

# Negative Regulation of dE2F1 by Cyclin-Dependent Kinases Controls Cell Cycle Timing

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## Summary

Many types of cells compensate for induced alterations in the length of one cell cycle phase (G1, S, or G2) by altering the lengths of the other phases. Here we show that, when cells in *Drosophila* wing discs are delayed in G1, they maintain normal division rates by accelerating passage through S and G2. Similarly, when G2→M progression is retarded, G1→S progression accelerates. This compensation mechanism employs negative feedback in which the cyclin-dependent kinases Cdk1 and Cdk2 downregulate the transcription factor dE2F1. dE2F1, in turn, positively regulates *cyclin E* and *string/cdc25*, which activate the Cdk1 to drive cell cycle progression. This homeostatic mechanism coordinates rates of G1→S and G2→M progression, maintaining normal rates of proliferation when cell cycle controls are perturbed (e.g., by ectopic Dacapo, dWee1, dMyc, or Rheb). Without dE2F1, the compensatory mechanism fails, and treatments that alter Cdk activity cause aberrant cell cycle timing and cell death.

## Introduction

Cells replicate their chromosomes in S phase and separate them during mitosis. The intervening gap phases G1 and G2 are preparative for S phase and mitosis, respectively. In metazoa, the activation of cyclin E/cyclin-dependent kinase 2 (CycE/Cdk2) triggers the onset of DNA replication (Ohtsubo and Roberts, 1993; Knoblich et al., 1994; Resnitzky et al., 1994; Duronio and O'Farrell, 1995; Ohtsubo et al., 1995; Neufeld et al., 1998). During G2, the activation of Cdk1 complexed with A- and B-type cyclins drives progression into mitosis (Gautier et al., 1989; Lehner and O'Farrell, 1989; Murray and Kirschner, 1989; Murray et al., 1989). Although much has been learned about how the cyclin/Cdk complexes are regulated, relatively little is known about how rates of progression through the different phase transitions (G1→S and G2→M) are coordinated to set overall cell cycle length. In both animal and plant cells, overexpression of G1 cyclins results in cell cycles with shorter G1 phases but relatively normal division rates (Ohtsubo and Rob-

erts, 1993; Resnitzky et al., 1994; A. Samland and J.A.H. Murray, personal communication). Experiments with regulators of mitosis in fission yeast showed that alterations in G2→M progression also do not necessarily alter overall cell cycle duration (Nurse and Thuriaux, 1980; Russell and Nurse, 1986). Similarly, in the *Drosophila* wing, overexpression of rate-limiting positive regulators for G1→S progression (CycE) or G2→M progression (String [Stg]/Cdc25) causes specific cell cycle phases (G1 or G2) to be truncated but does not substantially alter overall rates of cell division (Neufeld et al., 1998). The observation that Cdk activity and phase length can be modulated without affecting overall division rates indicates that a regulatory connection exists between G1→S and G2→M controls.

We considered two possible explanations for this compensatory phenomenon. By accelerating progress through one phase of the cell cycle, all the required preparative events for the following phase might not be completed on time, and cells entering the next phase would consequently be delayed until the required machinery was assembled. For example, if DNA polymerases normally accumulate in G1, cells entering S phase precociously might suffer delayed rates of DNA replication, thereby elongating S phase. Alternatively, cells might actively monitor the duration of each phase of the cycle in order to maintain overall cycle duration. If this timing was disturbed, a compensatory mechanism intrinsic to the cell cycle control apparatus would ensure optimal cell cycle timing.

We sought to distinguish between these two possibilities by inducing elongation of a specific phase of the cell cycle (G1 or G2) and monitoring the effects on the cell cycle as a whole. If the former hypothesis were correct, prolonging a specific phase of the cycle should not affect subsequent phases and would result in an overall elongation of the cell cycle. If the latter explanation were correct, elongation of a specific phase would trigger a compensatory shortening of another phase(s), thereby allowing normal division rates. Our experiments were performed *in vivo* in the asynchronous cell cycles of *Drosophila* wing discs. During larval development, these epithelial cells proliferate exponentially with asynchronous G1→S→G2→M cell cycles. Regulation of these cell cycles has been characterized in depth (Garcia-Bellido and Merriam, 1971; Madhavan and Schneiderman, 1977; Milan et al., 1996a, 1996b; Kylsten and Saint, 1997; Weigmann et al., 1997; Neufeld et al., 1998). CycE is required and rate limiting for G1→S progression, whereas the Cdk1-specific tyrosine phosphatase Stg/Cdc25 is required and rate limiting for G2→M progression. Both genes can be transcriptionally activated by overexpression of the *Drosophila* E2F1/DP (dE2F1/dDP) transcription factor, a treatment which, as one would predict, accelerates both phase transitions and shortens the overall cell cycle duration (Neufeld et al., 1998). Like its human orthologs, dE2F1 is subject to cell cycle-dependent positive feedback from CycE/Cdk2, which relieves repression of dE2F1 by RBF1, a *retinoblastoma* family member (Du et al., 1996). Additionally, E2F protein

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levels may be regulated. Human E2F1 protein is periodically targeted for ubiquitin-mediated degradation after G1→S transitions and thus appears to be subject to cell cycle-dependent negative feedback (Marti et al., 1999). A similar mechanism may apply to *Drosophila* E2F1, which is absent during S phase (Asano et al., 1996; Heriche et al., 2003). Considering that E2F can regulate both G1→S and G2→M phase transitions (Neufeld et al., 1998; Ishida et al., 2001) and is also subject to cell cycle-dependent regulation, it is a good candidate for a coordinator of overall cell cycle duration.

In this study, we specifically inhibit in developing wing cells either G1→S or G2→M progression and characterize the response of the overall cell cycle timing, phasing, and activity of regulatory factors. Our results indicate that compensatory feedback between G1→S and G2→M progression is an active mechanism that derives from inherent properties of the cell cycle control apparatus. Our data indicate that dE2F1 both regulates and responds to levels of Cdk activity, allowing the coordinate regulation of G1→S and G2→M progression.

**Results**

**Inhibition of Cyclin E/Cdk2 Elongates G1, but Cells Compensate by Shortening G2**

Previous studies showed that wing disc cells compensate for truncation of either G1 or G2 by elongating other phases (Neufeld et al., 1998). To further examine this compensatory effect, we specifically elongated G1 or G2. If an active mechanism of cell cycle compensation exists, cells would be expected to compensate not only when phases are shortened but also when they are elongated.

We used the Flp/Gal4 method (Pignoni and Zipursky, 1997; Neufeld et al., 1998) to activate UAS-linked transgenes in random cells at defined times in development. UAS-GFP was coexpressed as a marker. First, we induced expression of the *CycE/Cdk2*-specific inhibitor *dacapo* (*dap*), the fly homolog of human p21<sup>cip1</sup> and p27<sup>kip1</sup> (de Nooij et al., 1996; Lane et al., 1996), in young larval tissues and analyzed DNA profiles of wing disc cells by flow cytometry (FACS) 2 days later. As expected, ectopic *dap* caused cells to accumulate in G1 (Figure 1C). Next, we calculated cell division rates by determining the median number of cells per clone in clones that were allowed to grow during a defined time period (Neufeld et al., 1998). Analysis of division rates for wild-type (wt) and *dap*-overexpressing cells revealed that cell doubling times (CDT) were not significantly different in the two cell types (Figures 1B and 1D;  $p = 0.19$ ), despite the profound difference in cell cycle phasing. Wild-type cells spent on average 4.4 hr in G1, 4.0 hr in S, and 6.4 hr in G2, whereas cells overexpressing *dap* (line II.4) spent 7.3 hr in G1, 2.9 hr in S, and 4.8 hr in G2.

We considered the possibility that Dap might be differentially affecting these asynchronously dividing cells and arresting a fraction of them in G1. To label proliferating cells, we fed bromodeoxyuridine (BrdU) to larvae over a 24 hr period, longer than the average 15 hr cell cycle. By assaying BrdU incorporation, we confirmed that, as in the wt, ~90% of the *dap* (line II.4)-overexpressing cells were dividing (data not shown). Several other UAS-*dap* transgenes providing different levels of

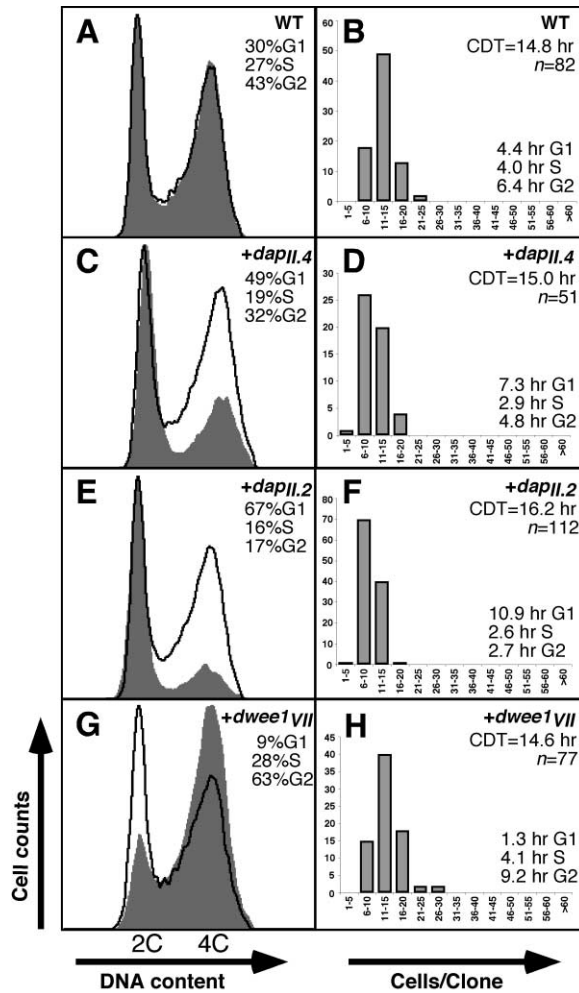


Figure 1. Effects of *dacapo* or *dwee1* Overexpression on Wing Disc Cell Cycles

(Left column) DNA profiles of wing disc cells overexpressing GFP alone (A) or coexpressing GFP and *dap* (C and E) or *dwee1* (G) are represented in gray, filled. Endogenous GFP-negative wt cells are represented by a solid black line. Percentages of G1, S, and G2 refer to GFP-positive cells only. (Right column) Distribution of the number of cells per clone and derived median CDT in hours. (B) Cells expressing GFP only. (D and F) *dap*-overexpressing cells. (H) *dwee1*-overexpressing cells. n, number of clones analyzed. Each experiment was performed at least three times for four different alleles of UAS-*dap* and at least twice for two different alleles of UAS-*dwee1*. Shown is one representative result.

expression were also analyzed (lines II.2, II.3, and III). In situations of extreme G1 elongation (Figure 1E), CDT were longer than wt (Figures 1B and 1F;  $p < 0.0001$ ), resulting in smaller clones. Some cells in these clones had a cell cycle longer than 24 hr, as evidenced by lack of BrdU incorporation (data not shown), and were probably arrested. We conclude that, within limits, elongation of G1 does not lead to a lengthened cell cycle. Rather, cells compensate by shortening their S and G2 phases.

***dacapo* Induces Accumulation of String, dE2F1, and Cyclin E**

Because G2 is shortened when *dap* is overexpressed, we asked whether Stg, the rate-limiting factor for G2→M

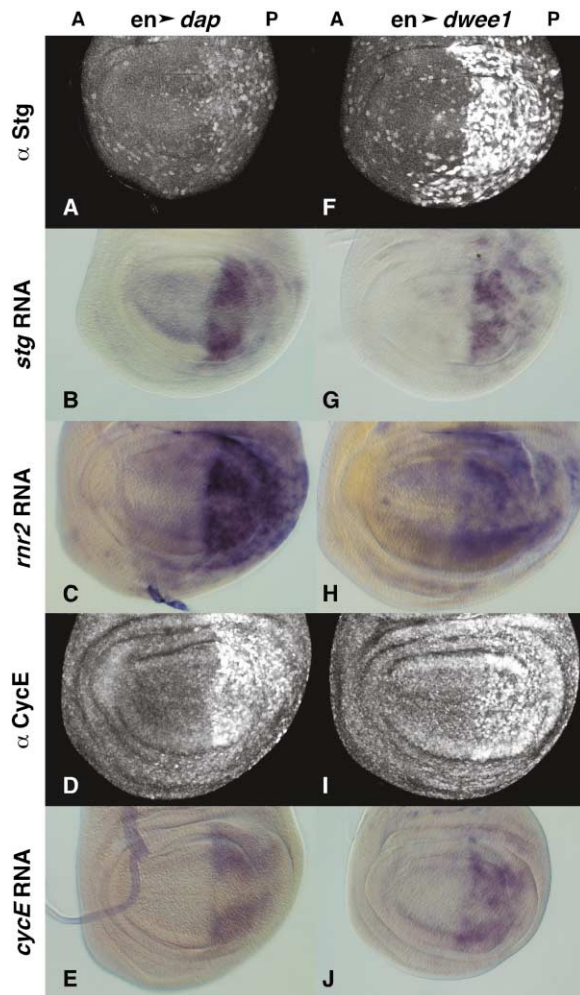


Figure 2. Effects of *dap* or *dwee1* Overexpression on Stg, CycE, and dE2F1 Activity

*en*-Gal4 was used to drive expression of *dap* (A–E) or *dwee1* (F–J) in the posterior (P; right half) compartments of wing discs. Wing discs were analyzed for levels of Stg protein (A and F) or *stg* RNA (B and G). (C and H) RNA in situ hybridization against the dE2F1 target gene *rnr2*. Levels of CycE protein (D and I) and *cycE* RNA (E and J) were also examined.

progression (Edgar and O'Farrell, 1989, 1990; Milan et al., 1996b; Kylsten and Saint, 1997; Neufeld et al., 1998), was involved in this compensatory G2 truncation. We used the *engrailed*-Gal4 (*en*-Gal4) driver to overexpress UAS-*dap* and UAS-GFP specifically in posterior (P) compartments of wing discs. This driver facilitates the comparison of wt control (anterior [A] compartment) and experimental cells (P compartment), since these cells exist in adjacent regions of the same tissue. Immunostaining of third instar (L3) larval wing discs revealed increased Stg protein in P cells overexpressing Dap (Figure 2A). Stg protein was not present in every P cell, suggesting that its previously described periodic cell cycle regulation (Edgar and Datar, 1996) was maintained. To determine whether the observed accumulation of Stg was due to increased transcription, we performed RNA in situ hybridization for *stg* mRNA (Figure 2B). *stg* mRNA accumulated in cells overexpressing *dap*, demonstrating that *stg* transcription was increased.

Previously, it was shown that *stg* transcription could be induced in wing disc cells by overexpressed dE2F1 (Neufeld et al., 1998). To determine whether dE2F1 was responsible for the accumulation of *stg* mRNA in *dap*-overexpressing cells, we assayed the expression of *ribonucleotide reductase 2/ribonucleotide reductase S* (*rnr2/rnrS*), a well-defined transcriptional target of dE2F1 activity (Duronio et al., 1995). As shown by RNA in situ hybridization, P cells overexpressing *dap* also upregulated *rnr2* transcription (Figure 2C). CycE RNA and protein accumulation were also observed in these cells (Figures 2E and 2D), consistent with *cycE* being a transcriptional target of dE2F1 (Duronio et al., 1996; Royzman et al., 1997; Neufeld et al., 1998; Dimova et al., 2003). Since G1 was extended, the upregulation of CycE was apparently insufficient to overcome the inhibitory effect of *dap* overexpression on Cdk2 activity.

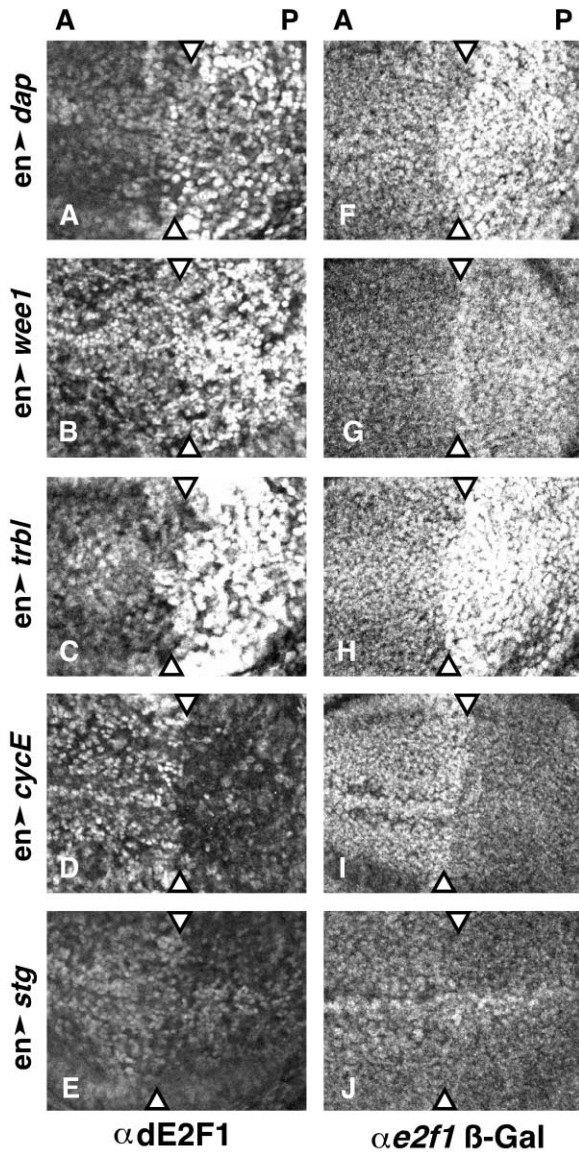
To address how dE2F1 activity was increased, we assayed the effect of overexpressed Dap on dE2F1 protein levels. Immunostaining revealed increased levels of dE2F1 protein in cells overexpressing Dap (Figure 3A). The increased dE2F1 protein correlated with elevated expression of dE2F1 targets, one of which, *stg*, is likely responsible for the observed compensatory shortening of G2.

#### Inhibition of Cdk1 Elongates G2, but Cells Compensate by Shortening G1

To address the effects on cell cycle timing when G2 is elongated, we overexpressed the Cdk1 inhibitory kinase *Drosophila wee1* (*dwee1*) (Campbell et al., 1995; Price et al., 2000). dWee1 inhibits G2→M progression by phosphorylating Cdk1 at tyrosine 15, an effect that is counteracted by Stg phosphatase activity (Edgar et al., 1994; Campbell et al., 1995). FACS analysis of cells overexpressing *dwee1* showed accumulation of cells in G2 (Figure 1G). Two other UAS-*dwee1* transgenics were analyzed, with similar results (data not shown). These clones showed rates of division similar to wt controls (Figures 1B and 1H;  $p = 0.20$ ), indicating that elongating G2 did not extend the entire cell cycle. Instead, G1 was shorter. *dwee1*-overexpressing cells spent 1.3 hr in G1, 4.1 hr in S phase, and 9.2 hr in G2, whereas wt cells spent 4.4 hr in G1, 4.0 hr in S phase, and 6.4 hr in G2. To confirm that all cells in the clones were cycling, animals were fed BrdU for 24 hr. As in the wt, ~90% of *dwee1*-overexpressing cells incorporated BrdU, indicating that dWee1 overexpression did not arrest a significant subpopulation of cells in G2 (data not shown). Thus, cells with higher levels of dWee1, despite elongated G2 phases, divide at wt rates by compensating and shortening their G1 phases.

#### Cdk1 Inhibition Induces Accumulation of Cyclin E, dE2F1, and String

The observed compensatory truncation of G1 in cells overexpressing *dwee1* could result from altered regulation of the limiting factor responsible for the G1→S transition, CycE. To test this, we performed immunostainings in L3 larval wing discs expressing UAS-*dwee1* under the control of the *en*-Gal4 driver. Cells in the P compartments of these discs showed higher levels of CycE protein and mRNA (Figures 2I and 2J), which probably account for the faster transit of these cells through



**Figure 3. Cdk Effects on dE2F1 Protein and Transcript Levels**  
L3 wing discs expressing the indicated transgenes in P (right) compartments, labeled for dE2F1 protein (A–E) or for  $\beta$ -Gal, which reports activity at the *de2f1* promoter (F–J). Overexpression of *dap* (A and F), *dwee1* (B and G), *trbl* (C and H), *cycE* (D and I), and *stg* (E and J). Arrowheads indicate A/P border.

G1 into S phase. Since it has been shown that dE2F1 can induce transcription of *cycE* (Duronio et al., 1996; Royzman et al., 1997; Neufeld et al., 1998; Dimova et al., 2003), we tested whether elevated dE2F1 activity occurred in this compensatory situation. *mnr2* mRNA accumulated in the *dwee1*-expressing P cells (Figure 2H), indicating increased dE2F1 transcriptional activity. In agreement with this, mRNA and protein accumulation of Stg (Figures 2G and 2F), another target of dE2F1 (Neufeld et al., 1998; Dimova et al., 2003), was also observed. Finally, we observed higher levels of dE2F1 protein in cells that overexpressed *dwee1* (Figure 3B).

To confirm that the observed outcome of elongating G2 was not specific to dWee1 overexpression, we overexpressed Tribbles (Trbl), a protein involved in promot-

ing Stg degradation (Grosshans and Wieschaus, 2000; Mata et al., 2000). Cells with high levels of Trbl have less of the Cdk1-activating phosphatase Stg and as a consequence have very low Cdk1 activity, an elongated G2, a severely truncated G1, and very slow rates of division (data not shown; Mata et al., 2000). Consistent with the magnitude of this effect on cell cycle profiles, cells overexpressing *trbl* showed stronger accumulation of *cycE* RNA and protein than cells overexpressing dWee1 (data not shown). Accumulation of the dE2F1 transcription factor was also evident (Figure 3C). Accordingly, high levels of *stg* transcript were observed (data not shown); however, as expected given the role of Trbl in Stg protein stability, no obvious change in Stg protein levels was observed (data not shown). These results demonstrate that cells with reduced Cdk1 activity accumulate dE2F1 protein and activity. Since *cycE* is a transcriptional target of dE2F1 (Duronio et al., 1996; Royzman et al., 1997; Neufeld et al., 1998; Dimova et al., 2003), we propose that the observed increase in dE2F1 levels caused increased *cycE* transcription and that CycE in turn effected the observed compensatory shortening of G1.

#### Increased Cdk Activity Downregulates dE2F1 Levels

In these two different compensatory situations, a common theme arises: reduction of Cdk activity extends a specific gap phase, causes upregulation of dE2F1, and leads to the compensatory truncation of the complementary gap phase. This suggests a link between Cdk activity and dE2F1. To address this possibility, we assayed the effects of increased Cdk activity on dE2F1 protein levels. We increased Cdk2 activity by driving *cycE* overexpression in the P compartment using *en-Gal4* and examined dE2F1 levels by immunofluorescence. As seen in Figure 3D, *cycE*-expressing (P) cells contained lower levels of dE2F1 protein than control (A) cells. To determine if this effect was general to Cdk activity, we increased Cdk1 activity by overexpressing *stg*. We observed slightly lower levels of dE2F1 in this case (Figure 3E), but the effect was less pronounced than when Cdk2 was stimulated. In summary, Cdk activity can regulate dE2F1 protein levels: high Cdk activity (caused by *cycE* or *stg* overexpression) decreased dE2F1 levels, whereas low Cdk activity (caused by overexpression of *dap*, *dwee1*, or *trbl*) increased dE2F1 levels.

#### Cdk Activity Regulates *de2f1* Transcription

The regulation of dE2F1 by Cdk2s may be transcriptional and/or posttranscriptional. To examine effects on *de2f1* transcription, we took advantage of the reporter *dE2F1<sup>729</sup>*, an enhancer trap line with LacZ inserted downstream of the *de2f1* promoter, which has been used as a reporter for *de2f1* transcription (Duronio et al., 1995; Brook et al., 1996). As evidenced by  $\beta$ -Gal immunostaining of *en-Gal4* UAS-*dap* discs, transcription of *de2f1* was increased in cells with higher levels of the Cdk2 inhibitor Dap (Figure 3F). Conversely, when Cdk2 activity was stimulated by *cycE* overexpression, transcription of *de2f1* was reduced (Figure 3I). Surprisingly, this relationship is opposite to that documented for E2F1 in mammalian tissue culture, in which G1 cyclin/Cdk activity was

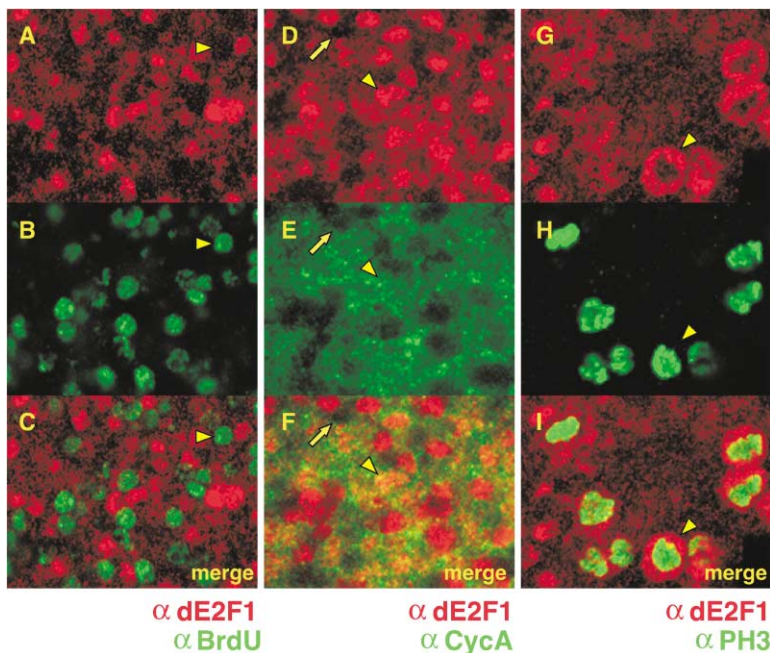


Figure 4. Cell Cycle Distribution of dE2F1 Protein

Early L3 wt wing discs were stained for dE2F1 protein (shown in red) and (in green) (A–C) BrdU incorporation, arrowheads indicate a dE2F1-negative BrdU-positive cell; (D–F) CycA protein, arrowheads indicate a dE2F1-positive CycA-positive cell, arrows indicate a dE2F1-negative CycA-negative cell; and (G–I) PH3, arrowheads indicate a dE2F1-positive and PH3-positive cell. Note: this scan was taken at the apical region of the discs, where cells are undergoing mitosis.

shown to activate the *e2f1* promoter (Johnson et al., 1994). Moreover, dE2F1 did not stimulate its own transcription when overexpressed using *en-Gal4* but instead downregulated transcription from the endogenous *de2f1* promoter (data not shown).

We were unable to detect a difference in *de2f1* promoter activity when Cdk1 was stimulated by *stg* overexpression (Figure 3J), leading us to suggest that the effect of Stg on dE2F1 protein levels is largely posttranscriptional. Inhibiting Cdk1 by *dWee1* or *Trbl* overexpression, however, did result in elevated *de2f1* transcription (Figures 3G and 3H). In summary, Cdk activity negatively regulates *de2f1* transcription.

#### dE2F1 Protein Oscillates with the Cell Cycle

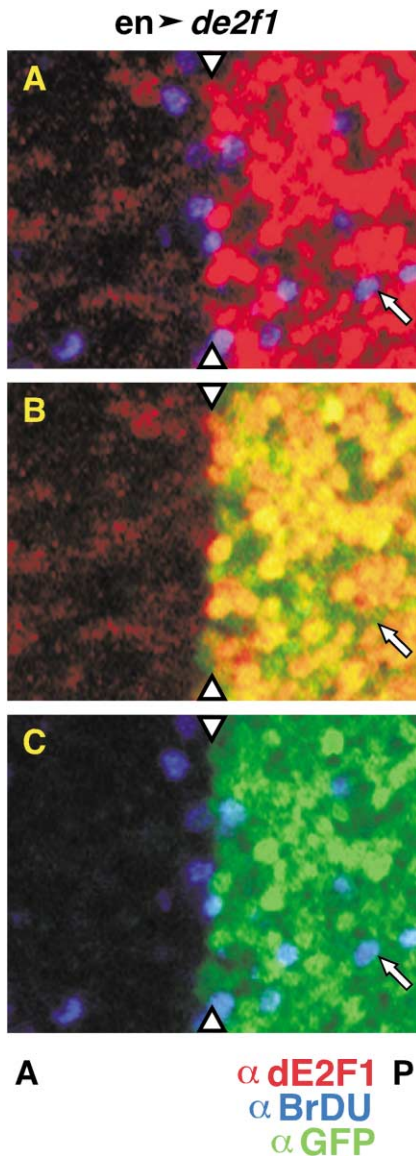
Because dE2F1 protein levels were altered by changes in the levels of Cdk activity, we next examined dE2F1 protein levels in a wt cell cycle, where it is expected that Cdk activity normally oscillates. We double labeled wt larval wing discs for dE2F1 protein together with different combinations of cell cycle markers. To identify S phase cells, we pulse labeled discs with BrdU for 30 min and then costained for BrdU (Figure 4B) and dE2F1 protein (Figure 4A). As shown in Figure 4C and Supplemental Table S1 (at <http://www.cell.com/cgi/content/full/117/2/253/DC1>) and reported by Asano et al. (1996) and Heriche et al. (2003), S phase cells had no detectable dE2F1 protein. Additionally, 31% of cells were negative for both dE2F1 and BrdU (Figure 4C; Supplemental Table S1). When labeling for dE2F1 (Figure 4D) and cyclin A (CycA) (Figure 4E), a marker for S and G2 phases (Lehner and O'Farrell, 1989), we identified four distinct populations of cells: positive only for dE2F1 (31%), positive only for CycA (32%), positive for both dE2F1 and CycA (35%), and negative for both proteins (3%) (Figure 4F; Supplemental Table S1). In combination with the previous results, we conclude that cells positive for dE2F1 only are in G1, cells positive for CycA only are in S phase, and cells positive for both dE2F1 and CycA

are in G2 or early mitosis. Cells lacking both dE2F1 and CycA could be in early S phase or in late mitosis/early G1. When examining cells positive for the mitotic marker phosphohistone H3 (PH3) (Su et al., 1998) (Figure 4H), we observed that dE2F1 (Figure 4G) was also present in these cells but did not colocalize subcellularly with this marker (Figure 4I). This indicates that dE2F1 does not associate with chromatin in mitosis. In sum, these experiments demonstrate that dE2F1 is present in G1, G2, and M phase cells but absent in S phase. Additionally, the presence of cells negative for CycA, dE2F1, and BrdU (data not shown) suggests that dE2F1 may also be absent during a short interval in early G1.

To determine if differential cell cycle regulation of dE2F1 protein plays a role in cell cycle compensation, we performed the same double labelings for dE2F1 and BrdU, dE2F1 and CycA, or dE2F1 and PH3 in discs in which *dap*, *wee1*, *trbl*, *cycE*, or *stg* were overexpressed using the *en-Gal4* driver. We observed that the cell cycle periodicity of dE2F1 protein expression was maintained (see Supplemental Figures S1A–S1F at <http://www.cell.com/cgi/content/full/117/2/253/DC1>; data not shown) and that dE2F1 protein accumulated to levels higher than wt during the cell cycle phases that were specifically elongated (e.g., G1 for *dap* overexpression, Supplemental Figures S1A and S1B). In the phases that were specifically truncated (e.g., G1 for *cycE* overexpression), dE2F1 levels were lower than wt (Supplemental Figure S1E). This indicates that altering Cdk2 or Cdk1 activity does not interfere with the normal cell cycle distribution of dE2F1 protein and suggests that the threshold of Cdk activity that triggers S phase might be the same as that required for dE2F1 degradation.

#### dE2F1 Is Regulated Posttranscriptionally

We wanted to determine if the observed effects on dE2F1 protein (upregulation by *Dap*, *dWee1*, or *Trbl* and downregulation by *CycE* or *Stg*) were consequences of transcriptional or posttranscriptional regulation. We



**Figure 5. dE2F1 Posttranscriptional Regulation**  
(A–C) Early L3 wing discs from *en*-Gal4 UAS-GFP, UAS-*de2f1* larvae were stained for dE2F1 protein (shown in red) and incorporated BrdU (blue). GFP is shown in green. Arrows indicate a dE2F1-negative, GFP-positive, BrdU-positive cell. Arrowheads indicate the A/P border.

expressed *de2f1* ubiquitously under *en*-Gal4 control to override the endogenous *de2f1* transcriptional regulation and then assayed the effects on dE2F1 protein distribution during the cell cycle. GFP was coexpressed with *en*-Gal4 so that the activity of *en*-Gal4 could be easily assessed, and S phase cells were labeled with BrdU. As shown in Figure 5A, dE2F1 protein still oscillated in cells that overexpressed the UAS-*de2f1* gene, and most cells that were positive for BrdU were negative for dE2F1. Furthermore, we found many S phase cells that were GFP positive but dE2F1 negative (Figures 5B and 5C). These cells must express the Gal4 protein and thus probably also express high levels of *de2f1* mRNA. Thus, dE2F1 protein can be cleared from S phase cells

even when its mRNA is expressed at high levels throughout the cell cycle.

### dE2F1 Is Required for Cell Cycle Compensation

We hypothesized that in *dap*- or *dwee1*-overexpressing cells, upregulation of dE2F1 induced the compensatory upregulation of the target genes *stg* or *cycE*, which accelerated passage through subsequent phases. To address the requirement for dE2F1 as a mediator of cell cycle compensation, we overexpressed *dap* or *dwee1* in *dDP* mutant (*dDP<sup>a1/a2</sup>*) animals. dDP is an essential coactivator for dE2Fs, and cells lacking dDP have no dE2F1 or dE2F2 activity (Royzman et al., 1997; Duronio et al., 1998; Frolov et al., 2001). We predicted that induction of *dap* or *dwee1* in animals lacking dE2F1 activity should fail to promote the compensatory accumulation of *stg* or *cycE* and that the cell cycle compensatory effect should not occur. We induced expression of Dap in wt or *dDP<sup>a1/a2</sup>* backgrounds using the Flp/Gal4 technique and analyzed FACS profiles and calculated CDT. The severe G2 truncation upon *dap* overexpression in wt cells was no longer observed in cells lacking dDP (Figure 6C). Analysis of CDT indicated that, in the *dDP<sup>a1/a2</sup>* mutant discs, *dap*-overexpressing cells divided slower than controls (Figures 6B and 6D) and thus failed to compensate. *dDP<sup>a1/a2</sup>* control cells spent 4.1 hr in G1, 4.8 hr in S, and 7.3 hr in G2, whereas *dDP<sup>a1/a2</sup>* cells that overexpressed Dap spent 5.6 hr in G1, 5.7 hr in S, and 7.8 hr in G2. No marked change in the number of apoptotic cells was observed in this situation (data not shown). Additionally, we induced nearly ubiquitous expression of Dap in wt or *dDP<sup>a1/a2</sup>* animals, isolated wing disc RNA, and performed reverse transcription (RT) followed by PCR to detect *mr2*, *stg*, and *cycE* mRNAs. As shown in Figure 6, wing discs overexpressing Dap had higher levels of *mr2* (Figure 6K), *stg* (Figure 6L), and *cycE* (Figure 6M), confirming our in situ data (Figure 2). When Dap was induced in a *dDP<sup>a1/a2</sup>* background, however, the induction of these dE2F1 target genes was not observed (Figures 6K–6M). These data strongly support the requirement for dE2F activity in the upregulation of these transcripts and the resulting cell cycle compensation when Dap is overexpressed.

We performed analogous experiments in which dWee1 was overexpressed in *dDP<sup>a1/a2</sup>* mutants. DNA profiles showed that the G1 truncation in cells overexpressing dWee1 in a *dDP<sup>a1/a2</sup>* background was less pronounced than in wt cells overexpressing dWee1 (Figure 6E). Moreover, *dDP<sup>a1/a2</sup>* mutant cells overexpressing dWee1 had slower rates of division (Figures 6B and 6F) and high rates of apoptosis (data not shown). When dWee1 was induced in wt cells, we observed by RT-PCR the expected induction of dE2F1 target genes (data not shown), consistent with our in situ data (Figure 2). In the *dDP<sup>a1/a2</sup>* background, however, dWee1-overexpressing cells were subviable, and effects on dE2F1 target gene expression could not be measured accurately. The decrease in viability of these cells may be due to the slower rates of division resulting from the loss of cell cycle compensation. We attempted similar experiments with *dE2F2<sup>G5.1176Q1</sup>*, *dE2F1<sup>91/179</sup>* mutants, but in our hands these animals failed to reach late larval stages, making the experiments technically unfeasible.

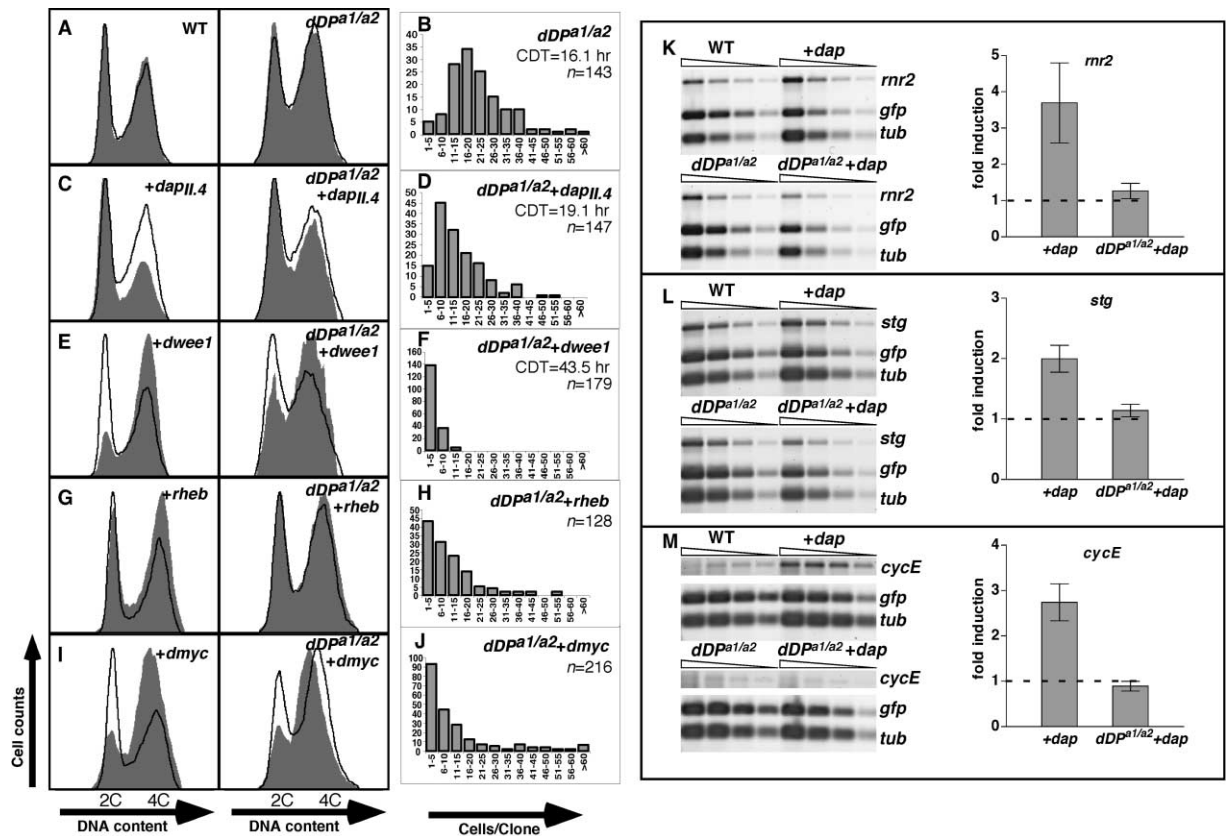


Figure 6. dE2F1 Activity Is Required for Cell Cycle Compensation

(Left) DNA profiles in solid gray represent cells expressing GFP alone (A) or in combination with Dap (C), dWee1 (E), Rheb (G), or dMyc (I) in a wt (left column) or *dDP<sup>a1/a2</sup>* (right column) background. Solid black lines represent endogenous controls. (Center) Distribution of the number of cells per clone and derived median CDT for the indicated genotypes. n, number of clones. Each experiment was performed at least three times. Shown is one representative result. (Right) Gels show levels of *rnr2* (K), *stg* (L), and *cycE* (M) mRNA together with *gfp* and *tub* after RT-PCR performed in wt or *dDP<sup>a1/a2</sup>* wing discs. Wild-type and *dDP<sup>a1/a2</sup>* control animals overexpress *gfp* only, and experimental animals coexpressed *gfp* and *dap*. PCR was performed using 4-fold dilutions of the obtained cDNAs. Top lanes show wt (four left lanes) and *dap*-overexpressing conditions (four right lanes). Bottom lanes represent *dDP<sup>a1/a2</sup>* (four left lanes) and *dDP<sup>a1/a2</sup> + dap* conditions (four right lanes). Graphs show the induction of *rnr2* (K), *stg* (L), and *cycE* (M) in response to *dap* overexpression in a wt or *dDP<sup>a1/a2</sup>* background. Inductions are normalized for level of overexpression using *gfp* signal. Shown are average inductions with SEM for four independent RT experiments.

In summary, our data show that dE2F1 activity is required for the observed upregulation of *rnr2*, *stg*, and *cycE* in situations of Cdk2 inhibition and for cycle compensation when Cdk1 or Cdk2 is inhibited.

#### dE2F1 Is Required for Cell Survival under Overgrowth Conditions

Genes that promote cellular growth, such as dMyc, Ras, PI3K, Akt, and Rheb, accelerate G1→S progression but fail to speed up overall cell division rates in the wing (Johnston et al., 1999; Verdu et al., 1999; Weinkove et al., 1999; Prober and Edgar, 2000; Saucedo et al., 2003; Stocker et al., 2003). An associated increase in CycE protein is likely responsible for G1→S acceleration in several of these situations (Prober and Edgar, 2000; L. Saucedo and B.A.E., unpublished data). This suggests that cell cycle compensation might be a protective mechanism against increased division rates. To determine the importance of dE2F-mediated cell cycle compensation in this context, we overexpressed *rheb* or *dmyc* in a *dDP<sup>a1/a2</sup>* mutant background, analyzed cell

cycle profiles by FACS, and calculated CDT. We predicted that in the absence of dDP, G2 elongation would not occur, and cells overexpressing these growth drivers would divide faster. Indeed, the compensatory elongation of G2, which is observed in wt cells upon *rheb* or *dmyc* overexpression, was not as pronounced in *dDP<sup>a1/a2</sup>* mutant cells (Figures 6G and 6I). This indicates that dE2F1 is also important for cell cycle compensation in these situations. When analyzing CDT, however, we observed that *dDP<sup>a1/a2</sup>* cells overexpressing dMyc or Rheb formed smaller clones than wt, which translated into longer CDT (Figures 6B, 6H, and 6J). Staining for a marker of apoptosis revealed that these clones contained greatly increased numbers of dead or dying cells (Figure 7D, see Supplemental Table S2 at <http://www.cell.com/cgi/content/full/117/2/253/DC1>). In addition, we noted that many fewer *rheb*- or *dmyc*-expressing clones were present in the *dDP<sup>a1/a2</sup>* background as compared to wt (Figure 7D; Supplemental Table S2). This suggests that many of these clones were completely culled by apoptosis. These high rates of cell death probably account for the apparent increase in CDT, which

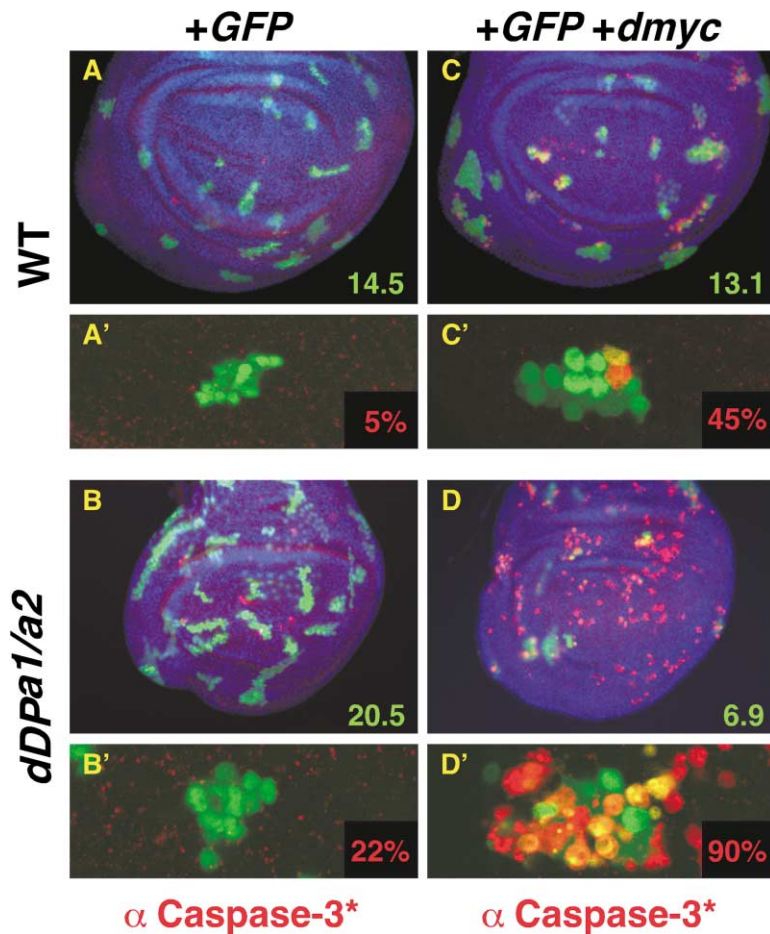


Figure 7. dE2F1 Is Required for Cell Survival after Overexpression of dMyc

Wing discs stained for the apoptotic marker cleaved Caspase-3 (red). GFP-marked clones (green) overexpressing GFP alone (A and B) or GFP and dMyc (C and D) were generated in a wt (A and C) or *dDPA<sup>a1/a2</sup>* background. DNA is stained blue with H $\ddot{o}$ chst 33258. Numbers in green, average clones per disc; red, percentage of clones with apoptotic cells. Clones were induced by 10 min HS (wt backgrounds) or 15 min HS (*dDPA<sup>a1/a2</sup>* backgrounds) at 72 hr AED and analyzed at 120 hr (wt) or at 144 hr (*dDPA<sup>a1/a2</sup>*) AED.

are thus inaccurate. We conclude that, in the absence of dE2F activity, cells become intolerant to changes in cell cycle phasing induced by physiological growth promoters.

### Discussion

Previous observations in cultured mammalian cells and yeasts have suggested regulatory crosstalk between G1→S and G2→M regulation, but the mechanisms at play have never been explained (Nurse and Thuriaux, 1980; Russell and Nurse, 1986; Nash et al., 1988; Resnitzky et al., 1994; Ohtsubo et al., 1995; Neufeld et al., 1998). Our findings indicate that, in *Drosophila*, E2F1 is a central component of a regulatory circuit that allows cells to sustain alterations in the lengths of individual cell cycle phases without compromising overall cell cycle timing (Figure 8). In situations where Cdk2 activity is downregulated, G1 is elongated, but, because dE2F1 levels are normally limited by Cdk2 activity, dE2F1 accumulates. This additional dE2F1 causes increased expression of dE2F1 target genes, including *Stg*, which promotes faster progression through G2. An analogous mechanism can also upregulate dE2F1 and, by inducing *CycE*, accelerate G1→S passage when G2 is elongated (Figure 8).

The accumulation of dE2F1 protein caused by suppression of Cdk1 or Cdk2 is unlikely to be a mere conse-

quence of altering the distributions of cells in the various cell cycle phases, since treatments that have similar effects on cell cycle phasing (e.g., *dap* or *stg* overexpression) can have opposite effects on dE2F1 levels. Similarly, treatments that have the opposite effects on cell cycle phasing (e.g., *dap* or *dwee1* overexpression) can have similar effects on dE2F1 levels (Figures 1 and 3; Neufeld et al., 1998). Hence, we suggest that the Cdk

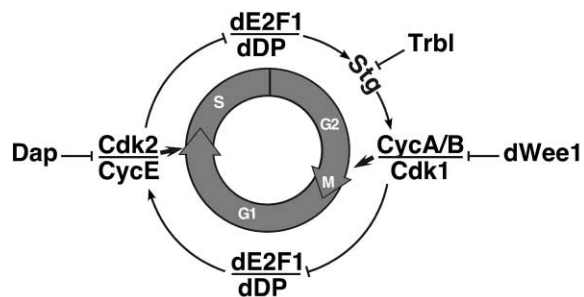


Figure 8. Model of Cell Cycle Regulation by dE2F1

Schematic depicting the homeostatic mechanism linking G1-S and G2-M control via dE2F1. Both Cdk1 and Cdk2 downregulate dE2F1, whereas dE2F1 stimulates transcription of *Stg* and *CycE*, which activate Cdk1 and Cdk2, respectively. Note that while dE2F1 is periodic, functional levels of the mRNAs encoding its limiting downstream targets, *Stg* and *CycE*, perdure for more than a single cycle. This allows crosstalk between cycle phases.

affect dE2F1 levels more directly, as discussed below. It is also important to note that the mechanism we propose could only function if the *stg* and *cycE* transcripts, which limit cycle progression, are not strictly periodic but persist through successive cell cycle phases. This condition is supported by experiments showing that *stg* and *cycE* mutant cells are able to divide two to three times after loss of the wt allele (Neufeld et al., 1998). Thus, functional levels of these cell cycle-regulatory transcripts persist through more than a single cell cycle.

While we based our work on overexpression studies, we also observed that dE2F1 levels are tightly regulated in normal cell cycles. dE2F1 protein oscillates, being present in the G<sub>2</sub>, M, and G<sub>1</sub> phases (Figure 4) but absent during S phase (Figure 4; Asano et al., 1996; Heriche et al., 2003). These oscillations may reflect fluxes in Cdk activity, which is expected to reach high thresholds at G<sub>1</sub>→S (for Cdk2) and G<sub>2</sub>→M transitions (for Cdk1). We believe that the loss of dE2F1 at the G<sub>1</sub>→S transition is Cdk2 dependent. Our data also suggest that Cdk1 may deplete dE2F1 during late mitosis or early G<sub>1</sub>. Previous reports support a correlation between Cdk2 activity and levels and activity of dE2F1 protein. Others have observed absence of dE2F1 in S phase (Asano et al., 1996; Heriche et al., 2003), and studies using a dE2F1 reporter gene suggest that dE2F1 activity oscillates during the wing disc cell cycle (Thacker et al., 2003). All of these effects could be attributed to downregulation of dE2F1 by CycE/Cdk2 at G<sub>1</sub>→S transitions.

Although CycE accumulates specifically during S phase in *Drosophila* endocycling cells (Weng et al., 2003), CycE protein has not been observed to oscillate during mitotic cell cycles in *Drosophila* embryos or wing discs. In the wing disc, CycE is a nuclear protein that varies in level from cell to cell but is not obviously absent during any specific cell cycle phase (our unpublished data). Thus, the mechanism controlling cyclic fluctuations in CycE/Cdk2 activity is unclear. dE2F1 levels could, in principle, oscillate even in situations where levels of total cellular Cdk activity remained constant through the cell cycle. Cell cycle-dependent fluxes in the subcellular distributions of the Cdks, cyclins, dE2F1, or associated factors might mediate the periodic depletion of dE2F1. In this regard, it is interesting to note that binding by the *retinoblastoma* protein (pRb) can protect human E2F1 from ubiquitin-mediated degradation (Hateboer et al., 1996; Hofmann et al., 1996; Campanero and Flemington, 1997; Martelli and Livingston, 1999). Indeed, we found that overexpression of *rbf1* in *Drosophila* wing discs caused accumulation of dE2F1 and suppressed the downregulation of dE2F1 that otherwise occurred upon *cycE* or *stg* overexpression (data not shown). Periodic Cdk2-dependent phosphorylation of RBF1, causing dissociation from dE2F1, might play a significant role in dE2F1 oscillation.

What other mechanisms might regulate the periodicity of dE2F1? Skp-cullin-F-box (SCF) ubiquitin-protein ligase complexes mediate the degradation of many cell cycle proteins (for review, see Krek, 1998; DeSalle and Pagano, 2001), including E2F1 (Marti et al., 1999; Heriche et al., 2003). Recently, SCF-mediated degradation of dE2F1 was shown to be important for its loss during S phase and was suggested to be dependent on the status of dE2F1 protein phosphorylation (Heriche et al., 2003).

Thus, dE2F1 may be targeted for SCF-mediated degradation following phosphorylation by Cdk2, Cdk1, or other kinases whose activity is Cdk dependent. Phosphorylation of mammalian E2F1/Dp1 complexes by CycA-dependent kinases has been reported, but a role in the degradation of E2F1 was not established (Krek et al., 1994; Dynlacht et al., 1997). Consistent with the idea that direct phosphorylation by Cdks may mediate dE2F1 degradation, dE2F1 has at least two consensus Cdk phosphorylation sites.

E2F's ability to promote G<sub>2</sub>→M transitions has been previously documented in flies (Neufeld et al., 1998) and suggested by expression studies in mammalian cells (Ishida et al., 2001). The additional feedback regulation described here adds to accumulating evidence that dE2F1 has functions beyond G<sub>1</sub>→S progression and implies a central role for dE2F1 in coordinating overall cell cycle timing. The feedback mechanism we describe also clarifies some unresolved issues, such as the effects on wing disc overgrowth induced by growth promoters. For instance, drivers of cellular growth, including dMyc, Ras, PI3K, Akt, and Rheb, fail to increase overall rates of proliferation despite profoundly accelerating G<sub>1</sub>→S progression (Johnston et al., 1999; Verdu et al., 1999; Weinkove et al., 1999; Prober and Edgar, 2000; Saucedo et al., 2003; Stocker et al., 2003). In several of these cases, posttranscriptionally driven increases in CycE protein are the probable cause of G<sub>1</sub>→S acceleration (Prober and Edgar, 2000; L. Saucedo and B.A.E., unpublished data). dE2F1-mediated crosstalk between G<sub>1</sub> and G<sub>2</sub> may explain why these growth drivers fail to speed up the cell cycle: when a factor such as dMyc, Rheb, or Ras promotes CycE accumulation, negative feedback in which increased CycE/Cdk2 activity depletes dE2F1 may suppress the expression of dE2F1 targets required for progression through S, G<sub>2</sub>, and mitosis. This would elongate the S and G<sub>2</sub> phases of the cycle and thereby maintain the normal overall rate of cell proliferation. In this capacity, downregulation of E2F1 may act to restrain unbridled proliferation caused by oncogenes like Myc, Ras, and Akt or tumor suppressors such as NF1 and PTEN, which feed into the cell cycle by promoting cell growth.

The scenario proposed above suggests that accelerated G<sub>1</sub>→S progression could lead to faster rates of cell division when a growth driver such as dMyc is overproduced in the absence of dE2F1-mediated feedback. Without the "catch-up" time afforded to wt cells by the compensatory elongation of phases, however, cells lacking dE2F1 might never accomplish essential tasks left unfinished during the truncated phase. This could have adverse effects on the cycle itself or on cell viability. Experimentally, we observed that cells lacking dE2F activity were intolerant of increases in cell growth caused by ectopic dMyc or Rheb and sustained high rates of apoptosis (Figures 6 and 7; see Supplemental Table S2 at <http://www.cell.com/cgi/content/full/117/2/253/DC1>). Although the cause of this cell death is unknown, one possibility is that rapid proliferation with a short G<sub>1</sub> compromises genomic stability. Origins are assembled in G<sub>1</sub>, and dE2F1 targets such as *dna-pol* $\alpha$ , *rnr2*, and *pcna* are important for the progression of DNA synthesis. Accelerated G<sub>1</sub>→S progression without compensatory elongation of G<sub>2</sub>, whereby the products of

dE2F1-mediated transcription could accumulate for the next G1, could result in incomplete or error-prone replication and the activation of cell cycle checkpoints. This would be expected to both elongate the cell cycle and increase rates of apoptosis, as we observed.

Thus, while dE2F1 is not essential for cell cycle gene expression or cell cycle progression in *Drosophila* (Royzman et al., 1997; Frolov et al., 2001), it appears to be a central component of a feedback mechanism that limits the effects of growth stimuli on rates of cell cycle progression and maintains the timing and perhaps also the fidelity of the replication cycle. This function might help to explain why loss of E2F1 in mice causes an increased incidence of cancer (Yamasaki et al., 1996) and why genetic lesions that deregulate E2F in humans (i.e., in *p16<sup>Ink4</sup>*, *pRB*, and *CycD1*) are so frequently involved in hyperproliferative disease (Bartkova et al., 1996), whereas lesions in phase-specific cell cycle regulators, such as *Cdk2* or *CycE*, are not. We suggest that, to promote unchecked proliferation in mitotic tissues, a growth stimulus must, besides promoting cellular growth, also overcome the crossregulation that exists between G1→S and G2→M controls or, alternatively, promote G2→M progression in addition to G1→S progression. Either strategy could potentially be achieved by affecting E2F1 function.

#### Experimental Procedures

##### Fly Stocks

*en-Gal4 UAS-GFP*, *act2>cd2>Gal4 UAS-GFP*, *y w flp<sup>122</sup>*, *UAS-cycE*, *UAS-stg*, *UAS-de2f1*, *UAS-rheb*, and *UAS-dmyc* were described previously (Neufeld et al., 1998; Johnston et al., 1999; Saucedo et al., 2003). *UAS-dap*, *UAS-dwee1*, and *UAS-trbl* flies were gifts from C. Lehner, S. Campbell, and P. Rörth, respectively. The *dE2F1<sup>729</sup>* reporter line has been described (Duronio et al., 1995; Brook et al., 1996). *dDP<sup>a1</sup>* and *dDP<sup>a2</sup>* are described elsewhere (Royzman et al., 1997). *y w flp<sup>122</sup>*; *dDP<sup>a1</sup> UAS-dap11.4/CyO-GFP*; +, *y w flp<sup>122</sup>*; *dDP<sup>a1</sup>/CyO-GFP*; *UAS-dwee1VIII*, *y w flp<sup>122</sup>*; *dDP<sup>a1</sup>/CyO-GFP*; *UAS-rheb*, *y w flp<sup>122</sup>*; *dDP<sup>a1</sup>/CyO-GFP*; *UAS-dmyc*; and *w*; *dDP<sup>a2</sup>/CyO-GFP*; *act2>cd2>Gal4 UAS-GFP* lines were made for use in the *dDP* mutant experiments.

##### Flow Cytometry

All clones were induced at 72 hr AED by a 1.5 hr heat shock (HS) at 37°C, and discs were dissected at 120 hr AED, in wt backgrounds. In the *dDP* mutant background, clones were induced at 72 hr AED by a 25 min HS in a 37°C water bath. *dDP<sup>a1/a2</sup>* animals were developmentally delayed. Dissections were thus performed at approximately 144 hr AED. FACS was performed as described (Neufeld et al., 1998). At least six independent experiments per genotype, with parallel controls, were performed with similar results.

##### Cell Doubling Times

Clones were induced at 72 hr AED by a 37°C HS for 19 min. Discs dissected from wandering larvae were fixed and analyzed as described (Neufeld et al., 1998) using the formula  $(\log_2/\log N) h$ , where *N* is median number of cells/clone, and *h* is time between HS and disc fixation. *p* values were calculated using one-tailed Student's *t* test. Three independent experiments with parallel wt controls were performed for *UAS-dap* transgenics and two for *UAS-dwee1*, with similar results. For *dDP* mutants, clones were induced by a 15 min 37°C HS at 72 hr AED. Dissections were performed at approximately 144 hr AED. Similar results were obtained from three independent experiments.

##### Histology

Discs were dissected from inverted larvae, fixed in 4% paraformaldehyde in PBS for 30 min, and washed in PBS, 0.1% Tween 20, at

least three times for 20 min. Discs were then used for immunostaining or RNA in situ hybridization.

##### Immunostaining

Discs were blocked with PBS 0.1% Triton X-100, 1% BSA for at least 2 hr at RT, incubated with primary antibody overnight at 4°C, washed with PBS 0.3% Triton X-100, 0.1% BSA, 2% NGS three times for 1 hr, and incubated with secondary antibody conjugated to Alexa-Fluor 568 or 633 1:5000 (Molecular Probes) for 2 hr at RT. Hoechst 33258 (Acros) was used to label nuclei.

##### Antibody Dilutions with Source

Antibody dilutions were α-Stg 1:20 (Edgar et al., 1994), α-CycE 1:800 (T. Orr-Weaver), α-dE2F1 1:500 (Bosco et al., 2001), α-BrdU 1:100 (Becton Dickinson), α-CycA 1:500 (D. Glover), α-PH3 1:4000 (Upstate), α-β-Gal 1:10000 (Cappel), and α-Cleaved Caspase-3 (Asp 175) 1:100 (Cell Signaling Technology). Images were obtained using a Leica TCSSP Confocal microscope.

##### RNA In Situ Hybridizations

RNA in situ hybridizations were performed as described (O'Neill and Bier, 1994). Images were obtained using a Leitz DMRD microscope.

##### BrdU Labeling of Discs

BrdU labeling of discs was assayed as described (Johnston and Schubiger, 1996), with a 30 min BrdU pulse and 45 min hydrolysis.

##### RT-PCR

Clones were induced at 48 hr AED for 30 min in a 37°C water bath. Larvae were dissected at wandering stages. RNA from 60 wing discs was isolated using Trizol Reagent (Invitrogen). Two independent RTs per RNA sample were performed using Superscript II Reverse Transcriptase (Invitrogen). cDNAs were diluted 4-fold, and 25 cycle multiplex PCR (94°C, 3 min; 25 cycles of [94°C, 1 min; 57°C (60°C for *cycE*) 45 s; 72°C, 2 min]; 72°C, 7 min) was performed for *mrr2* (primers: 5'-CAGTCAGGAGGTGCAGATCA-3', 5'-TGAATTCGGAAAAGGTACGC-3'), *stg* (5'-GGAGGAGCTGCTGTTCTACG-3', 5'-TCA GTCTGTTGGACGGTGAG-3'), or *cycE* (5'-GGCATGGCCAACTATTCTTA-3', 5'-CTTGGTTGTGAGAGCGTCA-3'), and *gfp* (5'-AGTGGAGAGGGTGAAGGTGA-3', 5'-AAAGGGCAGCTGTGTGGAC-3') and *tub* (5'-AACTTTGTGTTCCGCCAGTC-3', 5'-TGTCGATGCAGTAGG TCTCG-3'). PCR reactions included 67 mM Tris-HCl (pH 8.8), 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5% glycerol, 0.01% Tween 20, 200 μM each dNTP, 2.5 mM MgAc<sub>2</sub>, 3 U Platinum Taq Polymerase (Invitrogen), and 40 nM each of primers. Reactions were separated in 1.5% agarose gels and poststained with SYBR Green I (Molecular Probes) per the manufacturer's instructions. Signal intensities were quantitated using the Molecular Dynamics Typhoon 8600 and data analyzed using ImageQuant software. The ratio of *mrr2*, *stg*, or *cycE* to *gfp* mRNA was used to normalize for the amount of overexpressing cells in each sample. For each genotype, two independent RNA isolations were performed, followed by two independent RTs.

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