Dual Phosphorylation of Cdk1 Coordinates Cell Proliferation with Key Developmental Processes in *Drosophila*

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ABSTRACT Eukaryotic organisms use conserved checkpoint mechanisms that regulate Cdk1 by inhibitory phosphorylation to prevent mitosis from interfering with DNA replication or repair. In metazoans, this checkpoint mechanism is also used for coordinating mitosis with dynamic developmental processes. Inhibitory phosphorylation of Cdk1 is catalyzed by Wee1 kinases that phosphorylate tyrosine 15 (Y15) and dual-specificity Myt1 kinases found only in metazoans that phosphorylate Y15 and the adjacent threonine (T14) residue. Despite partially redundant roles in Cdk1 inhibitory phosphorylation, Wee1 and Myt1 serve specialized developmental functions that are not well understood. Here, we expressed wild-type and phospho-acceptor mutant Cdk1 proteins to investigate how biochemical differences in Cdk1 inhibitory phosphorylation influence *Drosophila* imaginal development. Phosphorylation of Cdk1 on Y15 appeared to be crucial for developmental and DNA damage-induced G2-phase checkpoint arrest, consistent with other evidence that Myt1 is the major Y15-directed Cdk1 inhibitory kinase at this stage of development. Expression of non-inhibitable Cdk1 also caused chromosome defects in larval neuroblasts that were not observed with Cdk1(Y15F) mutant proteins that were phosphorylated on T14, implicating Myt1 in a novel mechanism promoting genome stability. Collectively, these results suggest that dual inhibitory phosphorylation of Cdk1 by Myt1 serves at least two functions during development. Phosphorylation of Y15 is essential for the premitotic checkpoint mechanism, whereas T14 phosphorylation facilitates accumulation of dually inhibited Cdk1–Cyclin B complexes that can be rapidly activated once checkpoint-arrested G2-phase cells are ready for mitosis.

CONSERVED cell-cycle checkpoints delay entry into mitosis by inhibiting Cyclin-dependent kinase 1 (Cdk1), blocking cell-cycle progression when DNA replication or repair would threaten cell survival (Nurse 1990). In *Schizosaccharomyces pombe*, where the Wee1 inhibitory kinases were first discovered, phosphorylation of Cdk1 on a conserved tyrosine residue (Y15) is both necessary and sufficient for checkpoint arrest of cells in S or G2 phase until DNA replication and repair are completed and cells reach a critical size (Gould and Nurse 1989; O'Connell *et al.* 1997; Rhind and Russell 1998). Myt1 kinases are Wee1-related but metazoan-

specific Cdk1 inhibitory kinases (Mueller *et al.* 1995; Booher *et al.* 1997; Liu *et al.* 1997). Myt1 kinases regulate Cdk1 by dual inhibitory phosphorylation of Y15 and the adjacent threonine residue, T14 (Gu *et al.* 1992; Blasina *et al.* 1997; Poon *et al.* 1997). They have also been implicated in Cdk1/Cyclin B nucleo-cytoplasmic trafficking mechanisms coordinating the G2/M transition (Liu *et al.* 1999; Wells *et al.* 1999; Gavet and Pines 2010). These complexities have made it difficult to assign specific molecular functions to Wee1 and Myt1 kinases (Okamoto *et al.* 2002; Burrows *et al.* 2006; Oh *et al.* 2010).

During interphase, Cdk1 bound to mitotic cyclins can be detected in four distinct states with respect to inhibitory phosphorylation: T14-Y15, T14p-Y15, T14-Y15p, and T14p-Y15p (Edgar *et al.* 1994; Mayya *et al.* 2006; Coulonval *et al.* 2011). During *Drosophila* gastrulation, expression of Cdc25^{Stg} dual-specificity phosphatases removes Cdk1 inhibitory phosphorylation to activate Cdk1 in a dynamic developmental G2/M

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checkpoint mechanism used to coordinate mitosis with cell movements (Edgar and O'Farrell 1989, 1990). The expression of non-inhibitable Cdk1(T14A,Y15F) mutants (also called Cdk1AF) forces cells to bypass G2-phase checkpoint arrest by triggering auto-amplification of feedback mechanisms that activate endogenous Cdk1 (Krek and Nigg 1991a; Norbury *et al.* 1991; Jin *et al.* 1996; Su *et al.* 1998). In contrast, phospho-mimetic substitutions of Cdk1 on T14 and/or Y15 produce dominant negative, kinase-dead mutants that block cells in interphase, indicating that negative charges at either position inhibited Cdk1 activity (Krek *et al.* 1992). However, other studies of singly phosphorylated Cdk1 isoforms suggest that Y15 phosphorylation more potently inhibits Cdk1 activity than T14 phosphorylation (Fletcher *et al.* 2002; Potapova *et al.* 2009) or that T14 phosphorylation promotes T161-activating phosphorylation of Cdk1/Cyclin B complexes by Cdk1-activating kinase (CAK) kinases, perhaps because complexes phosphorylated only on Y15 are unstable during G2 phase (Coulonval *et al.* 2011). These findings suggest that biochemical differences in Wee1 and Myt1 phosphorylation mechanisms could impart distinct functional properties important for Cdk1 regulation at different stages of development.

Indeed, genetic studies in *Drosophila* have defined specialized developmental roles for Wee1 and Myt1 kinases, despite partial functional redundancy. Maternally expressed Wee1 is essential for checkpoint responses that delay mitosis to accommodate late-firing DNA replication origins and control chromosome condensation after DNA damage during the syncytial divisions of early embryogenesis (Price *et al.* 2000; Stumpff *et al.* 2004; Shermoen *et al.* 2010; Fasulo *et al.* 2012). Although zygotic Wee1 activity is dispensable for post-embryonic development, it is functionally redundant for viability when Myt1 becomes the major biochemically detectable Cdk1 inhibitory kinase, and *myt1* mutants are male-sterile with sensory bristle defects (Jin *et al.* 2005, 2008). Understanding the biochemically distinct mechanisms that Wee1 and Myt1 use to regulate Cdk1 by inhibitory phosphorylation could help us to understand specialized functions of these conserved cell-cycle kinases.

To study how biochemical differences in Cdk1 inhibitory phosphorylation affect *Drosophila* development, we expressed fluorescently tagged Cdk1 proteins to analyze phenotypic consequences during larval imaginal development. Expression of tagged wild-type Cdk1 functionally complemented conditional loss-of-function *cdc2* (*cdk1*) mutants. The fusion proteins interacted with endogenous mitotic cyclin A and B and were phosphorylated by endogenous inhibitory and activating kinases. Expression of tagged, non-inhibitable Cdk1 caused G2/M checkpoint bypass and chromosome defects, as expected. Distinct phenotypic effects were associated with expression of mutant versions of Cdk1 that could be phosphorylated only on T14 or Y15 residues, however. While other properties could also be a factor in these Cdk1 mutant proteins, our results provide new insights into developmental regulation of Cdk1 by inhibitory phosphorylation that may also be relevant to other systems.

Materials and Methods

Construction of Gal-4-inducible Cdk1-Venus fluorescent protein strains

Constructs were made from pSP64 plasmids carrying *Cdk1*^{WT}, *Cdk1*(T14A), *Cdk1*(Y15F), and *Cdk1*(T14A,Y15F) sequences that were amplified using high-fidelity Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) with the following primers: LPE 192 (5'-CACCATGGAGGATTTGAGAA-3') and RPE 192 (5'-ATTTCGAAGTAAGCCCGATTG-3'). The amplified DNA was subcloned into a pENTR directional TOPO vector (Invitrogen) according to the manufacturer's protocol with One Shot chemically competent *Escherichia coli* TOP10 cells (Invitrogen). Gateway recombination of the pENTR clones with the *Drosophila* destination vector pPWV (a UASp vector with 14 Gal4 UAS sites and a C-terminal Venus tag) was then used to generate Gal4-inducible, C-terminal, Venus fluorescent protein (VFP)-tagged expression constructs (Brand and Perrimon 1993). Each construct was verified by sequencing on both strands before being used for P-element transformation (Best Gene), with strains showing similar expression chosen for the experiments (further details available on request).

Analysis of wing imaginal disc development and checkpoint responses to radiation

Imaginal wing discs were dissected in phosphate-buffered saline (PBS) from larvae cultured at 22° on standard *Drosophila* media supplemented with 0.05% bromophenol. Late third instar larvae were selected by disappearance of bromophenol blue from the gut and initiation of wandering behavior. The discs were fixed for 15 min in 4% paraformaldehyde at room temperature, washed three times for 5 min each in PBT (1× PBS and 0.3% Triton X-100), and blocked for 30 min with 5% bovine serum albumin in 1× PBT before incubation with primary antibodies overnight at 4°. Antibodies were used at the following dilutions: rabbit anti-phosphohistone H3 (Upstate Biotechnology), 1:4000; rabbit anti-cleaved Caspase-3 (Cell Signaling Technology), 1:1000; mouse anti-Cyclin A, 1: 200; mouse anti-Cut, 1:200; and mouse anti-Wingless, 1:10 (all from Developmental Studies Hybridoma Bank). Secondary antibodies (Invitrogen) conjugated with Alexa Fluor-488 or Alexa Fluor-568 were used at a working dilution of 1:1000. Figure 4, Figure 5, and Figure 6 were composed from overlapping images to show the entire imaginal wing disc, deconvolved by iterative restoration using Volocity software, and then compiled with Adobe Photoshop software using identical manipulations for each experimental set.

Radiation-induced premitotic checkpoint assays were performed using established protocols (Brodsky *et al.* 2000). Briefly, staged late third instar larvae were irradiated with 40 Gy using a Co⁶⁰ gamma-ray source, and then allowed to recover for 1 hr at room temperature before dissecting, fixing, and labeling the wing discs with phospho-(S10)-histone H3 antibodies to mark mitotic cells.

Scanning electron microscopy of adult *Drosophila* eyes

Pharate adults were dissected from their pupal cases and fixed for 2 hr with 2% glutaraldehyde in PBS (adding a drop of 0.2% Tween-20 to reduce surface tension). Following fixation, the samples were rinsed with distilled water, dehydrated by passage through a graded ethanol series (once each for 30 min: 25, 50, and 75%, followed by twice with 100% ethanol), and mounted for imaging with a Philips/FEI LaB6 environmental scanning electron microscope.

Western blot analysis of protein extracts

Wing imaginal discs were dissected from late third instar larvae and kept on ice. Ten wing discs per sample were homogenized in SDS-PAGE sample buffer and the protein extracts were separated by 10% SDS-PAGE and transferred to Hybond P membranes (Amersham). After blocking with 5% bovine serum albumin in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) for 1 hr, the blots were incubated with primary antibodies overnight at 4°. Labeled proteins were then detected with anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Amersham), using a GE Healthcare ECL Plus chemiluminescence kit. The primary antibodies used for these experiments were rabbit anti-pT14-Cdk1 (1:500), pY15-Cdk1 (1:1000), rabbit pT161-Cdk1 (1:1000) (all from Cell Signaling Technology), mouse anti-GFP (1:5000; Clontech), and mouse anti-actin (1:5000; Chemicon).

Immunoprecipitation of transgenic Cdk1 protein and histone H1 kinase assays

Thirty to 35 wing discs from larvae expressing VFP-tagged Cdk1 transgenes under control of *engrailed-Gal4* were dissected for each sample in ice-cold PBS and stored at -80° until proceeding. After thawing, the tissue was homogenized in 200 μ l of ice-cold lysis buffer containing phosphatase and protease inhibitors (20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 2 mM EGTA, 0.1% NP-40, 1 mM benzamidine, 2 mM sodium orthovanadate, 80 mM glycerophosphate, 2.5 mM β -mercaptoethanol, 10 mM NaF, 2 mM PMSF, 10 mM pepstatin A, 0.5 μ g/ml leupeptin, and 2.5 mg/ml aprotinin). Lysates were kept at 4° throughout the remainder of the experiment. Cellular debris was pelleted by centrifugation at 14,000 $\times$ g for 15 min, and the supernatant was removed and precleared by incubation with 20 μ l of a 50% slurry of lysis buffer and protein A beads for 1 hr, followed by centrifugation at 3000 $\times$ g for 5 min. The precleared lysates were then incubated with 0.5 μ l of rabbit anti-GFP for 1 hr at 4°, and the GFP-fused proteins were immunoprecipitated by adding 20 μ l of a 50% slurry of protein A beads and incubated at 4° overnight. The precipitated proteins, the remaining supernatant (adsorbed) fractions, and aliquots of the initial precleared lysates were then analyzed on Western blots by probing with mouse anti-Cyclin B and Cyclin A antibodies (1:1 dilution) from the Developmental Studies Hybridoma Bank (Lehner and O'Farrell 1990) and then with horseradish peroxidase-coupled

secondary antibodies (Sigma) and ECL chemiluminescence to detect the labeled proteins.

For histone H1 kinase assays, immunoprecipitates were prepared as above except that 180 dissected third instar wing discs were used for each genotype. Precipitates were washed three times with lysis buffer and twice with H1 kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂) before incubation in a reaction mixture containing kinase buffer supplemented with 10 μ M unlabeled ATP, 1 mg/ml histone H1, and 30 μ Ci of γ -³²P-labeled ATP for 15 min at 30°. The reactions were quenched by addition of 1 vol of 2 $\times$ SDS loading buffer and incubated for 5 min at 100°. Reaction products were then separated on 10% SDS-PAGE gels, and the phosphorylated histone H1 was detected and quantified by scanning the dried gels with a Molecular Dynamics STORM Phosphorimager. To estimate the amount of transgenic protein in each sample, 20- μ l aliquots of each precipitate were Western-blotted and probed with anti-Cdk1 (PSTAIR) antibodies (diluted 1:5000) and then quantified using IMAGEJ software. Catalytic activity was determined by normalizing the H1 kinase activity of each sample to the amount of protein and shown relative to Cdk1(WT) activity set as 100%. Data from three independent experiments were compiled for Figure 1D.

Analysis of mitosis in larval neuroblasts

To study larval neuroblasts, we dissected third instar larvae in PBS to remove eye-antennal discs from the brains, which were incubated for 1.5 hr in 10⁻⁵ M colchicine at room temperature. This was followed by a 10-min incubation in hypotonic solution (0.5% sodium citrate), incubation for 2 min in 45% acetic acid, and incubation for 20 s in 60% acetic acid before squashing hard between a polylysine-coated slide and siliconized coverslip. The slides were then frozen immediately in liquid nitrogen before removing the coverslip, dehydrated in absolute ethanol at room temperature, and then left to air-dry before rehydration, staining with Hoechst 33258, and mounting in antifade glycerol-based medium. The slides were then examined for metaphase karyotypes to look for chromosome aberrations and to quantify the mitotic index by counting the number of cells with condensed mitotic chromosomes per microscopic field (the area observed with a $\times$ 40 Zeiss objective). To account for experimental variation, 10–20 fields were sampled from each of four brains per slide, with three slides per genotype examined.

Using an α -Tubulin-RFP transgene (Goshima *et al.* 2007), live analysis of mitosis was performed using published protocols to visualize VFP expressed from each of the Cdk1 transgenes and microtubules (Buffin *et al.* 2005). Data for fluorescent time-lapse movies were acquired with an inverted microscope (IX81; Olympus; $\times$ 60, numerical aperture 1.42, oil objective) equipped with a spinning-disc confocal head (CSU10; Yokogawa). Image capture with a CCD camera (ORCA-R2; Hamamatsu Photonics) was controlled by MetaMorph software (Molecular Devices). Stacks of five sections at 0.5- μ m intervals were collected every 30 sec. Images showing maximum intensity projections were adjusted for brightness and contrast,

Figure 1 Biochemical analysis of transgenic Cdk1 fusion proteins expressed in wing discs with *en-Gal*. In A and C, each sample was extracted from 10 late third instar wing discs. (A) Western blot of wing disc extracts probed with PSTAIRE antibodies (conserved Cdk1 epitope) detected both endogenous (34 kDa) and transgenic fusion (61 kDa) proteins, except in the nontransgenic yw control. Adjusting for exposure times, we estimated that levels of the transgenic Cdk1 proteins were approximately fourfold lower than endogenous Cdk1 in this experiment. (B) Western blot of transgenic Cdk1 that was immunoprecipitated from wing disc lysates (30–35 discs per sample) using rabbit anti-GFP. The blot was sequentially probed with mouse anti-GFP, *Drosophila* Cyclin B, and Cyclin A. Note that Cyclin A runs as a doublet (Lehner and O'Farrell 1990). (C) Western blot of wing-disc extracts with the top section (proteins above 48 kDa) probed sequentially with phospho-specific antibodies against the pT14-Cdk1, GFP, pY15-Cdk1, and pT161-Cdk1 phospho-isoforms, stripping between each reprobing. The bottom part of the blot containing 34-kDa endogenous Cdk1 was probed with PSTAIRE antibodies as a loading control. (D) Compilation of data from three independent experiments for each genotype that measured histone H1

kinase activity of Cdk1 fusion proteins immunoprecipitated from wing-disc extracts with anti-GFP antibodies (180 discs for each sample). We determined kinase activity for each Cdk1 variant by normalizing the H1 kinase activity per unit of total Cdk1 protein, estimated by probing Western blots of aliquots of the immunoprecipitates with anti-PSTAIRE for each experiment. Relative percentage kinase activity was plotted with respect to Cdk1(WT) activity, which was set as 100%. Error bars show standard deviation calculated from data from three experimental replicates for each genotype.

with mitosis defined as the interval between the appearance of centrosomes at opposite poles of the dividing cell to the end of cytokinesis.

Results

Functional characterization of Cdk1-VFP proteins expressed in wing imaginal discs

To test the possibility that specialized developmental roles of Wee1 and Myt1 kinases reflect different functional properties of Cdk1 proteins phosphorylated on T14 and/or Y15 residues, we made four Gal4-inducible transgenes for comparing the behavior of different phospho-isoforms *in vivo*: Cdk1 (WT)-VFP, Cdk1(T14A)-VFP, Cdk1(Y15F)-VFP, and Cdk1 (T14A,Y15F)-VFP. The fusion proteins were C-terminally tagged with VFP, allowing us to visualize protein expression. To characterize basic biochemical properties of these Cdk1 fusion proteins in a tissue where both Wee1 and Myt1 are expressed (Jin *et al.* 2008), we used genetic crosses to *en-grailed-Gal4* (*en-Gal4*) to induce transgene expression in the posterior regions of late third instar wing imaginal discs. Western blots of protein extracts (30–35 wing discs per sample) probed with anti-Cdk1 (PSTAIR) antibodies detected both endogenous Cdk1 (34 kD) and Cdk1-VFP (61 kDa) fusion proteins (Figure 1A), as expected. To determine if these Cdk1-VFP fusion proteins interacted with endogenous mitotic cyclins, we first optimized conditions for quantitatively immunoprecipitating Cdk1(WT)-VFP from wing disc extracts (Supporting Information, Figure S1A) using a non-transgenic yw strain as a negative control (Figure S1B). Using this protocol, Western blots of the immunoprecipitated Cdk1-

VFP proteins were then sequentially probed with mouse anti-*Drosophila* Cyclin B, Cyclin A, and mouse anti-GFP antibodies (Figure 1B). The results showed that Cyclin A and Cyclin B proteins coprecipitated with each of the transgenic Cdk1 fusion proteins, confirming that VFP-tagged Cdk1 proteins could physically interact with endogenous mitotic cyclins.

When Cdk1 proteins are bound to mitotic cyclins, they are subject to inhibitory phosphorylation by Wee1 and Myt1 kinases (Meijer *et al.* 1991) and activating phosphorylation on residue T161 by a Cdk-activating kinase, CAK (Solomon *et al.* 1992). We induced transgenic Cdk1 proteins in wing discs with *en-Gal4* to analyze phosphorylation status by sequentially probing Western blots with antibodies against phospho-T14-Cdk1 (pT14), phospho-Y15-Cdk1 (pY15), and phospho-T161-Cdk1 (pT161). All of the antibodies labeled Cdk1WT-VFP (Figure 1C) and detecting pT14, pY15, and pT161 Cdk1 phospho-isoforms showed that the proteins were recognized as substrates by endogenous Cdk1 inhibitory kinases. Cdk1(Y15F)-VFP was labeled only by pT14-Cdk1 antibodies whereas Cdk1(T14A)-VFP was labeled only by pY15-Cdk1 antibodies, confirming that the T14 and Y15 residues of transgenic Cdk1 were in fact mutated and that the remaining sites were independently phosphorylated by endogenous kinases. Cdk1(T14A-Y15F)-VFP was not recognized by antibodies against either pT14 or pY15, as expected.

Antibodies used to detect Cdk1 phosphorylation on the T161-activating residue labeled the Cdk1(WT)-VFP, Cdk1 (Y15F)-VFP, and Cdk1(T14A-Y15F)-VFP fusion proteins (Figure 1C). Curiously, these antibodies did not detect T161 phosphorylation of the Cdk1(T14A)-VFP sample. Similar results were observed when the Cdk1 transgenes were expressed in other tissues (data not shown), consistent with a proposal

that T14Y15p Cdk1 phospho-isoforms are compromised for T161 phosphorylation (Coulonval *et al.* 2011), although we cannot exclude alternative explanations.

To assess Cdk1 catalytic activity, the VFP-tagged fusion proteins were immunoprecipitated from wing disc extracts with GFP antibodies for histone H1 kinase assays, using 180 wing discs for each sample (Figure 1D and Figure S1B). The incorporation of radioactive phosphate into histone H1 was quantified by densitometry after normalizing each sample for protein concentration, setting Cdk1(WT)-VFP activity as 100%. Mock immunoprecipitates from a nontransgenic *yw* strain used as a negative control exhibited negligible background levels of histone H1 kinase activity (Figure S1B). Pooled data compiled from three independent experiments were used to prepare Figure 1D, which indicates that the relative levels of Cdk1WT-VFP and Cdk1(T14A)-VFP kinase activity were indistinguishable. In contrast, the Cdk1(Y15F)-VFP precipitates had roughly twofold higher activity than Cdk1WT-VFP whereas Cdk1(T14A,Y15F)-VFP precipitates had roughly threefold higher activity (Figure 1D).

The simplest interpretation of these results is that phosphorylation of Cdk1(Y15F)-VFP on T14 partially inhibits Cdk1 catalytic activity, as reported in other systems (Krek and Nigg 1991b; Fletcher *et al.* 2002), although we recognize that the Y15F mutation might also influence Cdk1 activity by affecting protein folding, stability, or interactions with other proteins. Interpreting the Cdk1(T14A)-VFP results was more difficult, however. The low activity of this mutant can be attributed to Y15 inhibitory phosphorylation and low levels of activating T161 phosphorylation (Figure 1C), in addition to other functional properties of the T14A mutant that could potentially be affecting Cdk1 activity.

To rigorously assay the functionality of the Cdk1 fusion proteins *in vivo*, we undertook genetic tests for complementation of *cdc2^{ts}* (*cdk1*) mutant lethality (Stern *et al.* 1993). Genetic crosses to *tubulin-Gal4* and *actin-Gal4* strains were used to ubiquitously express each transgene in a *cdc2^{E1-24}/cdc2^{B47}* mutant background that was temperature-sensitive for Cdk1 activity. The progeny were grown at 25° throughout development (the restrictive temperature for *cdc2^{E1-24}*). Adult progeny were then analyzed for phenotypic markers carried on the balancer chromosomes to score genotypes (Figure 2 and Figure S1, C–E). Cdk1(WT)-VFP expression completely rescued *cdc2* pupal lethality (Figure 2, B, B', and D; class A in Figure S1E). The rescued adults were morphologically indistinguishable from sibling heterozygous controls (Figure 2, A, A' and D; class D in Figure S1E), and Western blots (Figure S1, C and D) confirmed that the fusion proteins were highly expressed in both *cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1WT-VFP/tubulin-Gal4* and *cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1WT-VFP/actin-Gal4* flies. Surprisingly, ubiquitous expression of transgenic Cdk1(T14A)-VFP also rescued *cdc2* mutant lethality (Figure 2, C, C' and D; class A in Figure S1E), showing that the Cdk1 activity produced by this transgene was sufficient for complementation despite low T161 phosphorylation noted earlier (Figure 1C). In both cases, the percentage of rescued

progeny was not significantly different from the maximum expected for full genetic complementation (Figure 2D).

In contrast, expression of Cdk1(Y15F)-VFP or Cdk1(T14A,Y15F)-VFP did not rescue *cdc2* pupal lethality (class A, Figure S1E). Moreover, ubiquitous expression of either of these transgenes was lethal in an otherwise heterozygous *cdc2^{ts}/+* background (class C in Figure S1E). These results showing that ubiquitous expression of Cdk1 transgenic mutants that could not be phosphorylated on Y15 were dominant-lethal implies that regulation of this residue might be critical for normal *Drosophila* development.

Impact of Cdk1 inhibitory phosphorylation mutants on adult eye and wing development

Expression of non-inhibitable Cdk1 in *Drosophila* in an otherwise wild-type background induces G2-phase cells to prematurely enter mitosis, disrupting the developmental timing of cell division (Sprenger *et al.* 1997; Su *et al.* 1998; Jin *et al.* 2005). To assess whether Cdk1 phosphorylated only on T14 or Y15 residues caused similar defects, we used genetic crosses with *sd-Gal4* to induce expression during wing disc development in an otherwise wild-type genetic background (Campbell *et al.* 1992). The examples shown in Figure 3 were representative of at least 100 adult flies analyzed for each genotype. Wing development was not notably affected by expression of Cdk1(WT)-VFP, Cdk1(T14A)-VFP, or Cdk1(Y15F)-VFP (Figure 3, A–C); however, Cdk1(T14A,Y15F)-VFP expression caused wing-margin defects (Figure 3D).

We also analyzed transgene expression during eye-antennal disc development, using *ey-Gal4* (Hazelett *et al.* 1998). Expression of Cdk1(WT)-VFP, Cdk1(T14A)-VFP, or Cdk1(Y15F)-VFP resulted in adults with normal eyes (Figure 3, E–G); however, Cdk1(T14A,Y15F)-VFP expression produced eyeless, pharate adult flies that were unable to eclose (Figure 3H). These phenotypic defects induced by expression of non-inhibitable Cdk1 mutants that were not seen with singly phosphorylated Cdk1 isoforms suggested that phosphorylation of Cdk1 on either T14 or Y15 was sufficient for regulating transgene activity *in vivo*, although other factors in addition to phosphorylation could also be involved.

Expression of non-inhibitable Cdk1 mutants causes dominant phenotypic defects that can be enhanced by increasing levels of mitotic cyclins (Heald *et al.* 1993; Jin *et al.* 1998; Su *et al.* 1998). To assess this variable we co-expressed transgenic *Drosophila* Cyclin B with each Cdk1 variant by genetic crosses to *sd-Gal4*. Expression of Cyclin B alone (Figure 3I) had no detectable effect on the adult wing, nor did Cyclin B co-expressed with Cdk1WT-VFP (Figure 3J) or Cdk1(T14A)-VFP (Figure 3K). Wing-margin defects were observed, however, when Cyclin B was co-expressed with Cdk1(Y15F)-VFP (compare Figure 3C with Figure 3L). Co-expression of Cyclin B with Cdk1(T14A,Y15F)-VFP enhanced the severity of phenotypic defects compared to the mutant alone, resulting in complete loss of the adult wing (compare Figure 3D with Figure 3M). These results indicate that both Cdk1(T14A,Y15F)-VFP and Cdk1(Y15F)-VFP compete with endogenous

Figure 2 Temperature-sensitive *cdc2* (*cdk1*) mutants are rescued by ubiquitous expression of *Cdk1WT* and *Cdk1(T14A)*, but not by *Cdk1(Y15F)* or *Cdk1(T14AY15F)*. (A and A') Dorsal and side views of wild-type control flies, respectively. (B and B') Flies ubiquitously expressing the *Cdk1WT-VFP* transgene with *tubulin-Gal4* in temperature-sensitive *cdc2* mutant background (*cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1WT-VFP/tubulin-Gal4*). (C and C') Adult flies ubiquitously expressing the *Cdk1(T14A)-VFP* transgene with *tubulin-Gal4* (*cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1(T14A)-VFP/Gal4*). (D) Summary of progeny data tabulated in Figure S1E, showing the percentage of rescued flies relative to the expected maximum percentage rescue.

Cdk1 for mitotic cyclins as a limiting factor for Cdk1 activity. Our finding that co-expression of cyclin B with Cdk1(T14A, Y15F)-VFP caused more severe wing and eye defects than with Cdk1(Y15F)-VFP suggested that phosphorylation of Cdk1 (Y15F)-VFP on T14 partially inhibited Cdk1 activity *in vivo*, although other factors may also contribute.

To further test this hypothesis that T14 phosphorylation partially inhibited the activity of Cdk1 *in vivo* and prevented phenotypic defects, we expressed Cdk1(Y15F)-VFP in a *myt1* null mutant background by genetic crosses with *en-Gal4* and *neur-Gal4* (Jin *et al.* 2005). Previous analysis of these *myt1* mutants showed that Myt1 activity accounts for all detectable T14 phosphorylation of endogenous Cdk1 in *Drosophila* (Jin *et al.* 2008). We therefore predicted that loss of endogenous T14 kinase activity in a *myt1* mutant would mean that Cdk1(Y15F)-VFP expression would affect cells more like Cdk1(T14A,Y15F)-VFP if T14 phosphorylation alone partially inhibits transgene activity. In *myt1/+* heterozygote controls, adult progeny were observed with *en-Gal4* or *neur^{P72}-Gal4* expression of Cdk1(Y15F)-VFP (data not shown). These genotypes were not observed in a *myt1* mutant background, however, indicating that Cdk1(Y15F)-VFP expression was lethal. These results showed that Myt1 phosphorylation of transgenic Cdk1(Y15F)-VFP on T14 was responsible

for partially inhibiting Cdk1 activity *in vivo*, consistent with the *in vitro* H1 kinase measurements shown in Figure 1D.

***Cdk1(Y15F)* and *Cdk1(T14A,Y15F)* expression causes mitotic index and apoptosis defects**

Drosophila wing discs maintain tissue homeostasis by compensatory mechanisms controlling the balance of cell proliferation and apoptosis (Reis and Edgar 2004; Davidson and Duronio 2012). We examined wing discs for cell proliferation and ectopic apoptosis defects using *en-Gal4* to specifically express transgenes in the posterior compartment of third instar wing discs that were then probed with antibodies against activated cleaved-Caspase3 to label apoptotic cells (Jackman *et al.* 2002) or phosphorylated histone-H3 on S10 (PH3) to mark mitotic cells (Hendzel *et al.* 1997; Brodsky *et al.* 2000). Few apoptotic cells were observed in either compartment of discs expressing Cdk1WT-VFP or Cdk1(T14A)-VFP (Figure 4, A, E, B, and F; *n* = 10). In contrast, many apoptotic cells were observed specifically in the posterior regions of discs expressing Cdk1(Y15F)-VFP (Figure 4, C and G; *n* = 10) or Cdk1 (T14A,Y15F)-VFP (Figure 4, D and H; *n* = 10). Wing discs expressing Cdk1(T14A,Y15F)-VFP always appeared smaller than normal and abnormally shaped relative to the other genotypes, as shown (Figure 4, D and H). Expression of either

Figure 3 Phenotypic effects of expression of Cdk1 transgenes during adult wing and eye development are enhanced by co-expression of Cyclin B using *sd-Gal4* and *ey-Gal4*, respectively, with ~500 adults examined for each genotype. (A–C) Progeny expressing Cdk1(WT), Cdk1(T14A), or Cdk1(Y15F) in an otherwise wild-type background that have no defects in adult wing morphology. (D) Cdk1(T14A,Y15F) expression caused wing-margin defects. (E–G) Scanning electron micrographs of adult eyes from progeny expressing Cdk1(WT), Cdk1(T14A), or Cdk1(Y15F) that did not affect adult eye development. (H) Cdk1(T14A,Y15F) expression severely affected adult eye and head structures and caused pharate adult lethality ($n = 200$). (I–K) Expression of Cyclin B alone, co-expression of Cyclin B with Cdk1(WT), or co-expression of Cyclin B with Cdk1(T14A) had no detectable effect on adult wings. (L) Co-expression of Cdk1(Y15F) with Cyclin B caused extensive loss of adult wing margin. (M) Co-expression of Cdk1(T14A,Y15F) with Cyclin B resulted in complete loss of the adult wing.

Cdk1 transgene that could not be phosphorylated on the Y15 residue therefore promoted ectopic apoptosis.

Examination of PH3-labeled cells in wing discs expressing Cdk1WT-VFP (Figure 4, I and M; $n = 15$) or Cdk1(T14A)-VFP (Figure 4, J and N; $n = 15$) showed similar numbers of mitotic cells in the anterior and posterior (VFP-positive) compartments. In contrast, there were markedly more PH3-labeled cells in the posterior compartment of discs expressing Cdk1(Y15F)-VFP (Figure 4, K and O; $n = 15$) or Cdk1(T14A,Y15F)-VFP (Figure 4, L and P; $n = 9$). To quantify this phenotype, we obtained a (P/A: posterior/anterior) mitotic index ratio using deconvolved images to count PH3-labeled cells in the posterior (VFP-positive) and anterior (PH3-negative) compartments of these wing discs ($n = 7$ per genotype). The box plot data shown in Figure S2 confirmed that the P/A ratio of wing discs expressing Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F)-VFP was markedly higher than with the other two transgenes. Collectively, these results showed that Cdk1 transgenes that could not be phosphorylated on Y15 had similar effects on wing disc mitotic index and induction of apoptosis, even though expression of these transgenes had quite different ultimate effects on adult morphological development.

Expression of Cdk1(Y15F) alone can bypass developmentally regulated G2 phase arrest

Cdk1 activation by Cdc25^{stg}-catalyzed removal of dual inhibitory phosphorylation is an essential mechanism used for

coordinating mitosis with key developmental processes in *Drosophila* (Edgar and O'Farrell 1990; Fichelson and Gho 2004). Since our data suggested that Y15 inhibitory phosphorylation of Cdk1 was particularly important for this regulatory mechanism, we examined the zone of nonproliferating cells (ZNC) in late third instar wing discs, where cells of the presumptive dorsal-ventral wing margin undergo developmentally programmed cell-cycle arrest (O'Brochta and Bryant 1985). The ZNC is a tripartite structure consisting of a central row of G1-arrested cells flanked by G2-arrested cells (Johnston and Edgar 1998). We used antibody labeling to examine the wing-margin patterning proteins Cut or Wingless (Wg) when Cdk1 variants were expressed using genetic crosses with *sd-Gal4* (Jack *et al.* 1991; Williams *et al.* 1994). Expression of Cdk1WT-VFP resulted in discs with normal Cut expression at the presumptive wing margin (Figure 5, A and A'; $n = 10$), as did expression of Cdk1(T14A)-VFP (Figure 5, B and B', $n = 10$) or Cdk1(Y15F)-VFP (Figure 5, C and C', $n = 10$). Expression of Cdk1(T14A,Y15F)-VFP, however, resulted in reduced Cut labeling (Figure 5, D and D', $n = 10$) of discs that were smaller than normal and abnormally shaped, as noted earlier with *en-Gal4* (Figure 4). We observed similar results from analyzing Wingless, which was disrupted only by Cdk1(T14A,Y15F)-VFP expression (Figure S3).

To specifically assay developmental G2-phase arrest, we used genetic crosses with a *neur*^{p72} enhancer trap Gal4 insertion to express each transgene specifically in sensory organ precursor

Figure 4 Expression of Cdk1(Y15F) and Cdk1(T14A,Y15F) caused increased apoptosis and mitotic index in late third instar larval wing discs. VFP-tagged Cdk1 constructs (green label in E–H and M–P) were expressed with *en-Gal4* in the posterior compartment, and the discs were labeled with antibodies against activated Caspase-3 (red in E–H) as an apoptosis marker or antibodies against phosphorylated (S10) histone H3 (PH3; red in M, N, O, and P) as a mitotic marker. The VFP-negative anterior compartment serves as an internal control. (A, B, E, and F) Few apoptotic cells were observed in discs expressing Cdk1(WT) or Cdk1(T14A) ($n = 10$, each genotype). (C, D, G, and H) Expression of Cdk1(Y15F) or Cdk1(T14A,Y15F) resulted in elevated numbers of apoptotic cells in the posterior region of wing discs ($n = 10$). (I, J, M, and N) No notable differences in PH3-labeled cells from expression of Cdk1(WT) or Cdk1(T14A) in wing discs ($n = 15$, each genotype). (K, L, O, and P) More PH3-labeled cells were observed in posterior regions of Cdk1(Y15F)- and Cdk1(T14A,Y15F)-expressing discs ($n = 9$, each genotype). Wing discs expressing Cdk1(T14A,Y15F) were always smaller and morphologically abnormal relative to the other genotypes, as shown. Bar in A, 50 μm .

(SOP) cells of the ZNC (Fichelson and Gho 2004). VFP-positive SOP cells expressing either Cdk1(WT)-VFP (Figure 5, E and E', $n = 8$) or Cdk1(T14A)-VFP (Figure 5, F and F', $n = 8$) were not labeled by the PH3 antibodies that detect mitotic cells, consistent with these cells being arrested in G2 phase. In contrast, many of the SOP cells expressing Cdk1(Y15F)-VFP (Figure 5, G and G', $n = 8$) or Cdk1(T14A,Y15F)-VFP (Figure 5, H and H', $n = 7$) were PH3-positive, indicating that these cells had already initiated mitosis. These results indicated that Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F)-VFP were equally effective at bypassing the G2/M developmental checkpoint mechanism in SOP cells, even though only Cdk1(T14A,Y15F)-VFP expression caused adult wing defects. Although other factors may also contribute to differences between these mutants, the simplest interpretation is that Y15

phosphorylation is specifically required for the developmental G2-phase arrest checkpoint mechanism, and partial inhibition of Cdk1 by T14 phosphorylation prevented more serious defects.

Expression of Cdk1(Y15F) can bypass DNA damage checkpoint responses

Exposure to ionizing radiation causes DNA damage, eliciting interphase checkpoint responses that promote Cdk1 inhibitory phosphorylation to block cell-cycle progression and allow time for DNA repair before mitosis. In *S. pombe*, phosphorylation of Cdk1 on Y15 is both necessary and sufficient for the DNA damage checkpoint (O'Connell *et al.* 1997; Rhind *et al.* 1997). In human cells, T14 and Y15 residues of Cdk1 are both phosphorylated in response to DNA damage (Blasina

Figure 5 Expression of Cdk1 transgenes during wing development with *sd-Gal4* differentially affects wing-margin pattern and developmental regulation of G2-phase arrest. At least 10 labeled discs were examined for each genotype. (A–C) Wing discs from third instar larvae labeled with anti-Cut antibodies (red). Cut expression at the presumptive wing margin was unaffected by expression of Cdk1(WT), Cdk1(T14A), or Cdk1(Y15F), respectively. (D) Expression of Cdk1(T14A,Y15F) disrupted Cut expression. (A'–D') VFP-tagged transgenes were expressed throughout the wing pouch of each disc. (E–H) Wing discs from third instar larvae expressing transgenes driven by *neur⁷²-Gal4* in SOP cells (a subset of G2-phase-arrested ZNC cells) that were labeled with antibodies against phosphorylated (S10) histone H3 (PH3; red). (E and F) SOP cells expressing Cdk1(WT)-VFP and Cdk1(T14A) were PH3-negative. (G and H) SOP cells expressing Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F) were a mixture of smaller mitotic (PH3-positive) and nonmitotic cells. (E'–H') VFP-tagged transgenes were expressed in SOP cells.

et al. 1997), and, in *Drosophila*, Myt1 is required for the DNA damage checkpoint response in larval wing discs (Jin *et al.* 2008). Whether this involves Myt1 dual phosphorylation of Cdk1 or a specific role for T14 or Y15 inhibitory phosphorylation is not known, however. To investigate this issue, we expressed transgenic Cdk1 in the posterior compartment of wing discs using *en-Gal4* to examine DNA damage checkpoint responses caused by exposure to 40-Gy ionizing radiation (Brodsky *et al.* 2000). One hour after exposure, wing discs were dissected, fixed, and immunolabeled with PH3 antibodies. Mitotic (PH3-positive) cells were not observed in either anterior or posterior (VFP-positive) compartments of irradiated discs expressing Cdk1WT-VFP (Figure 6, A and A', *n* = 10) or Cdk1(T14A)-VFP (Figure 6, B and B', *n* = 10), demonstrating a functional checkpoint response. In contrast, many PH3-positive cells were observed specifically in the posterior compartment of discs from irradiated Cdk1(Y15F)-VFP (Figure 6, C and C', *n* = 10) and Cdk1(T14A,Y15F)-VFP larvae (Figure 6, D and D', *n* = 10). The simplest interpretation of these results is that phosphorylation of Cdk1 on Y15 is spe-

cifically required for premitotic checkpoint responses to DNA damage in late third instar wing discs, although other factors may also contribute.

Cdk1(T14AY15F) causes chromosome defects and mitotic delays, but Cdk1(Y15F) does not

The severe developmental outcomes associated with expression of Cdk1(T14AY15F) could reflect defects in mitotic progression, as reported recently for HeLa cells (Santos *et al.* 2012). To examine this possibility, we analyzed mitotic cells using *prospero-Gal4* to express the Cdk1 transgenes in type I larval brain neuroblasts. These are large neural stem cells with well-characterized metaphase checkpoint mechanisms (Basto *et al.* 2000; Donaldson *et al.* 2001; Royou *et al.* 2005). We first treated squashed brain preparations with colchicine, a microtubule poison used to arrest cells in mitosis, and then stained for DNA to quantify the mitotic index (Figure 7A). In larval brains expressing Cdk1WT-VFP or Cdk1(T14A)-VFP, we observed 10–20 mitotic cells per field. Cdk1(Y15F)-VFP expression resulted in a much higher mitotic

Figure 6 Expression of Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F) in wing discs causes dominant bypass of DNA-damage-induced G2/M checkpoint responses. VFP-tagged Cdk1 transgenes (green) were expressed with *en-Gal4* in the posterior compartment of each wing disc. Wing discs were dissected from late third instar larvae 60 min after exposure to 40 Gy of ionizing radiation and labeled with antibodies against phosphorylated (S10) histone H3 (PH3; white in A–D, red in A'–D') to mark mitotic cells. At least 10 labeled discs were examined for each genotype. (A, A', B, and B') No PH3-positive cells were in either compartment of wing discs expressing Cdk1(WT) or Cdk1(T14A), indicating a functional premitotic checkpoint response. (C, C', D, and D') Wing discs expressing Cdk1(Y15F) or Cdk1(T14A,Y15F), where PH3 labeling in the posterior compartment indicated a G2/M checkpoint defect. Bar in A, 50 μ m.

index, however, ranging from 50 to 80 mitotic cells per field. In contrast, the mitotic index of brains expressing Cdk1(T14A,Y15F)-VFP was much lower, ranging from 5 to 10 mitotic cells per field.

Cells use conserved metaphase checkpoint mechanisms for monitoring chromosome integrity and spindle attachment prior to anaphase that can be assayed by karyotype analysis of colchicine-treated neuroblasts (Hardwick *et al.* 1996). This results in cells arresting in a metaphase-like state, with paired sister chromatids (Gonzalez *et al.* 1988; Gatti and Baker 1989). Colchicine-treated neuroblasts expressing Cdk1(WT)-VFP or Cdk1(T14A)-VFP (Figure 7, B and C) had paired sister chromatids in 99.5% of the karyotypes, demonstrating a functional metaphase checkpoint response. Most neuroblasts expressing Cdk1(Y15F)-VFP (89.5%) also had karyotypes with paired sister chromatids (Figure 7D); however, in some cells the sister chromatids appeared to have lost cohesion (9.5%) (see Figure 7E).

Strikingly, almost half of colchicine-treated neuroblasts expressing Cdk1(T14A,Y15F)-VFP exhibited gross chromosome aberrations, including polyploidy (4%) (Figure 7F), abnormally thin, entangled chromosomes (5%) (Figure 7G), and chromosome breaks (35%) (Figure 7H). Since chromosomal aberrations are associated with genome instability and apoptosis, these defects could underlie the severe consequences of non-inhibitable Cdk1 expression that were not observed with Cdk1(Y15F)-VFP.

We also analyzed mitosis in live neuroblasts to further investigate the mitotic index differences described earlier. In particular, we wondered if differences in mitotic index between expression of Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F)-VFP

(Figure 7A) might be due to effects on mitotic progression causing an accumulation or deficit of mitotic cells, respectively. To test this idea, we co-expressed each of the Cdk1-VFP variants with *Tubulin-RFP* by genetic crosses with *prospero-Gal4* and used both fluorescent reporters for live imaging of mitosis, defined as the interval between appearance of centrosomes at opposite poles to the completion of cytokinesis (movement of VFP into the nucleus can also be observed at prophase).

Mitosis took $\sim 12.6 \pm 1.2$ min for Cdk1(WT)-VFP-expressing neuroblasts ($n = 5$) (Figure 8, A and E; [File S1](#)). Neuroblasts expressing Cdk1(T14A)-VFP or Cdk1(Y15F)-VFP showed similar results, with mitosis lasting 13.5 ± 1.3 min ($n = 5$) (Figure 8, B and E; [File S2](#)) and 13.0 ± 0.7 min, respectively ($n = 5$) (Figure 8, C and E; [File S3](#)). These results showed that the higher mitotic index associated with Cdk1(Y15F)-VFP expression was not caused by delays in M phase. Instead, these cells may undergo a faster cell cycle due to premature mitosis resulting from bypass of developmentally regulated G2-phase arrest, as observed with SOP cells of the presumptive wing margin (Figure 5).

Mitosis was much longer in Cdk1(T14A,Y15F)-VFP-expressing neuroblasts than with any of the other transgenes, and these cells were difficult to find (28.5 ± 2.3 min, $n = 3$) (Figure 8, D and E; [File S4](#)). These mitotic delays can be reconciled with the lower mitotic index that was also associated with Cdk1(T14A,Y15F)-VFP expression if there were simply fewer neuroblasts in these larval brains, perhaps because ectopic apoptosis had already removed many of the cells from this lineage earlier in development. Collectively, then, the mitotic defects, chromosome aberrations, and severe developmental defects caused by Cdk1(T14A,

Figure 7 Expression of Cdk1(Y15F)-VFP and Cdk1(T14A, Y15F) with *prospero-Gal4* differentially affects mitotic index measurements in type 1 larval neuroblasts; however, only Cdk1(T14A,Y15F) produced gross chromosome defects. Metaphase karyotypes of colchicine-treated brain squashes were labeled with Hoechst 33258 to identify mitotic chromosomes. At least 800 interpretable karyotypes were examined for each genotype. (A) Bar chart showing mitotic index associated with each Cdk1 transgene. (B and C) Neuroblasts expressing Cdk1(WT) or Cdk1(T14A) have chromosomes arrested in metaphase with cohered sister chromatids. (D) Approximately 90% of Cdk1(Y15F)-expressing mitotic neuroblasts also arrest in metaphase with cohered sister chromatids; however, defects in sister-chromatid cohesion were evident in 9.5% of the karyotypes (E, $n = 900$). Approximately 45% of Cdk1(T14A,Y15F)-expressing neuroblasts exhibited some form of chromosomal aberration ($n = 850$), including poly-ploidy (G); thin, poorly condensed chromosomes (H); or chromosome breaks (arrow).

Y15F)-VFP expression (but not Cdk1(Y15F)-VFP) provide strong evidence that inhibitory phosphorylation of T14 has a significant role in regulating Cdk1 activity *in vivo*.

Discussion

Premitotic checkpoint mechanisms coordinate mitosis with fundamental processes of metazoan development by controlling the activity of the master mitotic regulator Cdk1 via inhibitory phosphorylation. In this study we analyzed how differences in T14 and Y15 inhibitory phosphorylation of Cdk1 affected these premitotic checkpoint mechanisms by expressing transgenic Cdk1 phospho-acceptor mutants during *Drosophila* development. The Cdk1(Y15F) and Cdk1(T14AY15F) mutants could not be phosphorylated on Y15, and both were equally effective at dominantly bypassing both developmental and radiation-induced G2/M checkpoint arrest in wing discs. These results indicated that Y15 phosphorylation of Cdk1 was essential for G2/M checkpoint arrest, although other properties could also conceivably be affected in these mutants. Our interpretation is also consistent with genetic and biochemical data showing that Myt1 is the predominant Y15-directed Cdk1 kinase controlling premitotic checkpoint responses in wing discs, even though both Wee1 and Myt1 phosphorylate Cdk1 on Y15 and are functionally redundant for viability during post-embryonic development (Jin *et al.* 2008).

Although expression of Cdk1(Y15F) or Cdk1(T14A,Y15F) appeared equally effective at bypassing G2/M checkpoint arrest, only Cdk1(T14A,Y15F) caused profound developmental

abnormalities associated with chromosome aberrations and mitotic defects in larval neuroblasts. Although these mutants may differ in other respects in addition to their phosphorylation states, the simplest interpretation is that partial inhibition of Cdk1 activity by Myt1-mediated T14 phosphorylation serves a checkpoint-independent function that promotes cell survival. Studies of cultured mammalian cells suggest several possible mechanisms. Expression of non-inhibitable Cdk1 causes mis-regulation of the APC/C with defects in mitotic exit (Chow *et al.* 2011) and G1 phase (Ma *et al.* 2012). Myt1 has been implicated in checkpoint mechanisms used for coordinating Golgi and endoplasmic reticulum structural dynamics with mitosis (Nakajima *et al.* 2008; Villeneuve *et al.* 2013). The expression of Cdk1(T14A,Y15F) activity during S phase also causes DNA replication defects leading to genome instability and mitotic catastrophe (Heald *et al.* 1993). The idea that partial inhibition of Cdk1 by T14 phosphorylation might play a role during S phase is particularly attractive because Cdk activity levels required to trigger S phase are lower than those required for initiating mitosis (Coudreuse and Nurse 2010; Thomson *et al.* 2010). Further studies are clearly needed to define exactly how T14 phosphorylation of Cdk1 prevents chromosome instability and mitotic defects.

The ubiquitous expression of Cdk1(T14A)-VFP rescued *cdc2^{ts}* mutant lethality, demonstrating that regulation of Cdk1 by Y15 phosphorylation alone could be sufficient for normal development. Intriguingly, we observed effects on the steady-state levels of T161-activating phosphorylation associated with the Cdk1(T14A)-VFP mutant (Figure 1C) that were consistent with a proposed role for T14 phosphorylation in promoting

Figure 8 Live imaging of mitosis in larval neuroblasts driven by *prospero-Gal4* of Cdk1-VFP (green) and tubulin-RFP (red) shows that only Cdk1(T14A, Y15F) expression causes mitotic timing defects. Each set of panels represent still images taken at four time points from each of the movies in File S1, File S2, File S3, and File S4. (A–D) (Top) Merged images of the respective Cdk1-VFP (green) and tubulin-RFP (red) fluorescent reporters. (Bottom) Only tubulin-RFP. In A–D, the zero time point (first panels) marks the appearance of centrosomes at opposite poles ($t = 0$); the second panels show midpoint of bipolar spindle formation; and the third and fourth panels represent the beginning and end of cytokinesis, respectively. (A) Neuroblasts expressing Cdk1WT-VFP, where average time of mitosis was 12.56 ± 1.2 min ($n = 5$) (File S1). (B) Neuroblasts expressing Cdk1(T14A), where average time of mitosis was 13.52 ± 1.26 min ($n = 5$) (File S2). (C) Neuroblasts expressing Cdk1(Y15F), where average time of mitosis was 13.0 ± 0.7 min ($n = 5$) (File S3). (D) Neuroblasts expressing Cdk1(T14A,Y15F), where average time of mitosis was 28.54 ± 2.25 min, ($n = 3$) (File S4). (E) Quantification of the mitotic timing data for neuroblasts expressing Cdk1WT-VFP ($n = 5$), Cdk1(T14A)-VFP ($n = 5$), Cdk1(Y15F)-VFP ($n = 5$), and Cdk1(T14A,Y15F)-VFP ($n = 3$). The bar graph depicts the mean duration of mitosis $\pm$ SD for each genotype.

or stabilizing T161-activating phosphorylation of Cdk1/Cyclin complexes during G2 phase (Coulonval *et al.* 2011). Indeed, such a role for T14 phosphorylation of Cdk1 could explain requirements for Myt1 activity in cells that undergo prolonged developmentally regulated G2-phase arrest during *Drosophila* male meiosis or oocyte maturation in other systems (Karaiskou *et al.* 2004; Jin *et al.* 2005; Burrows *et al.* 2006). Thus, dual phosphorylation of Cdk1 by Myt1 kinases may have evolved as a dynamic mechanism for facilitating developmentally regulated G2-phase checkpoint arrest without compromising the cell's ability to initiate mitosis without delay when triggered by Cdc25 dephosphorylation.

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Supporting Information

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Dual Phosphorylation of Cdk1 Coordinates Cell Proliferation with Key Developmental Processes in *Drosophila*

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Figure S1 Data showing protocol for efficiently precipitating transgenic Cdk1(WT) proteins from wing disc extracts with rabbit antibodies against GFP. (S1A) Precipitate (P) and supernatant (S) fractions were probed with antibodies against Cyclin B which detect a single band of 63 kD, then stripped and re-probed with mouse anti-GFP antibodies, showing that Cdk1WT-VFP was efficiently precipitated (and depleted from the supernatant). The blot shows two experimental replicates. Panel S1B shows aliquots from one experiment, where Cdk1 fusion proteins were immunoprecipitated from larval wing discs with rabbit anti-GFP antibodies (yw as a negative control) and tested for kinase activity using histone H1 as a substrate. Anti-histone H1 and mouse anti-Cdk1 antibodies were used for labeling the blot as positive controls. Each sample represents immunoprecipitated protein from a protein extract representing 180 wing discs per genotype. The relative kinase activity for each Cdk1 variant was determined by normalizing kinase activity per unit of total immunoprecipitated protein. Panels S1C and S1D show western blots of protein extracts from *cdc2^{ts}* mutants rescued with Cdk1(WT)-VFP or Cdk1(T14A) expressed using *Tub-Gal4* and *Actin-Gal4*, respectively. Control extracts (*cdk1^{ts}/+*) probed with anti-GFP, anti-Cdk(PSTAIR) or anti-Actin antibody showed no transgenic protein, whereas extracts from *cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1WT-VFP/tubulin-Gal4*, *cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1WT-VFP/actin-Gal4*, *cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1A-VFP/tubulin-Gal4* or *cdc2^{B47}/cdc2^{E1-E24};UAS-Cdk1A-VFP/actin-Gal4* showed that the transgenic proteins were well expressed. Panel S1E shows a compilation of progeny data resulting from genetic crosses used to test whether the Cdk1-VFP transgenes ubiquitously expressed with either *tubulin-Gal4* or *actin-Gal4* could rescue pupal lethality in a *cdk1* temperature-sensitive mutant background (*w*; *cdc2^{B47}/cdc2^{E1-E24}; Cdk1-VFP/tubulin-Gal4*). Flies were cultured at 25 °C throughout development. The expected ratio of A:C:D classes of progeny that would represent full rescue was 1:2:4. Expression of Cdk1(WT)-VFP completely rescued *cdk1* lethality as indicated by progeny in Class A. Expression of Cdk1(T14A)-VFP also completely rescued the temperature sensitive *cdk1* lethality (see Class A). In contrast, neither expression of Cdk1(Y15F)-VFP nor Cdk1(T14A,Y15F)-VFP were capable of rescuing the lethality of *cdk1* mutants (no progeny in Class A).

Figure S2 Quantitation of mitotic index in third instar wing discs expressing Cdk1-VFP transgenes in the posterior compartment under *en-Gal4* control. PH3-labeled cells were counted in the posterior and anterior compartments of discs (N=7, per genotype) using de-convolved images to obtain a (P/A) mitotic index ratio, with the data illustrated as box plots representing the indicated wing discs genotypes shown also in Fig. 4 (panels I-L). Compared to Cdk1WT-VFP or Cdk1(T14A)-VFP, wing discs expressing either Cdk1(Y15F)-VFP or Cdk1(T14A,Y15F)-VFP had a significantly higher P/A ratio.

Figure S3 Expression of Cdk1(T14A,Y15F)-VFP using *sd-Gal4* disrupted Wingless (Wg) expression at the presumptive wing margin. Wing discs from third instar larvae were immuno-labeled with Wingless antibodies (red). At least 10 discs were examined for each genotype. Panels A, A' and B, B' show that Wg expression pattern was unaffected in wing discs expressing Cdk1(WT)-VFP and Cdk1(Y15F)-VFP. Panel C, C' shows that Wg expression was disrupted in wing discs expressing Cdk1(T14A,Y15F)-VFP.

Files S1-S4

Available for download as .avi files at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.156281/-/DC1>

File S1 Live analysis of mitotic timing in neuroblasts expressing *Cdk1(WT)-VFP* (green) and *Tubulin-RFP* (red). This movie is representative of five cells that were analyzed showing that the neuroblasts spent approximately 12.6 min $\pm$ 1.2 min in mitosis.

File S2 Live analysis of mitotic timing in neuroblasts expressing *Cdk1(T14A)-VFP* (green) and *Tubulin-RFP* (green). The movie is representative of five cells that were analyzed which showed that these neuroblasts spent approximately 13.5 min $\pm$ 1.3 min in mitosis.

File S3 Live analysis of mitotic timing in neuroblasts expressing *Cdk1(Y15F)-VFP* (green) and *Tubulin-RFP* (red). The movie is representative of five cells that were analyzed which showed that these neuroblasts spent approximately 13.0 min $\pm$ 0.7 min in mitosis.

File S4 Live analysis of mitotic timing in neuroblasts expressing *Cdk1(T14A, Y15F)-VFP* and *Tubulin-RFP*. The movie is representative of three cells analyzed showing that these neuroblasts spent 28.5min $\pm$ 2.3 min in mitosis, with apparent delays in spindle assembly.