Grapes(Chk1) prevents nuclear CDK1 activation by delaying cyclin B nuclear accumulation

Anne Royou, Derek McCusker, Douglas R. Kellogg, and William Sullivan

Department of Molecular Cell and Developmental Biology, University of California, Santa Cruz, Santa Cruz, CA 95064

Entry into mitosis is characterized by a dramatic remodeling of nuclear and cytoplasmic compartments. These changes are driven by cyclin-dependent kinase 1 (CDK1) activity, yet how cytoplasmic and nuclear CDK1 activities are coordinated is unclear. We injected cyclin B (CycB) into *Drosophila melanogaster* embryos during interphase of syncytial cycles and monitored effects on cytoplasmic and nuclear mitotic events. In untreated embryos or embryos arrested in interphase with a protein synthesis inhibitor, injection of CycB accelerates nuclear envelope breakdown and mitotic remodeling of the cytoskeleton. Upon activation of the Grapes(checkpoint kinase 1) (Grp(Chk1))-dependent S-phase checkpoint, increased levels of CycB drives cytoplasmic but not nuclear mitotic events. Grp(Chk1) prevents nuclear CDK1 activation by delaying CycB nuclear accumulation through Wee1-dependent and independent mechanisms.

Introduction

Entry into mitosis requires a dramatic reorganization of nuclear and cytoplasmic compartments. These changes are driven by the activity of CDK1 associated with mitotic cyclins, notably cyclin B (CycB; Morgan, 2006). Activation of CDK1 requires sufficient levels of CycB and the removal of CDK1 inhibitory phosphorylation. CDK1 phosphorylation is controlled by conserved kinases Wee1 and Myt1 and by the phosphatase Cdc25. The rapid onset of CDK1 activation at the end of G2 is driven by inactivation of Wee1 and activation of Cdc25. Active CDK1 contributes to these changes in Wee1 and Cdc25 activity, thus establishing a positive feedback loop that drives cells into mitosis (Ferrell, 2002; Morgan, 2006).

In spite of these insights, little is known about the mechanisms by which nuclear and cytoplasmic CDK1 activities are coordinated. Resolving this issue requires determining whether nuclear and cytoplasmic CDK1 pools are differentially regulated. Support for a differential regulation comes from the finding that subcellular localization of CycB plays a role in regulating CDK1 activity. In vertebrates, CycB is predominantly cytoplasmic at interphase because of Crm1-mediated nuclear exclusion (Hagting et al., 1998; Yang et al., 1998). At prophase, CDK1–CycB is activated in the cytoplasm before its entry into the nucleus (De Souza et al., 2000; Jackman et al., 2003), and the abrupt CDK1–CycB nuclear translocation is triggered by the phosphorylation of CycB on its cytoplasmic retention signal (Ookata et al., 1993; Pines and Hunter, 1994; Li et al., 1997; Hagting et al., 1999; Takizawa and Morgan, 2000).

Controlling the subcellular localization of CycB may also be involved in checkpoint function. Un-replicated or damaged DNA results in the activation of the conserved S-phase checkpoint kinase 1 (Chk1), which inhibits Cdc25 and activates Wee1 (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Lee et al., 2001). Consequently, the S-phase checkpoint delays the cell cycle in interphase by Chk1-mediated inhibition of CDK1 (Walworth, 2001; Melo and Toczyski, 2002). However, expression of $CDK1^{AF}$, a version of CDK1 lacking the phosphorylation inhibitory sites, only partially bypasses the interphase arrest induced upon DNA damage (Jin et al., 1996). This arrest is fully bypassed by coexpressing $CDK1^{AF}$ and nuclear-targeted CycB (Heald et al., 1993; Jin et al., 1998). These studies imply that prevention of CycB nuclear localization is one of the mechanisms by which the S-phase checkpoint delays nuclear entry into mitosis. Thus, the coordination of cytoplasmic and nuclear mitotic entry likely involves the control of CycB subcellular localization as well as the CDK1 phosphorylation state.

The late syncytial nuclear cycles of the *Drosophila melanogaster* embryo are regulated by levels of CycB and S-phase

Correspondence to Anne Royou: royou@biology.ucsc.edu

Abbreviations used in this paper: Aph, aphidicolin; AVD, average deviation; Chk1, checkpoint kinase 1; CHX, cycloheximide; CycB, cyclin B; Grp, Grapes; MTOC, microtubule-organizing center; NEB, nuclear envelope breakdown; NEF, nuclear envelope formation; RLC, regulatory light chain.

The online version of this article contains supplemental material.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 183 No. 1 63–75
www.jcb.org/cgi/doi/10.1083/jcb.200801153

checkpoint activity (Edgar et al., 1994; Fogarty et al., 1997; Sibon et al., 1997; Stiffler et al., 1999; Price et al., 2000; Stumpff et al., 2004; Crest et al., 2007). These cycles provide an excellent system to address the role of CycB subcellular localization in driving cytoplasmic and nuclear mitotic events during normal and S-phase checkpoint–activated conditions. We addressed this question by taking advantage of our ability to inject functional CycB in the syncytial embryo at precise times during the cell cycle and to monitor its effects on multiple cytoplasmic and nuclear events. Increasing the level of CycB during early interphase of cycle 13 induces premature nuclear envelope breakdown (NEB) and the reorganization of the cytoskeleton. Upon activation of the S-phase checkpoint, increased levels of CycB drives cytoplasmic but not nuclear mitotic events. We demonstrate that the S-phase checkpoint protects the nucleus from active cytoplasmic CDK1–CycB via two distinct mechanisms involving Grapes(Chk1)-dependent control of CycB nuclear localization and Wee1-dependent inhibition of nuclear CDK1.

Results

Injection of CycB prematurely drives NEB and spindle assembly

We injected recombinant CycB N-terminal GST fusion protein into living *Drosophila* embryos at precise times during interphase of the syncytial cycle 13. GST-CycB is able to induce CDK1 phosphorylation on T161 and promote its kinase activity in vitro (Edgar et al., 1994). We will refer to the recombinant protein as CycB.

Nuclear CDK1 activity is thought to promote chromosome condensation and NEB (Lamb et al., 1990; Peter et al., 1990; Enoch et al., 1991). Therefore, nuclear mitotic events were defined by chromosome condensation (monitored with GFP-H2Av) and NEB (monitored by nuclear infusion of injected rhodamine-conjugated tubulin). The central regions of embryos were injected with either 71 µM GST or 65 µM CycB at the onset of interphase of nuclear cycle 13. After GST injection, NEB occurred almost synchronously throughout the embryo at 14.3 min after injection, and the chromosomes entered anaphase shortly after NEB (Fig. 1 A, GST). After CycB injection, the timing of NEB was greatly accelerated near the site of injection compared with NEB in areas more distant from the site of injection (Fig. 1 A, CycB, white outlines). Premature NEB occurs as a wave probably caused by the slow diffusion of CycB from the injection site. These observations indicate that CycB injection at 65 µM is sufficient to induce a local activation of CDK1, which triggers premature NEB and spindle assembly. This result is consistent with work demonstrating that exogenous CycB promotes premature disassembly of the nuclear pore complex in syncytial *Drosophila* embryos (Onischenko et al., 2005). Although CycB induced premature NEB, it did not promote premature chromosome condensation before NEB. At NEB, the level of chromosome condensation was much greater in the control embryo than in the CycB-injected embryo (Fig. 1 B, top row). In most cases, once NEB occurred in the area near the site of CycB injection, the chromosomes rapidly condensed and, by metaphase, achieved the same state of condensation as control embryos (Fig. 1 B, bottom row).

To determine whether new rounds of CycB synthesis are required for each mitotic cycle, we investigated the effect of injecting CycB after treatment with the protein synthesis inhibitor cycloheximide (CHX). Injection of CHX at metaphase induced a cytoplasmic and nuclear interphase arrest in the next cycle (Fig. 1 C, left). Exogenous CycB locally overcame the CHX-induced interphase arrest as indicated by NEB and rapid spindle formation (Fig. 1 C, right; and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). The data suggest that new rounds of CycB synthesis are necessary to drive the next mitotic cycle. We determined by single embryo Western that the amount of injected CycB was equivalent to the total level of endogenous CycB at metaphase of cycle 13. Taking into account the limited diffusion of the injected CycB, we estimate that the 65 µM of exogenous CycB at the center of the gradient was less than fivefold the level of endogenous CycB at mitosis (see Materials and methods).

Injection of CycB prematurely drives the cytoplasm into mitosis

We next determined whether injected CycB prematurely drives the cytoplasm into mitosis. We addressed this issue by examining the dynamics of three cytoplasmic proteins fused with GFP that undergo dramatic changes in subcellular distribution during the cortical cycles: nonmuscle myosin II regulatory light chain ([RLC]; RLC-GFP; Fig. 2, A and D; Royou et al., 2004), moesin (GFP-moesin; Fig. 2 B and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1; Edwards et al., 1997), and Nuclear fallout (GFP-Nuf; Fig. 2 C and Video 3; Riggs et al., 2007).

In all embryos, CycB was injected at the onset of cycle 13 interphase. We monitored the timing of cytoplasmic marker dynamics and NEB in areas close to and distant from the site of injection. We will refer to these as injected and control areas, respectively. In control areas, RLC-GFP concentrated at the cortex throughout interphase and dispersed just before NEB. This dispersion occurred 12 min after the onset of interphase (n = 23, average deviation [AVD] = 1.8; Fig. 2, A and D; and see Fig. S1 for quantification of the RLC-GFP signal, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). In CycB-injected areas, RLC-GFP dispersed prematurely, 4 min after interphase onset (n = 23, AVD = 1.8; Fig. 2, A and D, blue outlines). Interestingly, in both areas, RLC-GFP disappearance always preceded NEB (Fig. 2, A and D, orange outlines). A previous study has shown that RLC-GFP dispersion is driven by CDK1 activity (Royou et al., 2002). Therefore, our results indicate that high levels of CycB promote premature cytoplasmic as well as nuclear CDK1 activation.

GFP-moesin, a marker for F-actin, reorganizes from caps at interphase into furrows in mitosis (Edwards et al., 1997). By focusing 5 µm below the plasma membrane, only furrows that have extended to this depth are visualized (Fig. 2 B). In control and CycB-injected regions, F-actin furrows progressed to this depth at 11.5 min. and 3.5 min after injection, respectively (Fig. 2 B, blue outlines). These experiments indicate that exogenous CycB drives premature reorganization of the actin cytoskeleton.

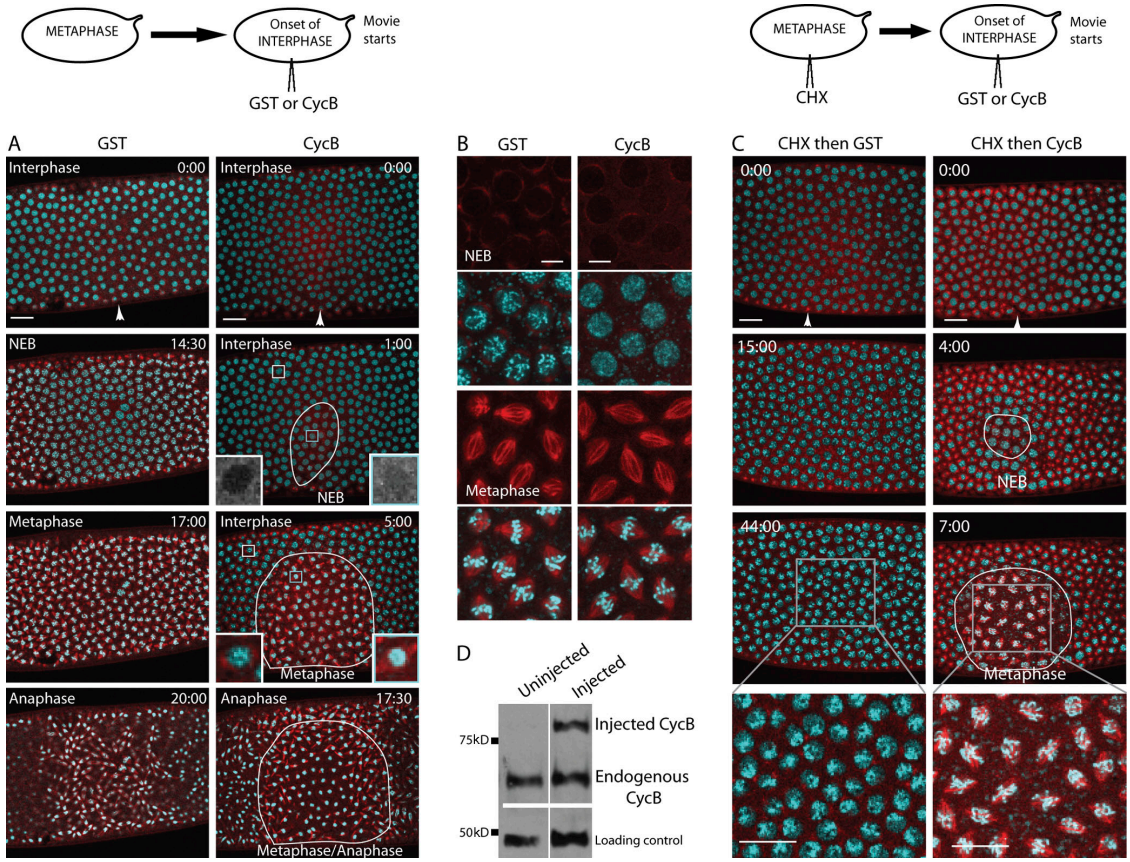


Figure 1. **CycB drives premature NEB and overrides CHX-induced interphase arrest.** (A) Injection of CycB induces premature NEB. GFP-H2Av (cyan) embryos were injected with rhodamine-tubulin (red) and with either GST or CycB at the onset of cycle 13 interphase. The schematic describes the injection and imaging sequence. Insets are enlarged images of the noninjected areas (white boxes) and the CycB-injected areas (blue boxes). The GST and CycB-injected embryos are representative of four injected embryos for each protein. The nuclear cell cycle stages of the noninjected and CycB-injected areas (white outlines) are indicated at the top left and the bottom of the images, respectively. NEB is detected when rhodamine-tubulin invades the nucleoplasm (compare left inset with right inset, second row). Time is given in minutes/seconds. Arrowheads mark the site of injection. Bars, 20 µm. (B) Injection of CycB does not promote premature chromosome condensation before NEB. GFP-H2Av (cyan) embryos were injected with rhodamine-tubulin (red) followed by GST or CycB injection at the beginning of cycle 13 interphase. The top two rows show the state of chromosome condensation at NEB, which was determined by the nucleoplasm being filled with rhodamine-tubulin. The bottom two rows show the state of chromosome condensation at metaphase. Bars, 5 µm. (C) CycB injections overcome the CHX-induced cytoplasmic and nuclear interphase arrest. GFP-H2Av (cyan) embryos were injected with rhodamine-tubulin (red) followed by CHX at mitosis. GST or CycB was injected at the onset of the following interphase. The schematic describes the injection and imaging sequence. Arrowheads mark the site of injection. CycB induced NEB, chromosome condensation, and mitotic spindle formation (white outlines; see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). The areas outlined in gray in the third row are enlarged in the bottom row. Time is given in minutes/seconds. Bars, 20 µm. (D) Single embryo Western blot of uninjected and CycB-injected embryos arrested at mitosis of cycle 13 with colchicine. Anti-CycB antibodies were used to detect endogenous and injected CycB. Anti-GFP was used to detect a marker for loading controls. The injected embryo extract reveals an additional 78-kD molecular mass GST-CycB band.

To confirm that the effect of CycB was not confined to actin and myosin, we monitored the effect of CycB injection on Nuf, a Rab11 effector that exhibits a cell cycle–dependent association with the microtubule- organizing center (MTOC; Fig. 2 C; Riggs et al., 2003; Cao et al., 2008). We find that GFP-Nuf is concentrated at the MTOC through interphase of cycle 13 in the control area. Upon CycB injection, Nuf prematurely disperses from the MTOC (Fig. 2 C, blue outlines). Collectively, these observations indicate that high levels of CycB induce local cytoplasmic CDK1 activation and trigger remodeling of the cytoskeleton.

CycB drives cytoplasmic events independently of the nuclear cycle

In the aforementioned studies, we observed that cytoplasmic mitotic events precede nuclear mitotic events. This observation raises the possibility that cytoplasmic and nuclear CDK1 activities are differentially regulated by CycB levels. To explore this possibility, we monitored nuclear (NEB) and cytoplasmic (myosin dispersion) mitotic events after injecting CycB at a lower concentration of 32 µM (Fig. 2 D). In six out of eight injected embryos, 32-µM CycB dilution induced cytoplasmic and nuclear CDK1 activation at a similar rate as 65 µM CycB. However, in two embryos, although CycB induced premature myosin dispersion, it did not induce premature NEB (Fig. 2 D). This uncoupling between the cytoplasmic and nuclear CDK1 activation rate suggests that different mechanisms regulate these two activities.

To further examine independent activation of cytoplasmic and nuclear CDK1, we took advantage of the prolonged interphase of nuclear cycle 14. During this cycle, the nuclei arrest in interphase while the cytoskeleton reorganizes to form furrows that elongate and encompass each nucleus in a process known

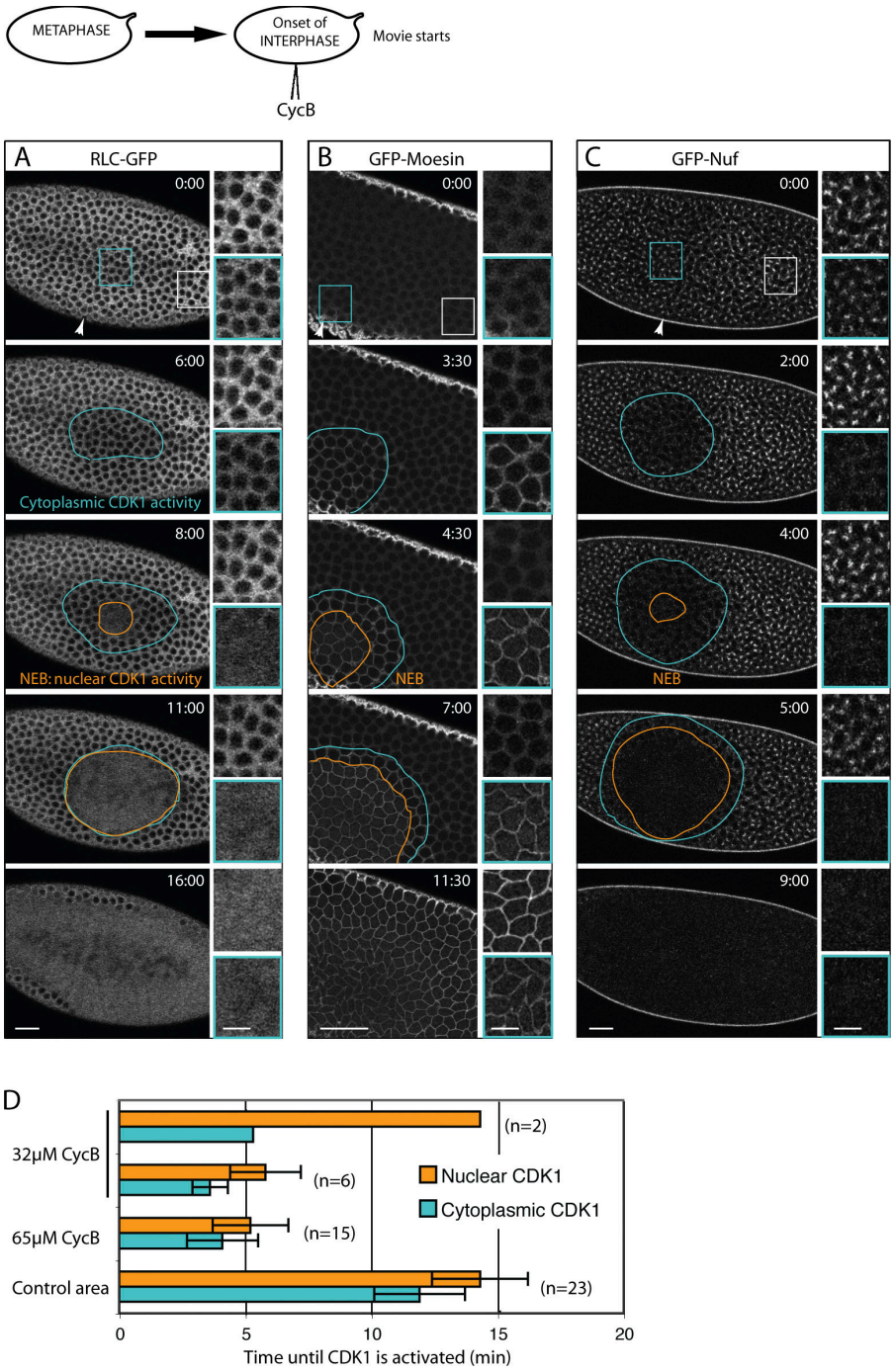


Figure 2. **CycB triggers reorganization of the cytoskeleton.** (A–C) RLC-GFP (A), GFP-moesin (B; see Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1), and GFP-Nuf (C; see Video 3) embryos were injected with CycB at early interphase of cycle 13. The schematic describes the injection and imaging sequence. The timing of CycB injection in GFP-Nuf was performed at a time when Nuf was concentrated at the MTOC (midinterphase). Arrowheads mark the sites of injection. Top row insets are enlarged images of the control area, which is distant from the site of injection (white boxes), and bottom row insets are enlarged images of the CycB-injected area (blue boxes). The RLC-GFP, GFP-moesin, and GFP-Nuf embryos are representative of 15, 4, and 3 injected embryos, respectively. NEB is determined when the GFP markers fill the nucleoplasm. The blue and orange outlines mark the areas where CycB has an effect on the cytoplasm and the nucleus, respectively. Time is given in minutes/seconds. Bars: (panels) 20 μm; (insets) 10 μm. (D) The timing of cytoplasmic and nuclear CDK1 activation after CycB injection was monitored with RLC-GFP embryos. CycB was injected at 65 or 32 μM at interphase onset, and the embryo was examined within 30 s after injection. The timing of cytoplasmic CDK1 activation was determined when RLC-GFP disappeared from the focal plane. The timing of nuclear CDK1 activation was determined when RLC-GFP filled the nucleoplasm (NEB). For each embryo, we monitored the timing of cytoplasmic and nuclear CDK1 activation in control and injected areas. Data are represented as mean ± AVD. n, number of embryos injected.

as cellularization. The mechanisms that trigger cellularization are not understood. To determine whether CycB is limiting for driving CDK1 activation at cycle 14, we injected CycB at different times during cellularization and monitored cytoplasmic and nuclear CDK1 activation. Cytoplasmic CDK1 activation was monitored by examining RLC-GFP, GFP-moesin, and GFP-Nuf dynamics (Fig. 3). Nuclear CDK1 activity was measured by monitoring NEB (when the cytoplasmic markers invade the nucleoplasm). When injected early during cellularization (3–5 min after nuclear envelope formation [NEF]), CycB triggered both cytoplasmic and nuclear entry into mitosis (Fig. 3, A and C, blue and orange outlines, respectively; and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). However, the timing of NEB was not coordinated with the timing of myosin dispersion. Although CycB injection induced myosin dispersion promptly (4.6 ± 0.6, n = 5; Fig. 3, A and C), NEB was delayed and occurred 5.5 min after myosin had dispersed (10.1 ± 2.1, n = 5; Fig. 3, A and C). This uncoupling between the cytoplasmic and nuclear cycle was even more dramatic when CycB was injected 6–9 min after the onset of cellularization. Injection of CycB at this later time failed to induce NEB (Fig. 3, B and C), yet the cytoplasm entered mitosis rapidly after CycB injection (Fig. 3, B and C, blue outlines; and Fig. S2). CycB also induced the reorganization of GFP-moesin into

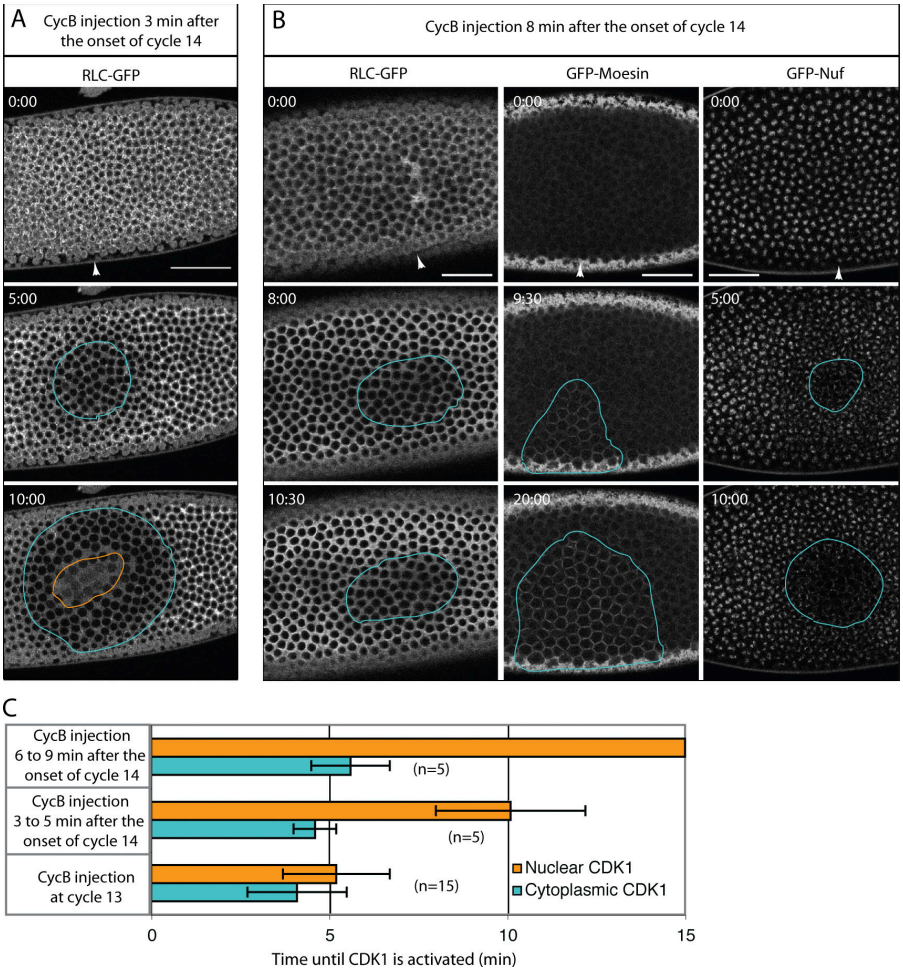


Figure 3. **CycB drives cytoplasmic mitotic events independently of the nuclear cycle during cellularization.** (A) CycB is limiting for nuclear and cytoplasmic CDK1 activity when injected at the onset of cycle 14 interphase. An RLC-GFP embryo was injected with CycB at cycle 14, 3 min after NEF. The arrowhead marks the site of injection. At the site of injection, RLC-GFP disappeared from the cortex 5 min after injection (blue outlines). NEB occurred 5 min later (orange outline; see Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). Time is given in minutes/seconds. Bar, 20 µm. (B) CycB drives the cytoplasm but not the nuclei into mitosis when injected later during interphase of cycle 14. RLC-GFP, GFP-moesin (see Video 5), and GFP-Nuf embryos were injected with CycB at cycle 14, 8 min after NEF. Arrowheads mark the sites of injection, and blue outlines mark the areas where the cytoplasm entered mitosis. Time is given in minutes/seconds. Bars, 20 µm. (C) The timing of cytoplasmic and nuclear CDK1 activation was determined as described in Fig. 2. Data are represented as mean ± AVD. n, number of embryos injected.

furrows and the dispersion of Nuf from the MTOC (Fig. 3 B, blue outlines; and Video 5). However, there were no cases in which NEB occurred. CycB injected 15 min after the onset of cellularization had no effect on the progression of nuclear or cytoplasmic events (n = 6; unpublished data). These experiments show that increasing the level of CycB is sufficient to induce an additional syncytial cycle. They demonstrate that CycB remains limiting for cytoplasmic and, to a lesser extent, nuclear CDK1 activation during early cellularization. Furthermore, they provide evidence that CDK1 can be activated in the cytoplasm independently of its activation in the nucleus.

The Grapes (Grp)-dependent S-phase checkpoint protects the nucleus from cytoplasmic CDK1 activity

We next addressed the effects of the S-phase checkpoint activity on cytoplasmic and nuclear CDK1 activation. To do so, we observed the effects of CycB injections in syncytial embryos arrested in interphase by injecting the DNA replication inhibitor aphidicolin (Aph). When Aph is injected during mitosis of cycle 12, it activates the S-phase checkpoint and induces a prolonged cytoplasmic and nuclear interphase arrest at the next cycle (unpublished data).

We next addressed whether CycB can overcome the S-phase checkpoint–induced cytoplasmic and nuclear interphase arrest. To perform this analysis in checkpoint-compromised mutants and normal embryos, we injected the embryos simultaneously with CHX and Aph. This provides time to perform the injections in the exceedingly short cell cycles of the checkpoint-compromised embryos. In wild-type embryos, the results are the same for injecting Aph alone or a combined injection of Aph and CHX (unpublished data). For both a wild-type and a *grp* mutant, only the combined injections are described.

In all experiments presented in Fig. 4, CHX and Aph were injected together at mitosis of cycle 12 in embryos expressing RLC-GFP, which allowed us to simultaneously monitor cytoplasmic CDK1 activity (myosin dispersion) and nuclear CDK1 activity (NEB). We injected CycB at two different time points during the next interphase, as represented by the schematic in Fig. 4. We injected CycB at the onset of interphase (early injection) and 10 min later (late injection). Injection of CycB during early interphase overcame the CHX + Aph–induced interphase arrest (Fig. 4 A). The timing of cytoplasmic and nuclear CDK1 activation was similar to embryos in which only CycB was injected (Fig. 4 D). The results were strikingly different when CycB was injected 10 min after the onset of interphase. NEB occurred in only 3 out of 15 embryos (Fig. 4, B and D; and Fig. S1). Furthermore, NEB was delayed relative to the timing of cytoplasmic CDK1 activation (9.7 ± 1.9 min vs. 3.8 ± 0.2 min, n = 3). CDK1 was activated in the cytoplasm but not in the nucleus in 7 out of 15 embryos (Fig. 4, B′ and D; and Fig. S1).

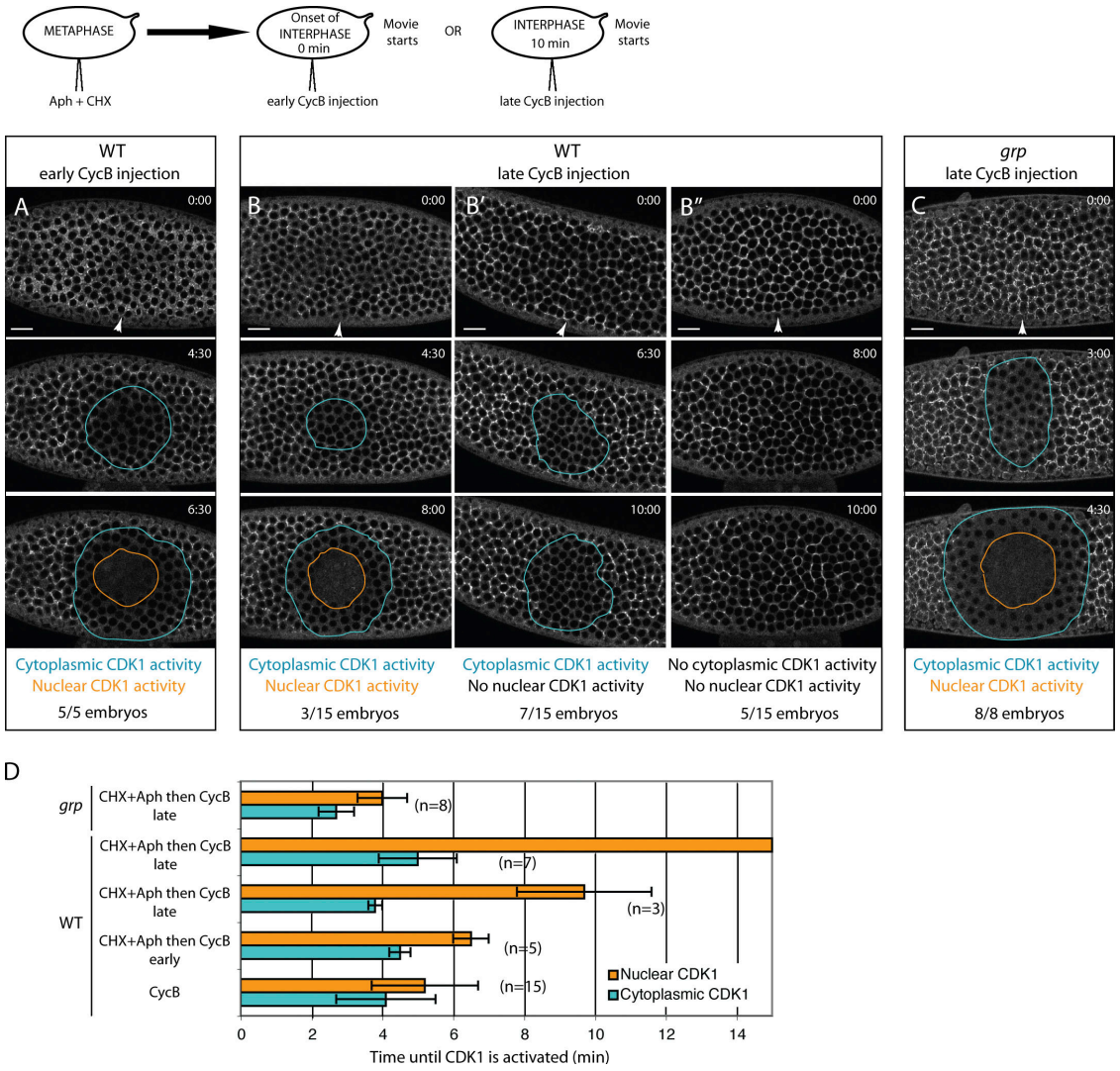


Figure 4. **CycB injections overcome the S-phase checkpoint–induced cytoplasmic cell cycle arrest, and to a lesser extent, nuclear cell cycle arrest.** (A–C) Wild-type (WT, A–B″) or *grp* mutant embryos (C) expressing RLC-GFP were injected with CHX and Aph together (CHX + Aph) at metaphase of cycle 12. The embryos were injected with CycB at the onset of interphase (A, early CycB injection) or 10 min after the onset of interphase (B–C, late CycB injection). The schematic describes the injection and imaging sequence. Arrowheads mark the sites of injection. Blue outlines mark areas with cytoplasmic CDK1 activity, and orange outlines mark areas where NEB occurs. Time is given in minutes/seconds. Bars, 20 µm. (D) Timing of cytoplasmic and nuclear CDK1 activation was measured as described in Fig. 2. Data are represented as mean ± AVD. n, number of embryos injected.

Finally, in 5 out of 15 embryos, neither cytoplasmic nor nuclear CDK1 activity was detected (Fig. 4 B″).

An explanation for the different results upon early and late injection of CycB into CHX + Aph–arrested embryos is that the S-phase checkpoint is activated in late but not in early injected embryos. We propose this idea because live analysis using a Grp-GFP fusion protein reveals that Grp is dispersed in the cytoplasm during mitosis and accumulates in the nucleus upon NEF (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). Thus, sufficient time after NEF is required to establish a functional S-phase checkpoint. To test this hypothesis, we performed the same experiments in the S-phase checkpoint–compromised mutant *grp* (Fig. 4 C). As with wild-type embryos, CHX + Aph arrested the nuclei and cytoplasm of *grp* embryos in interphase (unpublished data). In contrast to wild type, CycB injected late into *grp* embryos triggered consistent premature cytoplasmic and nuclear entry into mitosis at the site of injection (Fig. 4, C and D).

These results support our previous conclusion that cytoplasmic CDK1 activity is independent of its activity in the nucleus. In addition, they suggest that the S-phase checkpoint is more effective at inhibiting CDK1 in the nucleus than in the cytoplasm.

CycB has a dynamic localization through the syncytial cycle and associates with kinetochores before NEB and during mitosis

Given that the nucleus can be protected from active cytoplasmic CDK1–CycB by the Grp-dependent S-phase checkpoint, this raises the possibility that the checkpoint operates by regulating CycB nuclear import (Jin et al., 1998). To pursue this idea, we

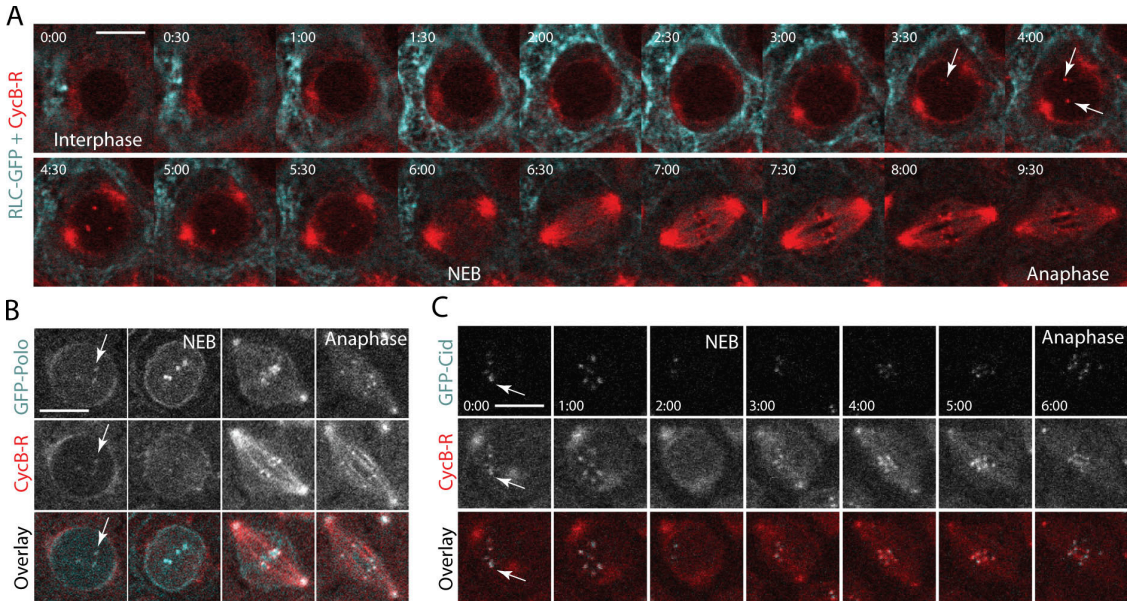


Figure 5. **CycB localizes to the kinetochores before NEB.** RLC-GFP (A, cyan), GFP-Polo (B, cyan), and GFP-Cid (C, cyan) embryos were injected with 13 µM CycB-R (red) at interphase of cycle 12 (A) or 13 (B and C). Arrows highlight the punctate nuclear localization of CycB-R (A) and its colocalization with GFP-Polo (B) and GFP-Cid (C) before NEB. Time is given in minutes/seconds. Bars, 5 µm.

analyzed the subcellular localization of CycB in living embryos by injecting rhodamine-labeled CycB (CycB-R).

We injected a low concentration of 13 µM CycB-R at the onset of interphase of cycles 12 and 13 in embryos expressing RLC-GFP (Fig. 5 A), the kinase Polo fused with GFP (GFP-Polo; Fig. 5 B; Moutinho-Santos et al., 1999), or the centromere-associated protein Cid fused with GFP (GFP-Cid; Fig. 5 C; Schuh et al., 2007). At this concentration, CycB-R did not induce premature entry into mitosis in five out of six wild-type embryos. CycB-R accumulates at the centrosome and is excluded from the nucleus during interphase, in agreement with our previous observations (Huang and Raff, 1999). During early prophase, before NEB, CycB-R maintains its association with the centrosome and accumulates in discrete puncta within the nucleus, indicating that the recombinant CycB used in these experiments is efficiently imported into the nucleus (Fig. 5 A, arrows). After NEB, CycB-R is primarily associated with the bipolar spindle, consistent with observations in other cell types (Pines and Hunter, 1991; Yang et al., 1998). In addition, a strong signal was also detected on the metaphase plate (Fig. 5). CycB-R colocalized with kinetochore GFP-Polo before NEB and throughout mitosis (Fig. 5 B, arrows). The CycB-R puncta in the nucleus before NEB colocalized with GFP-Cid (Fig. 5 C, arrows). This colocalization is observed during mitosis until late anaphase. Collectively, these observations suggest an association of CycB-R with the kinetochore that is initiated before NEB. The specific localization of CycB-R into kinetochores before NEB provides a means to monitor CycB nuclear import.

The S-phase checkpoint prevents CycB nuclear accumulation by maintaining CDK1 in an inactive state

To determine whether the S-phase checkpoint prevents nuclear CDK1 activation by controlling CycB subcellular localization, we injected CycB-R after CHX + Aph treatment into wild-type and *grp* mutant embryos expressing RLC-GFP (Fig. 6). We assayed three different CycB-R concentrations (100, 56, and 13 µM). We monitored the induction of cytoplasmic and nuclear CDK1 activity by observing cortical myosin dispersion and NEB, respectively. We also monitored the timing of nuclear CycB-R localization. Embryos were injected with CHX + Aph in mitosis of cycle 12 followed by injection of CycB-R 10 min after the onset of cycle 13 interphase (Fig. 6, schematic). In wild-type embryos injected with 100 µM CycB-R, the cytoplasm entered mitosis, as indicated by the disappearance of the RLC-GFP signal from the cortex (Fig. 6, A and F, blue outlines; Fig. S1; and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). However, up to 15 min after CycB-R injection, we did not observe NEB or CycB-R localization in the nucleus (Fig. 6, A and F; and Video 6). In contrast, the same injection in *grp* mutant embryos provoked a rapid CycB-R nuclear import concomitant with cytoplasmic entry into mitosis (Fig. 6, B and F, blue outlines; and Video 7) and was immediately followed by NEB (Fig. 6, B and F, orange outlines; and Video 7).

Injection of 56 or 13 µM CycB-R into wild-type embryos previously treated with CHX + Aph did not trigger cytoplasmic and nuclear entry into mitosis for up to 15 min (Fig. 6, C, G, and H). The first sign of CycB-R in nuclei (appearance of CycB-R puncta in at least three nuclei) was detected at a mean of 8.9 min for both CycB-R concentrations (Fig. 6, C and I, red arrow). Once in the nucleus, the CycB-R signal at the kinetochore increased progressively. This observation rules out the possibility that the S-phase checkpoint is preventing CycB nuclear accumulation by increasing the rate of nuclear CycB degradation (Fig. 6 C, compare the second row with the third row). In contrast, injection of 56 or 13 µM CycB-R in the *grp* mutant triggered

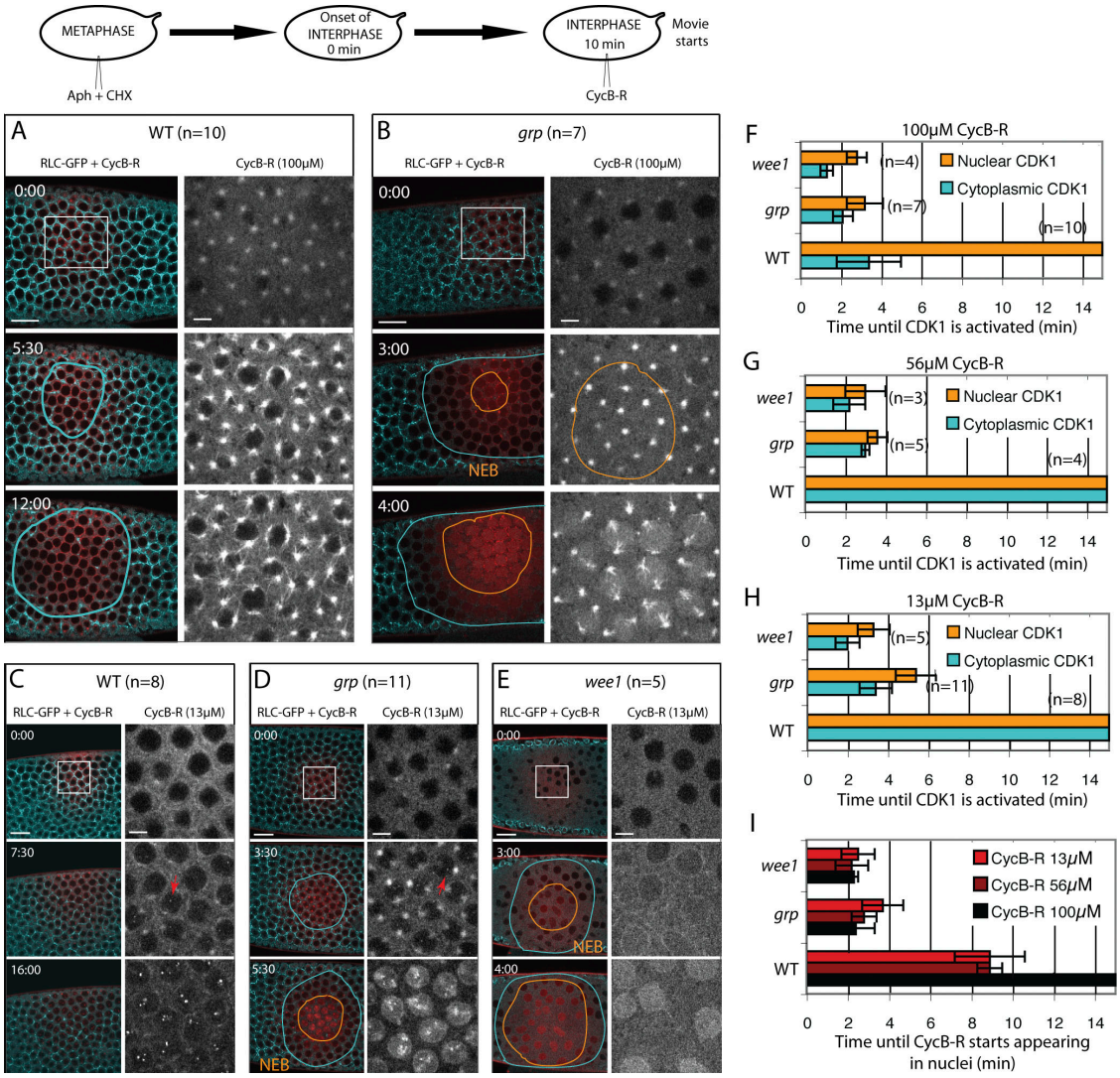
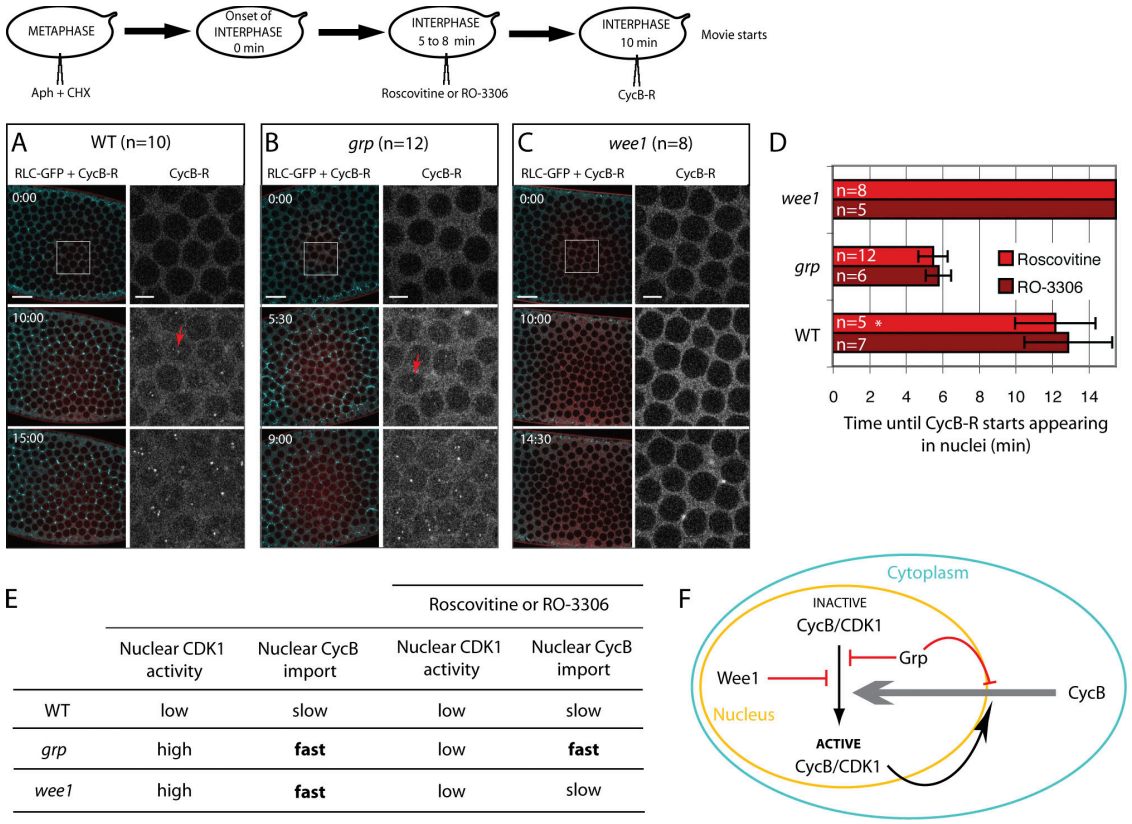


Figure 6. **The S-phase checkpoint delays nuclear CycB accumulation and CDK1 activation by a Grp and Wee1-dependent mechanism.** (A–E) Wild-type (A and C), *grp* (B and D), and *wee1* (E) embryos expressing RLC-GFP (cyan) were injected with a mix of Aph and CHX (CHX + Aph) at metaphase of cycle 12. 10 min after the onset of the next interphase, they were injected with CycB-R (red) at the specified concentrations. The schematic describes the injection and imaging sequence. Time is given in minutes/seconds (see Videos 6, 7, and 8 for A, B, and E, respectively, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). The right column of each panel is an enlargement of the area outlined with the white box shown on the first image of the left column. However, for A and B, the areas are at a focal plane 2 μm above the focal plane shown in the left columns. Blue outlines mark the areas with cytoplasmic CDK1 activity, and orange outlines mark the areas undergoing NEB. (C and D) Red arrows point to the nuclear localization of CycB-R. n, number of embryos injected. Bars: (left columns) 20 μm; (right columns) 5 μm. (F–H) 100 (F), 56 (G), and 13 μM (H) CycB-R was injected 10 min after the onset of interphase in CHX + Aph–treated embryos. The timing of cytoplasmic and nuclear CDK1 activation was determined as described in Fig. 2. (I) The timing of CycB-R accumulation in nuclei was determined when CycB-R appeared at kinetochores in at least three nuclei. (F–I) Data are represented as mean ± AVD. n, number of embryos injected.

cytoplasmic and nuclear entry into mitosis (Fig. 6, D, H, and I, blue and orange outlines, respectively). The first sign of CycB-R nuclear accumulation before NEB was detected at 2.2 min and 3.7 min after injection of 56 μM and 13 μM CycB-R, respectively (Fig. 6, D and I, red arrow).

These data indicate that the Grp S-phase checkpoint prevents mitotic entry by delaying nuclear CycB accumulation. It may be that a threshold of nuclear CDK1 activity is required to trigger CycB import. Activation of the S-phase checkpoint may prevent nuclear CDK1 from reaching this threshold. Support for this idea comes from experiments in *Xenopus laevis*, demonstrating that Chk1 phosphorylates and activates Wee1, a CDK1 inhibitor (Lee et al., 2001). Moreover, in mammalian cultured cells, Wee1 can protect the nucleus from active cytoplasmic CDK1 (Heald et al., 1993).

To test whether a threshold of nuclear CDK1 activity is required to promote CycB nuclear accumulation, we performed the same experiment as described in the previous paragraph in *wee1* mutant embryos. We monitored the accumulation of CycB-R in the nucleus and its effect on cytoplasmic and nuclear CDK1 activation. The injection of CHX + Aph during the previous mitosis induced an interphase arrest for at least 10 min in a *wee1* background. Injection of 100, 56, or 13 μM CycB-R provoked a rapid cytoplasmic and nuclear CDK1 activation (Fig. 6, E–H,



E

			Roscovitine or RO-3306	
	Nuclear CDK1 activity	Nuclear CycB import	Nuclear CDK1 activity	Nuclear CycB import
WT	low	slow	low	slow
grp	high	**fast**	low	**fast**
wee1	high	**fast**	low	slow

Figure 7. **Grp prevents CycB nuclear accumulation by a Wee1-independent mechanism.** (A–C) Wild-type (A), *grp* (B), and *wee1* (C) mutant embryos expressing RLC-GFP (cyan) were injected with a mix of Aph and CHX (CHX + Aph) at metaphase of cycle 12. 5–8 min after the onset of the next interphase, they were injected with 10 mM of the CDK1 inhibitor Roscovitine followed by 13 µM CycB-R (see Videos 9 and 10 for B and C, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). The schematic describes the injection and imaging sequence. The right column of each panel is an enlargement of the area outlined by the white box in the first images of the left column. Red arrows point to the nuclear localization of CycB-R. Time is given in minutes/seconds. Bars: (left) 20 µm; (right) 5 µm. (D) Histograms indicate the time until CycB-R starts accumulating in at least three nuclei in embryos treated with CHX + Aph and the CDK1 inhibitors Roscovitine or RO-3306. The embryos injected with RO-3306 and Roscovitine were subsequently injected with 56 µM and 13 µM, respectively. Data are represented as mean ± AVD. n, number of embryos injected. *, only 5 out of 10 wild-type embryos injected with Roscovitine showed nuclear CycB-R accumulation before 15 min, which is the time limit of each video for the Roscovitine assay. Therefore, the mean timing of nuclear CycB-R import represents only half of the embryos injected. (E) The table summarizes the results in Figs. 6 and 7. (F) The schematic presents a model for the control of CycB nuclear import. CycB nuclear accumulation is inhibited by Grp and activated by nuclear CDK1 activity, which is itself inhibited by Grp and Wee1.

blue and orange outlines, respectively; and Video 8, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). In each experiment, CycB-R nuclear import always occurred <3 min after injection (Fig. 6 I).

Collectively, these data reveal that high nuclear CDK1 activity correlates with rapid CycB nuclear import, and low nuclear CDK1 activity correlates with delayed CycB nuclear import (Fig. 7 E). The S-phase checkpoint, acting through Grp and Wee1, inhibits nuclear CDK1 activity and prevents CycB nuclear accumulation.

Grp also delays CycB nuclear accumulation via a mechanism independent of Wee1 and the state of nuclear CDK1 activity

To determine whether the S-phase checkpoint influences CycB nuclear import independently of the state of nuclear CDK1 activity, we monitored CycB-R dynamics in wild-type, *grp*, and *wee1* mutant embryos under conditions in which CDK1 is maintained inactive. This was achieved through injecting distinct, small molecule CDK1 inhibitors RO-3306 (Vassilev et al., 2006) and Roscovitine (Meijer et al., 1997). We performed the same experiments as described in Fig. 6, monitoring CycB nuclear import in wild-type, *grp*, and *wee1* embryos but with CDK1 maintained inactive through injection of CDK1 inhibitors (Fig. 7). In *grp* and *wee1* mutant embryos treated with CHX + Aph, CycB-R triggered rapid myosin dispersion from the cortex and NEB (Fig. 6, D–H). In contrast, the addition of Roscovitine or RO-3306 before CycB-R injection prevented both myosin dispersion and NEB (Fig. 7, B and C). This demonstrates that these compounds efficiently inhibit cytoplasmic and nuclear CDK1 activity.

In wild-type embryos treated with CHX + Aph and RO-3306, no nuclear CycB-R signal was detected in three out of seven embryos for up to 15 min. The first signs of CycB-R in the nuclei were detected at a mean of 12.9 min ($n = 7$; Fig. 7 D). A similar result was obtained with Roscovitine; no nuclear CycB-R signal was detected in 5 out of 10 embryos for up to 15 min (unpublished data). In the other five injected embryos, CycB-R started accumulating in the nucleus at a mean of 12.2 min (Fig. 7, A and D). In *grp* mutant embryos injected with CHX +

Aph and either RO-3306 or Roscovitine, no sign of cytoplasmic and nuclear CDK1 activity was observed. In contrast to wild-type embryos, CycB-R rapidly accumulated in the nucleus in all injected embryos. The first signs of CycB-R in the nucleus were detected at a mean of 5.5 min and 5.8 min in Roscovitine- and RO-3306–injected embryos, respectively (Fig. 7, B and D; and Video 9, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). In contrast, in the *wee1* mutant, no nuclear CycB-R was detected in any Roscovitine- or RO-3306–injected embryos for up to 15 min (Fig. 7, C and D; and Video 10). These data reveal that in addition to its role in maintaining nuclear CDK1 in an inactive state, Grp prevents nuclear accumulation of CycB through a separate mechanism not shared by Wee1 (Fig. 7 F).

Discussion

Increased levels of CycB induce premature cytoplasmic and nuclear CDK1 activation

In this study, we directly address the role of CycB in driving the syncytial divisions in the *Drosophila* embryo by demonstrating that increased levels of CycB are sufficient to trigger nuclear and cytoplasmic mitotic entry. Unlike more conventional cell cycles, the maternal pool of CycB is not fully degraded during these syncytial mitotic divisions; therefore, it was not clear whether CycB was limiting for CDK1 activation (Edgar et al., 1994). Our finding that inhibition of protein synthesis induced a prolonged interphase in wild-type and S-phase checkpoint–compromised embryos indicates that new rounds of protein synthesis are required to drive the late syncytial cycles. Of more significance is the fact that injection of CycB into these interphase-arrested embryos efficiently drives cytoplasmic and nuclear entry into mitosis. Our data imply that, even though CycB is not fully degraded at mitosis, new rounds of CycB synthesis are required to trigger the next mitosis. Support for this idea comes from the fact that increasing the maternal copies of CycB affects the timing of entry into mitosis during the late syncytial cycles (Ji et al., 2004). It may also be that the pool of endogenous CycB is maintained in a form that prevents CDK1 activation through posttranslational modifications and that the exogenous CycB lacks these modifications.

Careful analysis of the timing of cytoplasmic and nuclear mitotic events in the last syncytial cycle reveals that initiation of chromosome condensation and reorganization of the cytoskeleton occur before NEB. We have found that, even in the absence of protein synthesis, exogenous CycB can efficiently coordinate the reorganization of the cytoskeleton with NEB. However, exogenous CycB does not preserve the order of the nuclear mitotic events because the chromosomes did not condense before NEB. There are three maternal mitotic cyclins in a *Drosophila* embryo, cyclin A, B, and B3, with overlapping functions (Jacobs et al., 1998). One interesting idea is that the proper ratio of the three mitotic cyclins is required to maintain the order of the nuclear events.

We have observed that increased levels of CycB at the onset of cellularization during cycle 14 drive an additional round of syncytial mitosis. A recent study has shown that the knockdown of the three mitotic cyclins with RNAi blocks the syncytial cycles but does not affect the timing of cellularization (McCleland and O'Farrell, 2008). Both findings are compatible with the idea that low levels of CycB are required for the correct timing of cellularization. Earlier work demonstrated that inhibition of zygotic transcription also prevents cellularization and drives an additional round of syncytial mitosis (Edgar et al., 1986). Collectively, these results fit a model in which cellularization is triggered by thresholds of high levels of as yet unknown zygotic gene products and low levels of CycB. Although injecting CycB within the first 9 min of cycle 14 prevents cellularization, injecting CycB after this time point has no effect on cellularization. This suggests a narrow time window in which the embryo is not fully committed to cellularization.

CycB injections uncouple the cytoplasmic and nuclear cycles

Our results also demonstrate that CDK1 can be activated in the cytoplasm independent of its activation in the nucleus. The prolonged interphase of nuclear cycle 14 provided us with an opportunity to determine the effects of CycB injections later in interphase. Early injections of CycB (0–5 min after NEF) prematurely drive nuclear and cytoplasmic events. However, CycB injections later in interphase (6–9 min after NEF) drive the cytoplasm but not the nuclei into mitosis. Specifically, we observe myosin dispersion while the nuclear envelope remains intact. Given that myosin dispersion requires high CDK1 activity (Royou et al., 2002), these studies indicate that CDK1 is activated in the cytoplasm but not in the nucleus. This result is consistent with the observation that the cytoplasm continues to cycle in enucleated frog embryos (Wasserman and Smith, 1978). It is also in accord with studies in mammalian cultured cells and *Xenopus* egg extracts, indicating that the CDK1–CycB complex is activated in the cytoplasm before its nuclear activation (Heald et al., 1993; Jin et al., 1996; De Souza et al., 2000; Draviam et al., 2001; Jackman et al., 2003).

The S-phase checkpoint component Grp prevents nuclear CDK1 activation

In performing CycB injection in embryos treated with Aph and CHX, we observed that the effects of CycB on nuclear and cytoplasmic CDK1 activation varied depending on the timing of injection. CycB injections during early interphase drive both cytoplasmic and nuclear CDK1 activation, whereas later injections induced only cytoplasmic CDK1 activation. Moreover, this nuclear protection from premature cytoplasmic CDK1 activity upon late CycB injection depends on the S-phase checkpoint component Grp. Our observation that Grp must be imported into the nucleus at the beginning of interphase raises the possibility that time is required for sufficient quantities of Grp to accumulate in the nucleus and create a functional S-phase checkpoint. Injection of CycB induces mitosis in both the nuclei and cytoplasm early in interphase because the S-phase checkpoint is not fully established. However, later in interphase, Grp has accumulated sufficiently in the nucleus to completely prohibit nuclear CDK1 activation.

How does Grp protect the nucleus from active cytoplasmic CDK1? In response to damaged or incompletely replicated DNA, Chk1 phosphorylates Cdc25, altering its relative import/export rates such that it remains cytoplasmic (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Takizawa and Morgan, 2000). Additionally, Chk1 has been shown to phosphorylate and activate Wee1 (Lee et al., 2001). Although these findings remain to be demonstrated in *Drosophila*, it provides an explanation for the Grp-dependent protection of the nucleus from active cytoplasmic CDK1. Grp maintains the inhibitory tyrosine phosphorylation on nuclear CDK1 via activation of Wee1 and exclusion of Cdc25 from the nucleus. Support for this idea comes from a study in mammalian cultured cells in which overexpression of Wee1 prevents NEB in the presence of high cytoplasmic CDK1 activity (Heald et al., 1993). The fact that increased levels of cytoplasmic CycB overrides the cytoplasmic but not the nuclear S-phase checkpoint inhibitory signal suggests that the S-phase checkpoint is more efficient at inhibiting CDK1 in the nucleus. One idea is that the level of S-phase checkpoint activity is higher in the nucleus than in the cytoplasm. This notion is supported by experiments in binucleate yeast and sea urchin embryos in which one of the two nuclei has altered DNA. Entry into mitosis is delayed in the nucleus with altered DNA with respect to the other normal nucleus and the common cytoplasm (Sluder et al., 1995; Demeter et al., 2000). In addition, our observations, as well as others', that Grp and Wee1 are predominantly nuclear during S phase support this idea (Heald et al., 1993; Purdy et al., 2005).

Grp and Wee1 delay CycB nuclear accumulation

The subcellular localization of CycB plays an important role in regulating nuclear CDK1 activity (Takizawa and Morgan, 2000). Specifically, it has been shown in mammalian cultured cells that CycB is predominantly cytoplasmic during the G2 arrest caused by DNA damage, and a nuclear-targeted CycB can partially bypass this arrest (Smeets et al., 1994; Jin et al., 1998). To test the hypothesis that the S-phase checkpoint affects CycB subcellular localization, we injected CycB-R and monitored its dynamics after inhibition of DNA replication in wild-type embryos and embryos lacking Grp or Wee1. In a normal cycle, we observed that CycB-R is imported into the nucleus and forms discrete puncta, which colocalize with GFP-Polo and GFP-Cid, suggesting an association of CycB-R with kinetochores before NEB. This provides an excellent means to monitor CycB nuclear accumulation.

In embryos with high S-phase checkpoint activity, CycB nuclear accumulation was impaired. In contrast, in *grp* mutant embryos, CycB rapidly accumulated in the nucleus. One explanation is that in the absence of Grp, Wee1 activity is low, resulting in high nuclear CDK1 activity, which in turn promotes CycB import. Consistent with this hypothesis, we also found that CycB-R is rapidly imported into the nucleus and promotes NEB in *wee1* mutant embryos. Interestingly, the observation that high levels of CycB efficiently triggered cytoplasmic CDK1 activation but failed to localize in the nucleus reveals that cytoplasmic CDK1 activity is not sufficient to promote CycB nuclear import.

Grp also delays CycB nuclear accumulation by a mechanism independent of Wee1

Significantly, our observation that Grp-mediated inhibition of CycB nuclear import is independent of both Wee1 and the state of nuclear CDK1 activity reveals an additional mechanism by which the S-phase checkpoint prevents nuclear CDK1 activation. Monitoring CycB nuclear import in Aph and CHX-treated wild-type *wee1* and *grp* mutant embryos in which CDK1 is maintained in an inactive state reveals that CycB is rapidly imported into the nucleus only in the *grp* mutant. These results indicate a novel mechanism by which the S-phase checkpoint prevents mitotic entry: a Grp-dependent but Wee1-independent mechanism that prevents CycB nuclear accumulation. A satisfying aspect of this result is that it readily explains why bypassing interphase arrest caused by DNA damage not only requires a constitutively active form of CDK1 but also nuclear localized CycB (Jin et al., 1998). It would be of interest to determine whether Grp inhibits nuclear import of other cyclins as well.

The observation that CycB rapidly accumulates in the nucleus in the *wee1* mutant where Grp is fully active contrasts with our results that Grp delays CycB nuclear import. One idea is that, in the absence of *wee1*, the active form of nuclear CDK1 associated with basal levels of CycB promotes CycB (and more likely the CDK1–CycB complex) nuclear import, which in turn increases nuclear CDK1 activity. This triggers a positive feedback loop that efficiently bypasses the Grp inhibitory signal.

Model for the regulation of cytoplasmic and nuclear CDK1 activity

This study provides a model to explain the differential regulation of cytoplasmic and nuclear CDK1 in a normal cycle. We speculate that, at interphase, the slow rate of CycB synthesis in the cytoplasm provides sufficient time for cytoplasmic CDK1 inhibitors, such as Wee1 and Myt1, to prevent cytoplasmic CDK1 activation (Price et al., 2000; Stumpff et al., 2004; Jin et al., 2005). However, nuclear CDK1 inhibition is maintained by controlling CycB nuclear accumulation. At interphase, the rate of CycB nuclear translocation is low because of inhibition of nuclear CDK1 by Wee1 and Grp-dependent inhibition of CycB transport. Upon completion of S phase, Grp and Wee1 activity decreases. This triggers cytoplasmic CDK1 activation and initiation of cytoplasmic mitosis. This decrease in Grp and Wee1 activity also allows the abrupt CycB nuclear translocation catalyzed by nuclear CDK1 activity, thus increasing CDK1 activity to a level sufficient for triggering NEB.

Materials and methods

Drosophila stocks

The fly stocks GFP-H2Av (Clarkson and Saint, 1999), GFP-moesin (Edwards et al., 1997), RLC-GFP (Royou et al., 2004), GFP-Nuf (Riggs et al., 2007), GFP-Polo (Moutinho-Santos et al., 1999), and GFP-Cid (Schuh et al., 2007) were used in these studies. The *grp* embryos were derived from a female homozygote for the grp^{1} null allele (Fogarty et al., 1994). The *wee1* embryos were derived from females carrying the null allele $dwee1^{ES1}$ over Df(2L)$dwee1^{wo5}$ deficiency (Price et al., 2000).

GFP-Grp cloning

GFP-Grp–bearing transgenics were constructed by ligating a full-length *grp* cDNA into pEGFP-C3 (Clontech Laboratories, Inc.) upstream of the GFP

construct. The GFP-Grp subclone was ligated into the Germ-10 transformation vector. Transgenics were generated by standard methods.

CycB purification

Two independent GST-CycB purifications were performed and produced identical results. CycB cDNA was cloned at its N terminal to GST in pGEX-1 vector. The recombinant protein was overexpressed and purified from 4 liters of XL-10 bacteria culture (Stratagene) grown in 2XYT (tryptone, yeast extract, and NaCl). The culture was grown at RT until it reached an OD of 0.8. The cells were induced with 100 µM IPTG for 12 h at RT. The cells were harvested by spinning them for 5 min at 5,000 rpm. The pellet was ground in liquid nitrogen for 15 min. The powder was dissolved in 5 vol PBS that contained 0.5% Tween 20, 1 mM PMSF, and 1 M NaCl. The solution was sonicated three times for 20 s. 10 mM DTT was added to the solution before its ultracentrifugation at 40,000 rpm for 1 h in a 60.2 Ti rotor (Beckman Coulter). The supernatant was loaded onto a 9-ml glutathione-agarose column (Sigma-Aldrich) for a period of 2–4 h. The column was extensively washed with PBS, pH 7.4, 1 M NaCl, 0.05% Tween 20, and 0.5 mM DTT and rinsed with a 1-vol column with wash buffer without Tween 20. The protein was eluted with 50 mM Tris, pH 8.1, containing 500 mM KCl and 5 mM of reduced glutathione. The fractions were pooled and dialyzed extensively into 50 mM Hepes, pH 7.6, 500 mM KCl, and 30% glycerol. In all of the experiments shown in Figs. 1–4, CycB was injected at a concentration of 65 µM. A recent study used such purified recombinant GST-CycB and has demonstrated that it retains its ability to bind and activate CDK1 in vitro (Edgar et al., 1994). Another recent study has shown that GST-CycB is resistant to degradation in *Xenopus* embryo extracts (Su et al., 1998). Our studies have focused on mitotic entry, not exit, so the fact that it is not degraded should not affect the interpretation of our experiments.

CycB labeling

CycB was coupled with NHS-rhodamine (Thermo Fisher Scientific) in a 1:4 ratio. The solution was mixed gently and sat at RT for 75 min. The solution was loaded onto a 3-ml Sephadex G-25 column (GE Healthcare). The column was washed with 50 mM Hepes, pH 7.6, 500 mM KCl, and 30% glycerol. The absorbance of the fractions was measured with a NanoDrop (Eppendorf). The stochiometry of the labeling was calculated as 0.6 mol NHS-rhodamine per mole of CycB.

Microscopy

Microscopy was performed at RT with an inverted microscope (DMIRB; Leitz) equipped with a scanning laser confocal imaging system (TCS NT; Leica). Images were acquired with a 63× lens and confocal software (version 2.61; Leica). All embryos were monitored at three different z positions every 30 s for various amounts of time. Each image in all figures represents a single focal plane.

Microinjection

The embryos were prepared for microinjection as follows. They were dechorionated by hand on a double-sided sticky tape and aligned on a coverslip covered with glue. They were dehydrated and covered with halocarbon oil. The embryos were injected at the onset of interphase 2 min after NEF with either dialyzed buffer (50 mM Hepes, pH 7.6, 500 mM KCl, and 30% glycerol), 71 µM GST, or 65, 32, 13, and 6.5 µM CycB. The embryos were injected at the center lengthwise and one third heightwise toward the surface. The same hole was used for double and triple injections. The embryos were monitored within 30 s after injection. Injection of CycB at 13 and 6.5 µM did not have a significant effect on timing of cytoplasmic and nuclear entry into mitosis in wild-type embryos ($n = 6$ for each concentration). Aph and CHX (Sigma-Aldrich) were used at final concentrations of 300 µM and 1 mg/ml, respectively, in 2% DMSO and injected either on their own or mixed together during mitosis. CycB-R was injected at 13, 56, or 100 µM. Roscovitine and RO-3306 (EMD) were injected at 10 mM in 100% DMSO. Injection of 100% DMSO was used as a control. Rhodamine-conjugated tubulin (cytoskeleton) and Cy5-labeled histone (provided by E. Homola, University of Alberta, Edmonton, Canada) were injected at interphase of the previous cycle. The quantification of the RLC-GFP signal was performed by drawing two regions of interest of the same size, one near the site of CycB injection and one in a region not affected by CycB injection. The mean pixel intensity for each region was calculated using the Leica software.

Single-embryo Western blot and determination of the amount of injected CycB relative to the level of endogenous CycB

RLC-GFP embryos were prepared for microinjection as described in the previous paragraph. They were monitored with the confocal microscope until they reached interphase of cycle 13. They were injected with 0.5 mM colchicine and monitored until they reached metaphase. Each embryo was either collected and boiled in 10 µl of sample buffer or injected with 65 µM CycB and immediately collected and boiled in 10 µl of sample buffer. The samples were loaded on a 10% acrylamide gel and transferred onto the nylon membrane, and the membrane was probed with anti-CycB to reveal endogenous CycB and GST-CycB. Membranes were also probed with anti-GFP (Roche) to reveal RLC-GFP as a loading control. As expected, the injected embryo extract reveals an additional higher molecular weight GST-CycB band (Fig. 1 D, injected CycB). The intensities of the endogenous and injected CycB bands were estimated to be equivalent. Because CycB is injected at the center of the embryo lengthwise and diffuses very slowly through a fifth of the embryo, we estimated that, at the center of the gradient, the injected CycB is <10-fold the amount of endogenous CycB at mitosis of cycle 13.

Online supplemental material

Fig. S1 shows RLC-GFP signal intensities over time in control and CycB-injected areas of embryos previously injected or not injected with CHX and Aph. Fig. S2 shows a saggital view of an embryo expressing RLC-GFP injected with CycB 8 min after the onset of cellularization. Fig. S3 shows the dynamics of Grp-GFP from cycle 13 through cycle 14 in an embryo previously injected with rhodamine-tubulin. Video 1 corresponds to the embryo presented in Fig. 1 C. Video 2 corresponds to the embryo presented in Fig. 2 B. Video 3 corresponds to the embryo presented in Fig. 2 C. Video 4 corresponds to the embryo presented in Fig. 3 A. Video 5 corresponds to the embryo presented in Fig. 3 B. Video 6 corresponds to the embryo presented in Fig. 6 A. Video 7 corresponds to the embryo presented in Fig. 6 B. Video 8 corresponds to the embryo presented in Fig. 6 E. Video 9 corresponds to the embryo presented in Fig. 7 B, where only CycB-R is visualized. Video 10 corresponds to the embryo presented in Fig. 7 C, where only CycB-R is visualized. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1.

We thank K. Yu (Exploratorium, San Francisco, CA) for the GFP-Grp construct and transgenic stock, S. Campbell (University of Alberta, Edmonton, Canada) and E. Homola for the Cy5-histone and critical reading of the manuscript, and M. McCleland and P. O'Farrell for helpful discussions.

A. Royou and W. Sullivan were supported by grants from the California Institute for Regenerative Medicine and the National Institutes of Health (GM046409), respectively. D. McCusker and D. Kellogg were supported by a grant from the National Institutes of Health (GM69602).

Submitted: 24 January 2008
Accepted: 3 September 2008

References

Cao, J., R. Albertson, B. Riggs, C.M. Field, and W. Sullivan. 2008. Nuf, a Rab11 effector, maintains cytokinetic furrow integrity by promoting local actin polymerization. *J. Cell Biol.* 182:301–313.

Clarkson, M., and R. Saint. 1999. A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for *Drosophila* chromosome behavior. *DNA Cell Biol.* 18:457–462.

Crest, J., N. Oxnard, J.Y. Ji, and G. Schubiger. 2007. Onset of the DNA replication checkpoint in the early *Drosophila* embryo. *Genetics.* 175:567–584.

De Souza, C.P., K.A. Ellem, and B.G. Gabrielli. 2000. Centrosomal and cytoplasmic Cdc2/cyclin B1 activation precedes nuclear mitotic events. *Exp. Cell Res.* 257:11–21.

Demeter, J., S.E. Lee, J.E. Haber, and T. Stearns. 2000. The DNA damage checkpoint signal in budding yeast is nuclear limited. *Mol. Cell.* 6:487–492.

Draviam, V.M., S. Orrechia, M. Lowe, R. Pardi, and J. Pines. 2001. The localization of human cyclins B1 and B2 determines CDK1 substrate specificity and neither enzyme requires MEK to disassemble the Golgi apparatus. *J. Cell Biol.* 152:945–958.

Edgar, B.A., C.P. Kiehle, and G. Schubiger. 1986. Cell cycle control by the nucleocytoplasmic ratio in early *Drosophila* development. *Cell.* 44:365–372.

Edgar, B.A., F. Sprenger, R.J. Duronio, P. Leopold, and P.H. O'Farrell. 1994. Distinct molecular mechanism regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes Dev.* 8:440–452.

Edwards, K.A., M. Demsky, R.A. Montague, N. Weymouth, and D.P. Kiehart. 1997. GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in *Drosophila*. *Dev. Biol.* 191:103–117.

Enoch, T., M. Peter, P. Nurse, and E.A. Nigg. 1991. p34cdc2 acts as a lamin kinase in fission yeast. *J. Cell Biol.* 112:797–807.

Ferrell, J.E., Jr. 2002. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Curr. Opin. Cell Biol.* 14:140–148.

Fogarty, P., R.F. Kalpin, and W. Sullivan. 1994. The *Drosophila* maternal-effect mutation grapes causes a metaphase arrest at nuclear cycle 13. *Development.* 120:2131–2142.

Fogarty, P., S.D. Campbell, R. Abu-Shumays, B.S. Phalle, K.R. Yu, G.L. Uy, M.L. Goldberg, and W. Sullivan. 1997. The *Drosophila* grapes gene is related to checkpoint gene chk1/rad27 and is required for late syncytial division fidelity. *Curr. Biol.* 7:418–426.

Furnari, B., N. Rhind, and P. Russell. 1997. Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. *Science.* 277:1495–1497.

Hagting, A., C. Karlsson, P. Clute, M. Jackman, and J. Pines. 1998. MPF localization is controlled by nuclear export. *EMBO J.* 17:4127–4138.

Hagting, A., M. Jackman, K. Simpson, and J. Pines. 1999. Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. *Curr. Biol.* 9:680–689.

Heald, R., M. McLoughlin, and F. McKeon. 1993. Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. *Cell.* 74:463–474.

Huang, J., and J.W. Raff. 1999. The disappearance of cyclin B at the end of mitosis is regulated spatially in *Drosophila* cells. *EMBO J.* 18:2184–2195.

Jackman, M., C. Lindon, E.A. Nigg, and J. Pines. 2003. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat. Cell Biol.* 5:143–148.

Jacobs, H.W., J.A. Knoblich, and C.F. Lehner. 1998. *Drosophila* Cyclin B3 is required for female fertility and is dispensable for mitosis like Cyclin B. *Genes Dev.* 12:3741–3751.

Ji, J.Y., J.M. Squirrell, and G. Schubiger. 2004. Both cyclin B levels and DNA-replication checkpoint control the early embryonic mitoses in *Drosophila*. *Development.* 131:401–411.

Jin, P., Y. Gu, and D.O. Morgan. 1996. Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J. Cell Biol.* 134:963–970.

Jin, P., S. Hardy, and D.O. Morgan. 1998. Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. *J. Cell Biol.* 141:875–885.

Jin, Z., E.M. Homola, P. Goldbach, Y. Choi, J.A. Brill, and S.D. Campbell. 2005. *Drosophila* Myt1 is a Cdk1 inhibitory kinase that regulates multiple aspects of cell cycle behavior during gametogenesis. *Development.* 132:4075–4085.

Lamb, N.J., A. Fernandez, A. Watrin, J.C. Labbe, and J.C. Cavadore. 1990. Microinjection of p34cdc2 kinase induces marked changes in cell shape, cytoskeletal organization, and chromatin structure in mammalian fibroblasts. *Cell.* 60:151–165.

Lee, J., A. Kumagai, and W.G. Dunphy. 2001. Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. *Mol. Biol. Cell.* 12:551–563.

Li, J., A.N. Meyer, and D.J. Donoghue. 1997. Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. *Proc. Natl. Acad. Sci. USA.* 94:502–507.

McCleland, M.L., and P.H. O'Farrell. 2008. RNAi of mitotic cyclins in *Drosophila* uncouples the nuclear and centrosome cycle. *Curr. Biol.* 18:245–254.

Meijer, L., A. Borgne, O. Mulner, J.P. Chong, J.J. Blow, N. Inagaki, M. Inagaki, J.G. Delcros, and J.P. Moulinoux. 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* 243:527–536.

Melo, J., and D. Toczyski. 2002. A unified view of the DNA-damage checkpoint. *Curr. Opin. Cell Biol.* 14:237–245.

Morgan, D.O. 2006. The Cell Cycle: Principles of Control. New Science Press Ltd, London. 297 pp.

Moutinho-Santos, T., P. Sampaio, I. Amorim, M. Costa, and C.E. Sunkel. 1999. In vivo localisation of the mitotic POLO kinase shows a highly dynamic association with the mitotic apparatus during early embryogenesis in *Drosophila*. *Biol. Cell.* 91:585–596.

Onischenko, E.A., N.V. Gubanova, E.V. Kiseleva, and E. Hallberg. 2005. Cdk1 and okadaic acid-sensitive phosphatases control assembly of nuclear pore complexes in *Drosophila* embryos. *Mol. Biol. Cell.* 16:5152–5162.

Ookata, K., S. Hisanaga, E. Okumura, and T. Kishimoto. 1993. Association of p34cdc2/cyclin B complex with microtubules in starfish oocytes. *J. Cell Sci.* 105:873–881.

Peng, C.Y., P.R. Graves, R.S. Thoma, Z. Wu, A.S. Shaw, and H. Piwnica-Worms. 1997. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science.* 277:1501–1505.

Peter, M., J. Nakagawa, M. Doree, J.C. Labbe, and E.A. Nigg. 1990. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell.* 61:591–602.

Pines, J., and T. Hunter. 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J. Cell Biol.* 115:1–17.

Pines, J., and T. Hunter. 1994. The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. *EMBO J.* 13:3772–3781.

Price, D., S. Rabinovitch, P.H. O'Farrell, and S.D. Campbell. 2000. *Drosophila* wee1 has an essential role in the nuclear divisions of early embryogenesis. *Genetics.* 155:159–166.

Purdy, A., L. Uyetake, M.G. Cordeiro, and T.T. Su. 2005. Regulation of mitosis in response to damaged or incompletely replicated DNA require different levels of Grapes (*Drosophila* Chk1). *J. Cell Sci.* 118:3305–3315.

Riggs, B., W. Rothwell, S. Mische, G.R. Hickson, J. Matheson, T.S. Hays, G.W. Gould, and W. Sullivan. 2003. Actin cytoskeleton remodeling during early *Drosophila* furrow formation requires recycling endosomal components Nuclear-fallout and Rab11. *J. Cell Biol.* 163:143–154.

Riggs, B., B. Fasulo, A. Royou, S. Mische, J. Cao, T.S. Hays, and W. Sullivan. 2007. The concentration of Nuf, a Rab11 effector, at the microtubule-organizing center is cell cycle-regulated, dynein-dependent, and coincides with furrow formation. *Mol. Biol. Cell.* 18:3313–3322.

Royou, A., W. Sullivan, and R. Karess. 2002. Cortical recruitment of nonmuscle myosin II in early syncytial *Drosophila* embryos: its role in nuclear axial expansion and its regulation by Cdc2 activity. *J. Cell Biol.* 158:127–137.

Royou, A., C. Field, J.C. Sisson, W. Sullivan, and R. Karess. 2004. Reassessing the role and dynamics of nonmuscle myosin II during furrow formation in early *Drosophila* embryos. *Mol. Biol. Cell.* 15:838–850.

Sanchez, Y., C. Wong, R.S. Thoma, R. Richman, Z. Wu, H. Piwnica-Worms, and S.J. Elledge. 1997. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science.* 277:1497–1501.

Schuh, M., C.F. Lehner, and S. Heidmann. 2007. Incorporation of *Drosophila* CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. *Curr. Biol.* 17:237–243.

Sibon, O.C., V.A. Stevenson, and W.E. Theurkauf. 1997. DNA-replication checkpoint control at the *Drosophila* midblastula transition. *Nature.* 388:93–97.

Sluder, G., E.A. Thompson, C.L. Rieder, and F.J. Miller. 1995. Nuclear envelope breakdown is under nuclear not cytoplasmic control in sea urchin zygotes. *J. Cell Biol.* 129:1447–1458.

Smeets, M.F., E.H. Mooren, and A.C. Begg. 1994. The effect of radiation on G2 blocks, cyclin B expression and cdc2 expression in human squamous carcinoma cell lines with different radiosensitivities. *Radiother. Oncol.* 33:217–227.

Stiffler, L.A., J.Y. Ji, S. Trautmann, C. Trusty, and G. Schubiger. 1999. Cyclin A and B functions in the early *Drosophila* embryo. *Development.* 126:5505–5513.

Stumpff, J., T. Duncan, E. Homola, S.D. Campbell, and T.T. Su. 2004. *Drosophila* Wee1 kinase regulates Cdk1 and mitotic entry during embryogenesis. *Curr. Biol.* 14:2143–2148.

Su, T.T., F. Sprenger, P.J. DiGregorio, S.D. Campbell, and P.H. O'Farrell. 1998. Exit from mitosis in *Drosophila* syncytial embryos requires proteolysis and cyclin degradation, and is associated with localized dephosphorylation. *Genes Dev.* 12:1495–1503.

Takizawa, C.G., and D.O. Morgan. 2000. Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. *Curr. Opin. Cell Biol.* 12:658–665.

Vassilev, L.T., C. Tovar, S. Chen, D. Knezevic, X. Zhao, H. Sun, D.C. Heimbrook, and L. Chen. 2006. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc. Natl. Acad. Sci. USA.* 103:10660–10665.

Walworth, N.C. 2001. DNA damage: Chk1 and Cdc25, more than meets the eye. *Curr. Opin. Genet. Dev.* 11:78–82.

Wasserman, W.J., and L.D. Smith. 1978. The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown. *J. Cell Biol.* 78:R15–R22.

Yang, J., E.S. Bardes, J.D. Moore, J. Brennan, M.A. Powers, and S. Kornbluth. 1998. Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. *Genes Dev.* 12:2131–2143.