analysis that revealed cell-cycle defects, ectopic apoptosis, and abnormal responses to ionizing radiation in the *myt1* mutant imaginal wing discs that give rise to these mechanosensory organs. Cdk1 inhibitory phosphorylation was also aberrant in these *myt1* mutant imaginal wing discs, indicating that dMyt1 serves Cdk1 regulatory functions that are important both for normal cell-cycle progression and for coordinating mitosis with critical developmental processes.

Cdk1 is a conserved cyclin-dependent kinase, whose activity is responsible for promoting the dramatic cellular rearrangements associated with mitosis (Nigg *et al.* 1991; Masui 1992). During interphase, Cdk1 must be maintained in an inactive state by Wee1-related Cdk1 inhibitory kinases, otherwise premature initiation of mitotic events would disrupt essential cellular processes and cause cell lethality by mitotic catastrophe (Lundgren *et al.* 1991; Grosshans and Wieschaus 2000; Mata *et al.* 2000; Seher and Leptin 2000). Cell division must also be coordinated with critical developmental processes, such as cell movements and cell shape changes. This is accomplished during most Drosophila somatic cell cycles by regulating the expression of a Cdc25-related phosphatase that releases Cdk1 from inhibitory phosphorylation at the G2/M transition (Edgar and O'Farrell 1989, 1990; Lehman *et al.* 1999). Much less is known about specific developmental roles of the two types of Cdk1 inhibitory kinases, however, a question further complicated in many organisms by the presence of more than one Wee1 homolog (Wilson *et al.* 1999; Nakanishi *et al.* 2000; Leise and Mueller 2002; Okamoto *et al.* 2002). To address this issue, we have undertaken a genetic analysis of the Drosophila Cdk1 inhibitory kinases.

Drosophila has only one representative of each type of metazoan Cdk1 inhibitory kinase: designated dWee1 and dMyt1. We showed previously that dWee1 regulation of Cdk1 is essential for a premitotic checkpoint mechanism that prevents mitotic catastrophe during the rapid S/M nuclear cycles of early embryogenesis (Price *et al.* 2000; Stumpff *et al.* 2004). Zygotic *wee1* mutants are viable with no obvious developmental defects although they are sensitive to the DNA replication inhibitor hydroxyurea, suggesting they are impaired for a DNA replication checkpoint (Price *et al.* 2000). Loss-of-function studies of a mouse Wee1 homolog showed similar defects in rapidly cycling embryonic cells, indicating that this is a conserved developmental role for Wee1-like kinases (Price *et al.* 2000; Tominaga *et al.* 2006).

Myt1 was originally discovered in Xenopus as a membrane-associated Cdk1 inhibitory kinase capable of catalyzing Cdk1 inhibitory phosphorylation on both the Y15 and T14 residues (Kornbluth *et al.* 1994; Mueller *et al.* 1995). Myt1 kinases also physically interact with Cdk1 complexes through a protein motif that binds to B-type mitotic cyclins (Liu *et al.* 1999; Wells *et al.* 1999). This interaction is thought to be responsible for tethering inhibited Cdk1 complexes in the cytoplasm as they accumulate during G2 phase. Thus, Myt1 can potentially regulate Cdk1 by two distinct mechanisms, one of which is kinase independent.

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Myt1 activity is negatively regulated during oocyte maturation in many organisms, consistent with a role for Myt1 in inhibiting Cdk1 during meiotic G2 phase (Palmer *et al.* 1998; Lamitina and L'Hernault 2002; Leise and Mueller 2002; Okumura *et al.* 2002; Peter *et al.* 2002; Inoue and Sagata 2005; Burrows *et al.* 2006). Whether Myt1 has a role in Drosophila oocyte maturation remains unclear (Ivanovska *et al.* 2004), however, Drosophila *myt1* mutants exhibit pleiotropic cell-cycle defects during male and female gametogenesis, which suggest that dMyt1 has a role in developmentally regulated G2 phase arrest and in cell-cycle exit mechanisms that are normally coupled with terminal differentiation (Jin *et al.* 2005).

Studies in cultured mammalian cells have also recently implicated Myt1 in a novel Cdk1 regulatory mechanism that is important for proper assembly of the Golgi network and endoplasmic reticulum during mitotic exit (Nakajima *et al.* 2008). The generality of this mechanism and its possible relevance to specialized developmental functions of Myt1 kinases has not yet been established, however.

In this study, we characterized mutant phenotypes as well as Cdk1 inhibitory phosphorylation associated with loss of dMyt1 and dWee1 activity in larval imaginal wing discs and in adult structures derived from this tissue. The results identified dMyt1 as the major Cdk1 inhibitory kinase operating at these stages of development. In comparison, loss of dWee1 activity caused relatively minor cellular and developmental effects, unless dMyt1 functions were also compromised. We also found evidence that dMyt1 is required for normal cellular responses to ionizing radiation. These observations must be incorporated into models for understanding the role of dMyt1 in coordinating cell-cycle progression with critical developmental events.

MATERIALS AND METHODS

Genetic strains: In a previously published study of *myt1* mutants, we analyzed *myt1^{1}/Df(3L)64D-F* hemizygote and *myt1^{1}/myt1^{2}* transheterozygote genotypes (Garcia-Bellido *et al.* 1994; Jin *et al.* 2005). These genotypes exhibited identical gametogenesis and macrochaetae defects, indicating that *myt1^{1}* and *myt1^{2}* were null alleles. Both of these alleles were later found to have identical mutations in the *myt1* coding region, indicating they were likely preexisting mutations that were isolated on chromosomes that had subsequently acquired different second-site lethal alleles during EMS mutagenesis (Jin *et al.* 2005). To make genetic manipulations easier for subsequent analysis of *myt1* mutant phenotypes, we used meiotic recombination to remove secondary lethal mutations from the original *myt1^{1}* mutant chromosome, and thereby reisolated a homozygous viable *myt1^{1}* allele. The phenotype of homozygous viable *myt1^{1}/myt1^{1}* mutants was identical to what was observed for *myt1^{1}/Df(3L)64D-F* and *myt1^{1}/Df(3L)CH39* hemizygotes, two different chromosomal deletions that uncover the *myt1* locus (Extavour and Garcia-Bellido 2001). Genetic interactions with *wee1* were analyzed with a null allele (*wee^{ES1}*), a hypomorphic allele (*wee1^{DS1}*), and a deletion, *Df(2L)wee1^{W05}*, all of which were previously described (Price *et al.* 2000).

Immunofluorescent analysis of larval wing discs: For the larval checkpoint assays, wandering third instar larvae were transferred into fresh vials and then irradiated by a Co^{60} γ-ray source, calibrated to administer a dosage of 40 Gy. In these experiments, *myt1* mutants were identified as nonbalancer larvae, by the *Tubby* marker on the *TM6B* balancer chromosome, whereas *wee1* mutants were identified by the *actin-GFP* transgene inserted on the *SM6* balancer chromosome. An otherwise wild-type *yw* stock was used as the control genotype for these experiments. Wing imaginal discs were dissected from the larvae for fixation in 3.7% formaldehyde (buffered with 1× PBS) at room temperature for 20 min, washed twice with 1× PBS, and then permeabilized in 1× PBS containing 0.5% Triton X-100 for 30 min. The discs were then processed for immunofluorescent labeling by standard procedures. Primary antibodies and concentrations were: rabbit anti-phospho-S10-histone 3 (1/4000; Upstate), rabbit anti-cleaved caspase-3 (1/500, Cell Signaling Technology), mouse MAb 2B10 anti-Cut (1/200, Developmental Studies Hybridoma Bank). Secondary antibodies conjugated with Alexa Fluor-488 or Alexa Fluor-568 were used at a 1/1000 working dilution (Molecular Probes). Microscopy images were acquired with either a Zeiss Axioskop or a Leica TCS-SP2 multiphoton confocal laser scanning microscope (TCS-MP). The imaginal discs shown in Figures 2 and 3 were composed of more than one overlapping image to include the whole disc and these images were deconvolved using iterative restoration by Volocity software. All of the figures were compiled using Adobe Photoshop software; identical image manipulations were applied to control and experimental panels to prepare them for printing. PH3-positive nuclei of the widefield images used to compare *yw*, *wee1*, *myt1*, and *wee1; myt1*, as shown in Figure 2, were counted manually. The particle analysis function of ImageJ software (NIH) was used to count PH3-positive nuclei of the confocal images to generate the data shown in Figure 5. The area of activated caspase-3 staining in the wing disc was also determined using ImageJ software. All quantification was performed on a 200–273 μM^2 area centered on the wing pouch of 3–7 imaginal discs.

Scanning electron microscopy of adult structures: Adult flies were fixed for 2 hr in 1% glutaraldehyde:1% formaldehyde in 1 M sodium cacodylate, pH 7.2, with a drop of 0.2% Tween-20 to reduce the surface tension. Following fixation, samples were rinsed with distilled water and dehydrated by passage through a graded ethanol series (once each with 25, 50, and 75%, twice with absolute ethanol). The samples were mounted, gold coated, and then imaged using a Philips/FEI LaB6 environmental scanning electron microscope (ESEM).

Biochemical analysis of Cdk1 inhibitory phosphorylation: Third instar imaginal wing discs were dissected from the appropriate genotypes and placed in 1× PBS on ice. For each sample, 10 wing discs were homogenized in SDS–PAGE sample buffer and boiled for 5 min. The proteins were separated by electrophoresis on a 10% acrylamide gel containing 2 mM vanadate and 10 mM NaF as phosphatase inhibitors, then transferred to a Hybond P membrane blot (Amersham). The blot was probed with a 1:1000 dilution of rabbit antibodies directed against pT14-Cdk1 (Cell Signaling Technology) overnight at 4°. As a loading control, the blot was reprobed with a 1/1000 dilution of antibodies against actin (Mab1501, Chemicon). The blot was stripped according to manufacturer's instructions and then reprobed with a 1:1000 dilution of rabbit antibodies directed against pY15-CdK1 (Cell Signaling Technology) overnight at 4°. Proteins were detected using anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase diluted 1:10,000 (Amersham) and a GE Healthcare ECL Plus chemiluminescence kit.

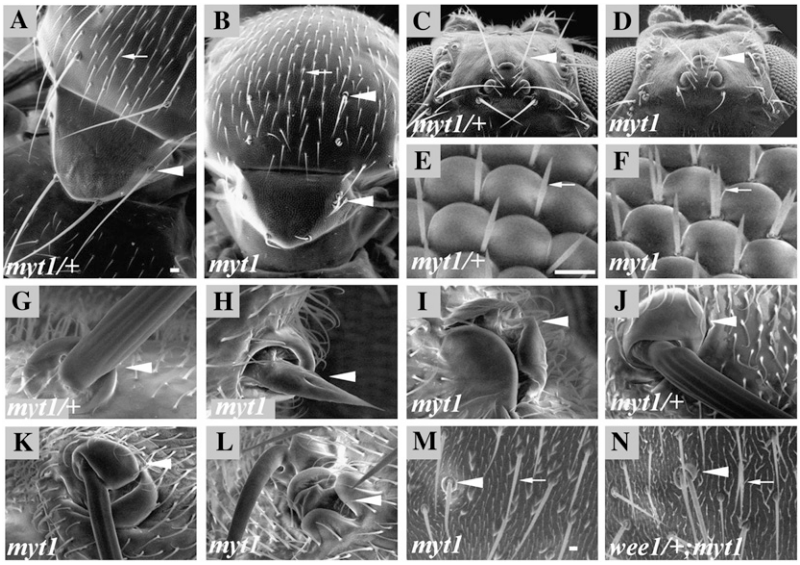

FIGURE 1.—Adult macrochaetae defects observed in *myt1* mutant adults. Scanning electron micrographs are shown of (A, C, E, G, and J) wild-type controls (*myt1*^{1}/+ heterozygotes), (B, D, F, H, I, and K–M) *myt1*^{1}/*Df(3L)64D-F* mutant or (N) *wee1*^{ES1}/*CyO*; *myt1*/*myt1*^{11} mutant adult flies. In (A, B, and G–N), arrowheads denote macrochaetae and arrows denote microchaetae. (A and B) Adult notum. (C and D) Adult head. (E and F) Interommatidial bristles (arrows) in the compound eye, showing duplicated interommatidial bristles in the *myt1* mutant. (G–I) Unlike the heterozygous controls (G), the posterior scutellar bristles of *myt1* mutants often exhibit bristles with shortened shafts (H) and/or multiple socket cells (I). (J–L) Comparison of macrochaetae on wild-type and mutant adult heads, showing that *myt1* mutants exhibit similar macrochaetae defects on the head as were seen on the notum. (M) The notum of a *myt1* mutant, shown at higher magnification to show normal appearing microchaetae. (N) The removal of one copy of *wee1* in a *myt1* mutant background resulted in frequent microchaetae duplications (white arrow). Bar for A–D, 40 μm; for E–L, 10 μm; and for M and N, 20 μm.

RESULTS

Drosophila Myt1 is required for head and thoracic macrochaetae development: In a previous study of *myt1*^{1}/*Df(3L)64D-F* hemizygous mutants that focused on male and female gametogenesis (JIN *et al.* 2005), we noted additional developmental defects affecting large sensory bristles called macrochaetae, located on the adult thorax and head. Further examination revealed similar bristle phenotypes in *Df(3L)CH39/myt1*^{1} hemizygotes, as well as recombinant, homozygous viable *myt1*^{1} mutants that were reisolated for this study (see MATERIALS AND METHODS). These *myt1* mutant macrochaetae defects were complemented by a *P{w*$^{+}$, *myt1*$^{+}$*}* genomic rescue transgene (JIN *et al.* 2005) and by expressing a *P{UASp-dMyt1*$^{+}$*}* cDNA transgene (PRICE *et al.* 2002) with a *Neu-Gal4* transgene driver line (not shown), confirming that they were due to loss of dMyt1 activity.

To further investigate the role of dMyt1 in macrochaetae development, we first classified the types and frequencies of defects observed in the thoracic macrochaetae of *myt1* mutants. These structures occupy specific locations on the head and thorax of heterozygous control adults (Figure 1, A, C, and G). The pattern and morphology of these macrochaetae was disrupted in *myt1*^{1}/*Df(3L)64D-F* hemizygotes. Similar macrochaetae defects were also seen, to similar degrees, in *myt1*^{1} homozygous viable mutants. The observed defects included bristle shafts that were shorter and thinner than normal (Figure 1, B, D, and H), bristle duplications (Figure 1B), missing macrochaetae (Figure 1, B, D, and I), and multiple socket cells (Figure 1, I and K). The *myt1* mutant adults also had duplicated eye ommatidial bristles that were not seen in controls (compare Figure 1, E and F). We also observed wing blister defects in *myt1* mutant adults (not shown). The macrochaetae located along the anterior wing margin appeared normal, however.

Since the arrangement and morphology of adult thoracic macrochaetae is highly stereotyped (NEEL 1940), we decided to quantify these morphological defects by comparing homozygous *myt1*^{1} mutants and heterozygous controls with respect to each of seven different classes of shaft and socket defects, as described in Table 1. Eight thoracic macrochaetae were scored for 25 adult flies of each sex (50 adults), representing a total of 400 macrochaetae analyzed for each genotype (the one indicated exception was due to insufficient numbers of progeny). In heterozygous *myt1*^{1}/*TM6* controls, shaft and socket defects were rarely observed (2%, $N =$ 400 macrochaetae). Homozygous viable *myt1*^{1} mutants exhibited a very high frequency of macrochaetae defects however (91%, $N = 400$), confirming that dMyt1 activity is important for normal thoracic macrochaetae development.

The only known enzymatic activity of Myt1 kinases is inhibitory phosphorylation of Cdk1 (KORNBLUTH *et al.* 1994; MUELLER *et al.* 1995; BOOHER *et al.* 1997; LIU *et al.* 1997). If the *myt1* mutant bristle phenotype was caused by a defect in Cdk1 inhibitory phosphorylation, we reasoned that a partial loss of dWee1 activity should enhance these defects. To test this hypothesis, we used *wee1* alleles that were previously isolated in our laboratory to manipulate dWee1 activity levels (PRICE *et al.* 2000). As shown in Table 1, very few macrochaetae defects were observed in *wee1*^{ES1}/+ heterozygote controls, using a representative null allele of *wee1* (<1%, $N = 400$). We also observed very few bristle or socket defects in *wee1*^{ES1}/+; *myt1*^{1}/+ double heterozygote controls (1%, $N = 400$). In *wee1*^{ES1}/*Df(2L)wee1*^{WO5},

TABLE 1

Frequency of macrochaetae defects in *myt1*, *wee1*, and double mutants

Genotype	Short shaft	2 Sha., 2 Soc.	0 Sha., 0 Soc.	0 Sha., 1 Soc.	2 Sha., 1 Soc.	1 Sha., 2 Soc.	0 Sha., 2 Soc.	Total bristles	Total defects	% defective
wee^{ES1}/CyO	0	1	0	0	0	0	0	400	1	<1
$myt1^{1}/TM6$	0	5	1	0	0	0	0	400	6	2
$wee^{ES1}/CyO;\ myt1^{1}/TM6$	0	2	0	2	0	0	0	400	4	1
$wee^{ES1}/Df(2L)wee^{WO5}$	8	11	2	9	2	1	0	700	33	8
$myt1^{1}/myt1^{1}$	251	66	31	12	3	2	0	400	365	91
$wee^{ES1}/CyO;\ myt1^{1}/myt1^{1}$	278	31	24	16	40	28	2	400	419	105[a]
$wee^{ES1}/Df;\ myt1^{1}/TM6$	4	1	0	0	0	0	0	72	5	12.5

The four dorsocentral bristles on the scutum, the two anterior scutellar bristles, and the two posterior scutellar bristles (eight total) were scored for 25 males and 25 females of each genotype. The only exception was the genotype $wee^{ES1}/DF(2L)wee^{WO5};\ myt1^{1}/TM6$, because only five females and four males of this genotype were recovered. Normal macrochaetae have a single socket (1 Soc.) and a single shaft (1 Sha.), which are consistent in length. Defects included shafts that were <75% of normal length (short shafts), complete bristle duplications (2 Sha., 2 Soc.), absent bristles (0 Sha., 0 Soc.), socket only (0 Sha., 1 Soc.), shaft duplications with single socket (2 Sha., 1 Soc.), socket duplications with a single shaft (1 Sha., 2 Soc.), and two sockets without shafts (0 Sha., 2 Soc.)

[a] Some bristles had multiple defects, resulting in an observed frequency of >100%.

however, thoracic macrochaetae defects were observed in ~8% of these flies ($N = 400$), a developmental defect that had not previously been noticed for this genotype (Price *et al.* 2000). Removal of one functional copy of *wee1* also enhanced the macrochaetae defects observed in *myt1* mutants (105%, $N = 400$). Note that the frequency of macrochaetae defects summed to >100% in this genotype because macrochaetae with more than one type of defect were often observed. Collectively, these results showed that abnormalities in Cdk1 regulation by inhibitory phosphorylation resulted in thoracic macrochaetae defects, consistent with observations from earlier studies (Milan *et al.* 1996; Lehman *et al.* 1999; Tio *et al.* 2001; Fichelson and Gho 2004).

The wing blister defects observed in *myt1* mutants were also enhanced by loss of one functional copy of *wee1*; however, we did not quantify this effect. A new type of defect was also observed in *wee1/+; myt1* mutants, involving duplication of small bristles called microchaetae. Although microchaetae duplications were rarely observed in *myt1* mutants alone (Figure 1M), they were common in homozygous *myt1* mutants that were heterozygous for either $wee1^{ES1}$ or $wee1^{DS1}$ (Figure 1N). To quantify this mutant phenotype, we analyzed the microchaetae in a region of the thorax defined by the four dorsocentral macrochaetae. A total of 20 adults of each genotype (10 males and 10 females) were analyzed in this experiment, for four classes of microchaetae defects (Table 2). Unlike *myt1* mutants alone, where only ~1% of the microchaetae were affected, ~20% of the microchaetae were duplicated in $wee1^{ES1}/+;\ myt1^{1}$ mutants. The magnitude of phenotypic enhancement seen in this experiment was therefore considerably greater than that observed for the macrochaetae (Table 1). This discrepancy could mean that microchaetae development is more sensitive to lowered Cdk1 inhibitory phosphorylation than macrochaetae development. Another possibility is that the macrochaetae defects were already so severe in *myt1* mutants that they could not be made much worse by further loss of dWee1 activity.

Drosophila Cdk1 inhibitory kinases are functionally redundant for zygotic viability: Cdk1 inhibitory phosphorylation is not only essential for normal Drosophila development; it is also essential for viability (Edgar and O'Farrell 1990; Price *et al.* 2000; Jin *et al.* 2005; Prokopenko and Chia 2005). Zygotic *wee1* and *myt1* mutants are both relatively viable, however (Price *et al.* 2000; Jin *et al.* 2005), suggesting that dWee1 and dMyt1 may be partially redundant for essential functions. Studies of fission yeast (*Schizosaccharomyces pombe*) provide a precedent for such a relationship, where loss of both Cdk1 inhibitory kinases (Wee1 and Mik1) causes lethal mitotic catastrophe due to defects in the DNA replication checkpoint (Lundgren *et al.* 1991). These redundant functions are distinct from the G2 phase Wee1-regulated cell size checkpoint for which this Cdk1 inhibitory kinase was originally named (Nurse and Thuriaux 1980). Partial functional redundancies have also been inferred for other Cdk1 inhibitory kinases, although not directly demonstrated (Wilson *et al.* 1999; Nakanishi *et al.* 2000; Lamitina and L'Hernault 2002; Leise and Mueller 2002; Okamoto *et al.* 2002).

To determine whether dWee1 and dMyt1 serve partially redundant Cdk1 regulatory functions, we quantified adult viability in different mutant genotypes. As expected, most zygotic *myt1* and *wee1* mutant progeny developed to adulthood, at only slightly lower frequencies than their heterozygous siblings (Table 3). There was a significant reduction in viability observed when *myt1* mutants were also heterozygous for *wee1*, however. These phenotypic interactions were influenced by the relative levels of dWee1 function, as the viability of *myt1* mutants that were heterozygous for a hypomorphic *wee1*

TABLE 2

Frequency of microchaetae defects in *myt1* and *wee1/+; myt1* mutants

Genotype	2 Sha., 1 Soc.	1 Sha., 2 Soc.	2 Sha., 2 Soc.	0 Sha., 1 Soc.	Total bristles	Normal bristles	Total defects	% Defective
$myt1^1/myt1^1$	2	1	0	1	367	363	4	1
$wee^{ESI}/CyO; myt1^1/myt1^1$	50	21	10	2	358	275	83	23

Microchaetae are small bristles, found on most of the adult cuticle, with a single shaft and a single socket. These are normal in $myt1^1/myt1^1$ single mutants but exhibit defects in $wee1/+; myt1^1/myt1^1$ mutants. To quantify these defects, all of the microchaetae were scored within the area bounded by the four central thoracic macrochaetae on the scutum of 10 males and 10 females of each of these genotypes. Sha., shaft; Soc., socket.

allele ($wee1^{DS1}$) was lowered by only ~5-fold, whereas *myt1* mutants that were heterozygous for a null allele [either $wee1^{ES1}$ or a chromosomal deletion, $Df(2L)wee1^{W05}$] were ~10-fold less viable. Adult *wee1; myt1* double mutants were never recovered from these genetic crosses (Table 3). Dominant genetic markers carried on the balancer chromosomes were used to identify rare *wee1; myt1* double mutant homozygotes that survived until the third instar larvae and pupal stages, suggesting that synthetic lethality occurred as maternally provided dWee1 and dMyt1 gene products were progressively depleted during zygotic development. These results indicate that dWee1 and dMyt1 are partially redundant for essential Cdk1 regulatory functions, during zygotic development.

Novel cell-cycle defects associated with deficient Cdk1 inhibitory phosphorylation: The cells that eventually develop into the thoracic macrochaetae originate from the wing discs, so we examined this tissue for earlier phenotypic defects in *myt1* mutants. We used antibodies against a phosphorylated form of histone H3 (PH3) that labels mitotic cells to examine late third instar wing discs (Hendzel *et al.* 1997; Brodsky *et al.* 2000). Using confocal microscopy, we observed normal numbers of PH3-labeled cells in discs from *yw* control larvae (Figure 2A). We consistently observed more PH3-labeled cells in the *myt1* mutant discs (Figure 2D), however. We quantified this observation by counting PH3-labeled cells in predefined areas of third instar wing discs, imaged by wide-field microscopy (see materials and methods). We compared *yw* controls (Figure 2B′), *wee1* mutant (Figure 2C′), *myt1* mutant (Figure 2E′), and *wee1; myt1* double mutant larvae (Figure 2F′). In the *yw* controls, the average (Avg.) number and standard deviation of PH3-labeled cells counted per unit area were 68 ± 10 cells/200 μM^2; $N = 7$. In *wee1*

TABLE 3

Genetic interactions between *wee1* and *myt1*

Genotypes	Observed (%)	Expected (%)	*P*-value
$myt1^1/TM6B$	565 (75)	503 (67)	
$myt1^1/myt1^1$	189 (25)	251 (33)	**
	754 (100)	754 (100)	$P < 0.001$
$wee1^{ESI}$ or $Df(2L)wee1^{W05}/CyO$	380 (76)	333 (67)	
$wee1^{ESI}/Df(2L)wee1^{W05}$	119 (24)	166 (33)	**
	449 (100)	499 (100)	$P < 0.001$
$wee1^{DSI}/CyO; myt1^1/TM6B$	375 (63)	263 (44)	
$wee1^{DSI}/wee1^{DSI}; myt1^1/TM6B$	187 (31)	132 (22)	
$wee1^{DSI}/CyO; myt1^1/myt1^1$	30 (5)	131 (22)	
$wee1^{DSI}/wee1^{DSI}; myt1^1/myt1^1$	0 (0)	66 (11)	**
	592 (100)	592 (100)	$P < 0.001$
$wee1^{ESI}$ or $Df(2L)wee1^{W05}/CyO; myt1^1/TM6B$	549 (67)	361 (44)	
$wee1^{ESI}/Df(2L)wee1^{W05}; myt1^1/TM6B$	251 (30)	181 (22)	
$wee1^{ESI}/CyO; myt1^1/myt1^1$	13 (2)	181 (22)	
$wee1^{ESI}/Df(2L)wee1^{W05}; myt1^1/myt1^1$	0 (0)	90 (11)	**
	813 (100)	813 (100)	$P < 0.001$

Genetic crosses were set up to generate the indicated progeny genotype as a means of investigating genetic interactions between *wee1* and *myt1*. Σ is the sum of individuals from all genotypes in an experiment. The indicated *P*-values represent the probability that the observed distribution of genotypes is not significantly different from the expected values, according to Pearson's chi-square test. **Highly significant difference.

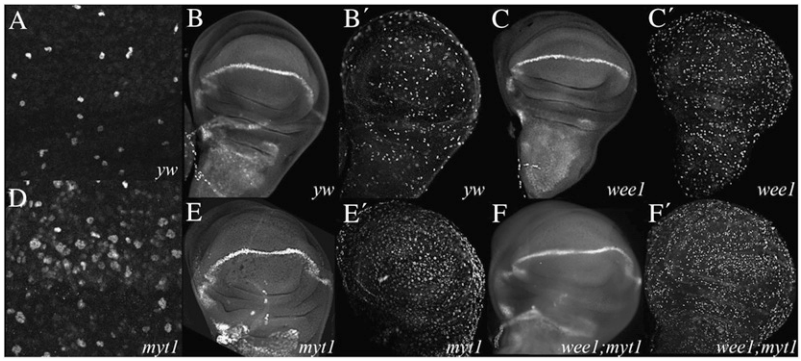

FIGURE 2.—*myt1* mutants have increased incidence of mitosis in imaginal wing discs. Wing discs from wandering third instar larvae were fixed and stained with rabbit anti-phospho-histone 3 (PH3) antibodies (A, B′, C′, D, E′, and F′) and mouse anti-Cut antibodies (B, C, E, and F). Sections A and D show differences in PH3 staining between wild-type *yw* controls (A) and homozygous viable $myt1^{1}$ mutants (D), using confocal microscopy. In wide-field images, Cut and PH3 labeling were shown for *yw* controls (B and B′), for $wee1^{ES1}/Df(2L)wee^{W05}$ mutants (C and C′), for homozygous viable $myt1^{1}/myt1^{1}$ mutants (E and E′), and for $wee1^{ES1}/wee1^{DS1}$; $myt1^{1}/myt1^{1}$ mutants (F and F′).

mutant wing discs we observed slightly more PH3-labeled cells than in the controls (Avg. = 108 ± 23 cells/200 μM^2; $N = 6$). A notable increase in numbers of PH3-labeled cells was observed in *myt1* mutants (Avg. = 179 ± 26 cells/200 μM^2; $N = 5$) and *wee1; myt1* double mutants (Avg. = 210 ± 31 cells/200 μM^2; $N = 3$). These results suggested that loss of dMyt1 activity caused a novel cell-cycle defect in third instar imaginal wing discs.

We also noticed that the chromatin appeared to be relatively less condensed in many of the *myt1* mutant PH3-labeled cells, compared with controls (compare Figure 2, A and D). This observation suggests that loss of dMyt1 activity caused defects in chromatin condensation or decondensation during mitosis, suggesting problems in a mechanism for coupling chromatin condensation with progression through mitosis.

Imaginal wing disc cells proliferate asynchronously during larval development until the late third instar, when the cells that will form the presumptive wing margin withdraw from the cell cycle and begin to differentiate (O'BROCHTA and BRYANT 1985). Some of these cells can be identified by expression of the Cut homeodomain protein (JACK and DELOTTO 1992; BLAIR 1993). To determine if loss of dMyt1 activity affected these cells, we examined Cut expression in third instar wing discs by immunolabeling. In *yw* controls, Cut antibody labeling marked the presumptive wing margin, as expected (Figure 2B). We observed similar results in *wee1* mutants (Figure 2C), in *myt1* mutants (Figure 2E), and in *wee1; myt1* double mutants (Figure 2F). Thus, it appears that Cdk1 inhibitory phosphorylation is not required for differentiation of Cut expressing cells in the presumptive wing margin.

Increased levels of apoptosis in *myt1* mutant wing discs: Tissue homeostasis is partly regulated by mechanisms that balance excessive cell proliferation with elevated apoptotic cell death (NEUFELD *et al.* 1998; ABRAMS 2002). Since our PH3 labeling experiments indicated there was a novel cell-cycle defect in *myt1* mutants, we investigated whether apoptosis might also be affected in these mutants. To label apoptotic cells we used antibodies against the active, cleaved form of caspase-3 (YU *et al.* 2002), comparing *yw* controls (Figure 3A), *wee1* mutants (Figure 3B), *myt1* mutants (Figure 3C), and *wee1; myt1* double mutants (Figure 3D). In the *yw* controls, apoptotic cells were rarely observed. Loss of Myt1 (and to a lesser extent, dWee1) was associated with increased apoptosis, however. We quantified this mutant phenotype by analyzing predefined regions of late third instar imaginal wing discs (MATERIALS AND METHODS). The results are presented as the average area labeled by the activated caspase-3 antibodies ± SD, expressed as a percentage of the total area. As expected, very low levels of apoptosis were observed in *yw* control imaginal wing discs (Avg. = 0.21 ± 0.90% of total area; $N = 4$). There was a small increase in the area of cells undergoing apoptosis in *wee1* mutant wing discs (Avg. = 1.09 ± 0.44% of total area; $N = 7$), relative to controls. In *myt1* wing discs, there were considerably more apoptotic cells, relative to the controls (Avg. = 6.09 ± 2.77% of total area; $N = 5$). This cellular defect was even more apparent in *wee1; myt1* double mutant wing discs (Avg. =12.55 ± 6.02% of total area; $N = 5$). Thus, we have identified novel cellular defects associated with deficiencies in Cdk1 regulation by inhibitory phosphorylation. Whether these cellular defects are relevant to the morphological defects observed in adults, remains unclear.

dMyt1 activity is required for normal responses to ionizing radiation: Conserved cell-cycle checkpoint responses promote Cdk1 inhibitory phosphorylation to prevent mitosis when cells sense ongoing DNA replication or repair of damaged DNA (O'CONNELL *et al.* 1997; RHIND and RUSSELL 2001). Although previous studies had implicated dWee1 in a DNA replication checkpoint response that is required for early embryonic development (PRICE *et al.* 2000), a role for dWee1 or dMyt1 in the DNA damage checkpoint had not yet been reported. To address this issue, we used PH3 antibody labeling to assay premitotic checkpoint responses in larvae that had been exposed to ionizing radiation (BRODSKY *et al.* 2000). When *yw* control larvae were exposed to 40 Gy of ionizing radiation (IR), we observed that PH3-labeled cells had nearly disappeared by 1 hour after exposure (compare Figure 4, A and D).

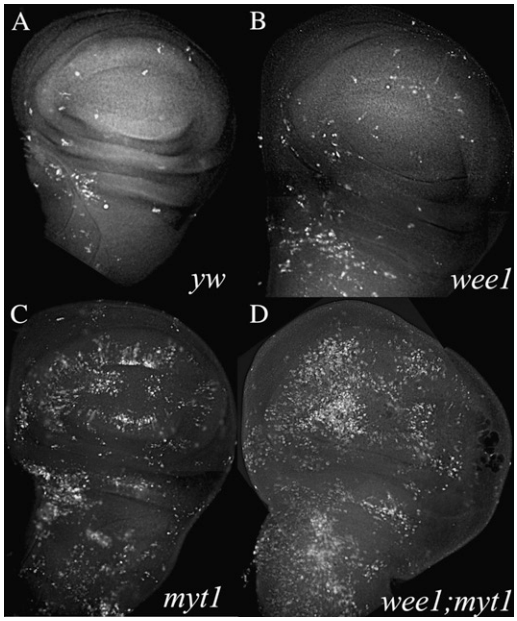

FIGURE 3.—*myt1* mutants exhibit increased incidence of apoptosis in imaginal wing discs. Shown are wing discs from wandering third instar larvae that were immunolabeled with antibodies against activated caspase-3 as a marker for apoptosis (YU *et al.* 2002). Relatively few apoptotic cells were observed in *yw* controls (A) or in $wee1^{ES1}/Df(2L)wee^{WO5}$ (B) mutants. There was a noticeable increase in apoptotic cells in $myt1^{1}/myt1^{1}$ (C) and $wee1^{ES1}/wee1^{DS1}$; $myt1^{1}/myt1^{1}$ double mutants (D).

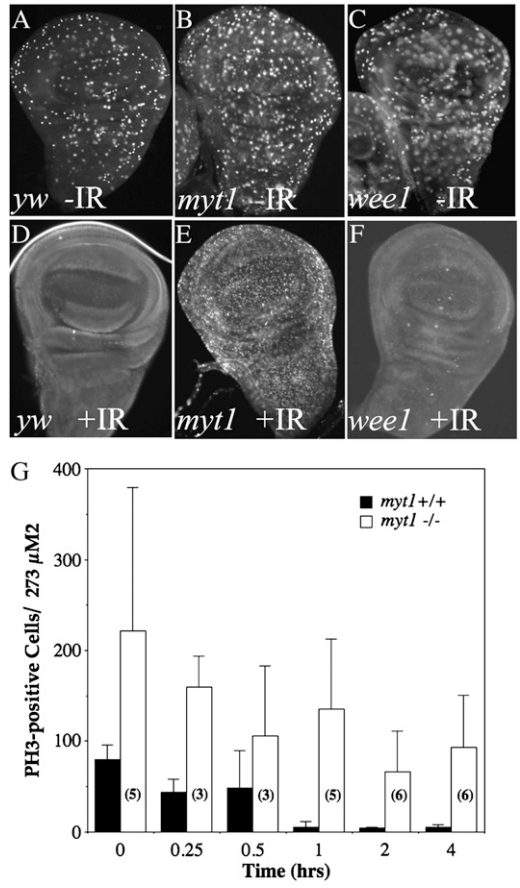

FIGURE 4.—*myt1* mutants are defective for normal cellular responses to ionizing radiation. Wing imaginal discs were dissected from wandering third instar larvae that had been immunolabeled with antibodies against phospho-histone H3 as a marker for mitotic cells. Comparisons of unirradiated and irradiated wing discs are shown for each of the following genotypes: *yw* controls (A and D), $myt1^{1}/myt1^{1}$ mutants (B and E), and $wee1^{ES1}/Df(2L)wee^{WO5}$ mutants (C and F). The irradiated (IR) larvae were dissected 60 min after exposure to 40 Gy of ionizing radiation, in D–F. PH3-positive cells were seen in all of the unirradiated controls: *yw* (A), homozygous viable $myt1^{1}$ mutants (B), and *wee1* mutant discs (C). There were almost no PH3-positive cells remaining in *yw* control discs or in *wee1* mutant discs by 60 min after exposure to IR (D and F), indicating the presence of a functional premitotic checkpoint in these genotypes. In *myt1* mutant discs (E) the PH3 antibody labeling persisted after IR exposure, suggesting a checkpoint defect. To quantify this defect, we counted the numbers of PH3-positive cells in *yw* control and *myt1* mutant wing discs at intervals after exposure to ionizing radiation (G). The numbers (3–6) shown for each open bar indicate the number of control and mutant discs that were analyzed for each time point, with error bars indicating standard deviation.

These results demonstrated that premitotic checkpoint responses were active in the controls, as expected. We also observed similar results in *wee1* mutant wing discs, indicating that dWee1 activity is not required for the premitotic checkpoint response to ionizing radiation (compare Figure 4, C and F). In contrast, when *myt1* mutant wing discs were examined there was only a slight reduction in the numbers of PH3-labeled cells by 1 hour after IR exposure, relative to unirradiated *myt1* mutant controls (Figure 4, B and E).

To quantify this apparent DNA damage-response defect, we counted PH3-labeled cells to compare *myt1* mutant and control imaginal discs at timed intervals after the larvae were exposed to ionizing radiation. There was a marked reduction in PH3-labeled cells in the irradiated *yw* control wing discs by 15 min after exposure, with a further decline in the numbers of PH3-labeled cells observed over time (Figure 4G). These results indicated that the premitotic checkpoint was fully engaged by 1 hr after IR exposure and remained so for several hours after irradiation, in the control wing discs (Figure 4G).

We observed a variable but greater than twofold average increase in PH3-labeled cells in the unirradiated *myt1* mutant wing discs (relative to unirradiated *yw* control discs), consistent with results presented earlier. Although there was a modest reduction in numbers of PH3 positive cells shortly after exposure to ionizing radiation (Figure 4G), labeled cells persisted in the *myt1* mutant wing discs for several hours after irradiation, long after they had disappeared in the *yw* controls. These results indicate that dMyt1 is important for normal cellular responses to ionizing radiation.

Cdk1 inhibitory phosphorylation is impaired in *myt1* mutant wing discs: Biochemical studies of Myt1 kinases have shown that they are capable of phosphorylating both the T14 and Y15 inhibitory residues of Cdk1 (MUELLER *et al.* 1995; BOOHER *et al.* 1997; LIU *et al.*

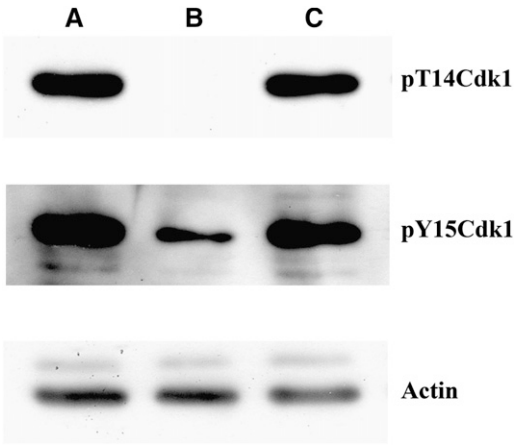

FIGURE 5.—Cdk1 inhibitory phosphorylation defects in *myt1* mutant wing discs. Protein extract samples were each prepared from 10 dissected wing discs and then separated by SDS–PAGE, then probed serially with antibodies specific to the pT14-Cdk1 isoform, Actin (as a loading control), and the pY15-Cdk1 isoform (after stripping). (A) The *yw* wild-type control extract was immunolabeled for both the pT14-Cdk1 and the pY15-Cdk1 isoforms, as expected. (B) In a $myt1^{1}/myt1^{1}$ mutant extract, the pT14-Cdk1 isoform was completely absent and the pY15-Cdk1 isoform was markedly reduced. (C) In a $wee1^{ES1}/Df(2L)wee^{WO5}$ mutant extract, Cdk1 inhibitory phosphorylation appeared normal for both isoforms.

1997). In contrast, Wee1 kinases appear to phosphorylate Cdk1 exclusively on the Y15 site (PARKER *et al.* 1995). Drosophila dWee1 also functions as a Y15-specific Cdk1 inhibitory kinase, *in vitro* (CAMPBELL *et al.* 1995) and early embryos (PRICE *et al.* 2000; STUMPFF *et al.* 2004).

To determine how loss of dWee1 or dMyt1 affected Cdk1 inhibitory phosphorylation in third instar imaginal wing discs, we used phospho-specific antibodies that specifically recognize T14- and Y15-phosphorylated Cdk1 isoforms to assay whole protein extracts on Western blots (see MATERIALS AND METHODS). The *yw* control extracts showed both T14- and Y15-phosphorylated Cdk1 isoforms, demonstrating that we could detect phosphorylation of each of the two Cdk1 inhibitory sites (Figure 5, lane A). In the *myt1* mutant wing disc protein extracts (Figure 5, lane B), phosphorylation of Cdk1 on the T14 residue was undetectable in the *myt1* mutant samples, even when the protein blots were overloaded and overexposed. This result demonstrated that dMyt1 is the only Cdk1 inhibitory kinase capable of phosphorylating the T14 residue in Drosophila. Phosphorylation of the Cdk1-Y15 site was also substantially reduced in the *myt1* mutants relative to the controls (Figure 5, lane B), with the remaining Y15 phosphoisoform that was observed presumably representing dWee1 activity. The simplest interpretation of these results is that dMyt1 functions as a dual specificity Cdk1 inhibitory kinase and is largely responsible for regulating Cdk1 at this stage of development.

In the *wee1* mutant wing disc protein extract samples, Cdk1 inhibitory phosphorylation was indistinguishable from the controls (Figure 5, lane C). These biochemical results were therefore consistent with phenotypic data described earlier suggesting that dWee1 was active, but largely dispensable, unless dMyt1 was absent. Collectively, these results indicate that dMyt1 is the major Cdk1 inhibitory kinase in third instar wing discs.

DISCUSSION

Multicellular organisms regulate Cdk1 by inhibitory phosphorylation to prevent mitosis when DNA is being replicated or repaired (POON *et al.* 1997) and to ensure that mitosis does not interfere with critical developmental processes that require remodeling of the cytoskeleton (EDGAR and O'FARRELL 1990; GROSSHANS and WIESCHAUS 2000; MATA *et al.* 2000; SEHER and LEPTIN 2000; MURAKAMI *et al.* 2004). Previous studies of Drosophila Wee1 and Myt1 revealed that these conserved Cdk1 inhibitory kinases were required during early embryogenesis and gametogenesis, respectively (PRICE *et al.* 2000; STUMPFF *et al.* 2004; JIN *et al.* 2005). We have now characterized imaginal and adult developmental defects caused by loss of dMyt1 activity (and to a much lesser extent, dWee1), that confirm the importance of Cdk1 inhibitory phosphorylation for coordinating cell-cycle events with critical developmental processes.

In Drosophila and other organisms, G2/M delays can be induced by overexpression of Myt1 kinases, suggesting a specific role for Myt1 in regulating this stage of the cell cycle (BOOHER *et al.* 1997; LIU *et al.* 1999; CORNWELL *et al.* 2002; LAMITINA and L'HERNAULT 2002; PRICE *et al.* 2002). Further evidence of a role for Myt1 in G2/M regulation comes from studies of oocyte maturation in frogs, starfish, and nematodes (PALMER *et al.* 1998; OKUMURA *et al.* 2002; PETER *et al.* 2002; BURROWS *et al.* 2006). Not all data indicate that Myt1 is required for G2 phase arrest, however, and there is no evidence that dMyt1 regulates oocyte maturation in Drosophila (IVANOVSKA *et al.* 2004; JIN *et al.* 2005). Nor is there evidence that dMyt1 activity is responsible for the timing of the G2/M meiotic transition that follows a prolonged 4-day-long G2 phase arrest, in Drosophila primary spermatocytes (D. GUHA MAJUMDAR, unpublished results). Moreover, a recent study showed that functional depletion of human Myt1 by siRNA did not affect the proportion of cells in G2 phase, but instead affected membrane dynamics during mitotic exit (NAKAJIMA *et al.* 2008). More clearly needs to be learned about Myt1 mediated regulatory mechanisms before these apparent discrepancies in Myt1 functions are resolved.

Previous work showed that Cdk1 inhibitory phosphorylation is required for proper development of thoracic mechanosensory organs (MILAN *et al.* 1996;

Lehman *et al.* 1999; Tio *et al.* 2001; Fichelson and Gho 2004). We have now identified dMyt1 as the primary Cdk1 inhibitory kinase for this developmental program. Several molecular mechanisms could explain the role of dMyt1 in mechanosensory bristle development. One obvious possibility is that *myt1* mutant sensory organ precursor (SOP) cells and their descendants might divide prematurely due to a defect in G2/M regulation, resulting in aberrant segregation of cell fate determinants. If there was a relatively narrow window for coordinating specific developmental events with the G2/M transition, disrupting this regulatory mechanism could account for the observed loss and duplication of bristles and socket cells in *myt1* mutants. Live analysis of mechanosensory organ development could test this possibility (Fichelson and Gho 2004).

Alternatively, *myt1* mutant phenotypes could reflect defects in Myt1-mediated regulatory mechanisms that are important for the control of intracellular membrane dynamics during mitosis, particularly the Golgi apparatus and endoplasmic reticulum (Cornwell *et al.* 2002; Nakajima *et al.* 2008). The Drosophila Golgi apparatus undergoes significant morphological changes that have been linked to specific developmental states and so the observed *myt1* mutant developmental defects might reflect problems in the structure or function of this organelle (Kondylis *et al.* 2001). Further support for this idea comes from a recent study showing that asymmetrical segregation of mouse Numb (a conserved cell fate determinant) requires the Golgi apparatus, leading the authors to suggest that Golgi fragmentation and reconstitution could represent a mechanism for coupling cell-fate specification and cell-cycle progression (Zhou *et al.* 2007).

Another possible explanation for *myt1* mutant defects concerns the large quantities of actin that are synthesized and packaged to form the large mechanosensory bristle shafts (Wulfkuhle *et al.* 1998). This process involves extensive reorganization of the endoplasmic reticulum and Golgi apparatus to accommodate increased membrane trafficking (Tilney and Derosier 2005; Lee and Cooley 2007). Defects in the structure or function of the Golgi apparatus and ER caused by loss of dMyt1 activity could therefore account for defects or diminution in these bristles. Resolving which of these potential mechanisms best explain the role of dMyt1 during mechanosensory organ development will be a major challenge of our future research.

We also observed intriguing cell-cycle defects (higher mitotic index, aberrant chromatin condensation, and ectopic apoptosis), as well as defects in responses to ionizing radiation in proliferating cells in *myt1* mutant imaginal wing discs. These observations suggest an important role for dMyt1 in conserved cell-cycle checkpoint responses that target Cdk1 by inhibitory phosphorylation (O'connell *et al.* 1997; Poon *et al.* 1997; Rhind *et al.* 1997; Niida and Nakanishi 2006). We had not anticipated that dMyt1 would serve such functions, since Wee1 kinases are generally assumed to be responsible for checkpoint responses that protect the nucleus from premature Cdk1 activity (Heald *et al.* 1993). It was not clear that *myt1* mutants were deficient in conventional premitotic checkpoint responses, however. Indeed, the partial decline in *myt1* mutant PH3-labeled cells observed immediately after exposure to ionizing radiation could reflect activation of an otherwise dispensable Wee1-regulated premitotic checkpoint mechanism. The remaining PH3-positive cells that persisted long after irradiation in *myt1* mutant discs could be arrested in mitosis by an alternative regulatory mechanism that was responsive to DNA damage (Sanchez *et al.* 1999; Royou *et al.* 2005; Kim and Burke 2008; Musaro *et al.* 2008). Further studies will be needed to clarify the respective roles of dMyt1 and dWee1 in cellular responses to DNA damage.

We also observed profound defects in Cdk1 inhibitory phosphorylation in *myt1* mutant imaginal discs. Phosphorylation of the T14 residue of Cdk1 was eliminated, demonstrating that dMyt1 is solely responsible for this regulatory modification, like Myt1 homologs described in other organisms (Mueller *et al.* 1995; Booher *et al.* 1997; Liu *et al.* 1997). We also observed that phosphorylation of the Y15 residue of Cdk1 was markedly reduced in *myt1* mutant extracts, demonstrating for the first time that dMyt1 functions as a dual specificity Cdk1 inhibitory kinase, *in vivo*. Why dWee1 activity is insufficient for maintaining normal levels of phosphorylation of the Y15 residue is not clear, since Cdk1 complexes are thought to shuttle between the nucleus and cytoplasm (Hagting *et al.* 1998; Wells *et al.* 1999; Yang *et al.* 2001). One possible explanation is that the doubly phosphorylated Cdk1 isoform may be more refractory to dephosphorylation by Cdc25 phosphatases, and hence more stably inhibited, than Cdk1 phosphorylated on a single residue (Liu *et al.* 1997). Another possibility is that the kinase-independent Myt1 mechanism proposed to tether phospho-inhibited Cdk1 complexes in the cytoplasm until cells are ready for mitosis might also protect them from dephosphorylation (Mueller *et al.* 1995; Liu *et al.* 1999; Wells *et al.* 1999). Loss of either of these regulatory mechanisms could therefore underlie the cell-cycle defects observed in *myt1* mutants. Testing these hypotheses promises to yield interesting new insights into cell-cycle regulation and the diverse developmental roles of dMyt1 and similar regulatory kinases in other organisms.

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