A robust cell cycle control mechanism limits E2F-induced proliferation of terminally differentiated cells in vivo

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Terminally differentiated cells in *Drosophila melanogaster* wings and eyes are largely resistant to proliferation upon deregulation of either E2F or cyclin E (CycE), but exogenous expression of both factors together can bypass cell cycle exit. In this study, we show this is the result of cooperation of cell cycle control mechanisms that limit E2F-CycE positive feedback and prevent cycling after terminal differentiation. Aberrant CycE activity after differentiation leads to the degradation of E2F activator complexes, which increases the proportion of CycE-resistant E2F repressor complexes, resulting in stable E2F target gene repression. If E2F-dependent repression is lost after differentiation, high anaphase-promoting complex/cyclosome (APC/C) activity degrades key E2F targets to limit cell cycle reentry. Providing both CycE and E2F activities bypasses exit by simultaneously inhibiting the APC/C and inducing a group of E2F target genes essential for cell cycle reentry after differentiation. These mechanisms are essential for proper development, as evading them leads to tissue outgrowths composed of dividing but terminally differentiated cells.

Introduction

Terminal differentiation is usually coupled with permanent exit from the cell cycle and represents the most common cellular state in adult animals. How terminal differentiation controls the cell cycle machinery to maintain a stable postmitotic state remains unclear. Evidence from several model organisms suggests that inhibition of the E2F transcription factor and/or the G1-S cyclin, cyclin E (CycE), is a key event for coordinating cell cycle exit and differentiation (for reviews see Buttitta and Edgar, 2007; Miller et al., 2007; Onoyama and Nakayama, 2008; Pajalunga et al., 2008; van den Heuvel and Dyson, 2008). Yet, activation of E2F or CycE in many contexts is insufficient to abrogate exit (Akli et al., 1999; Pajalunga et al., 1999; Latella et al., 2001; Camarda et al., 2004; Balsitis et al., 2006; Buttitta et al., 2007; Gibbs et al., 2008).

E2F transcription factors play key roles in regulating the cell cycle as many genes required for S, G2, and M phases are targets of E2F (Ishida et al., 2001; Polager et al., 2002; Ren et al., 2002; Dimova et al., 2003). Importantly, E2F complexes (made up of an E2F subunit and its dimerization partner [DP]) can act as both transcriptional activators and repressors of the same targets. Association with the retinoblastoma tumor suppressor proteins (RBs) can convert E2F–DP complexes to transcriptional repressors by recruiting repressive chromatin-modifying complexes (Dyson, 1998), whereas the G1 cyclin–Cdk complexes, CycD–Cdk4 and CycE–Cdk2, promote E2F activity by phosphorylating and inhibiting the association of RBs with E2F–DP complexes (Du and Pogoriler, 2006). CycE–Cdk2 activity is also directly essential for S-phase entry, as it regulates critical components to initiate S phase in both kinase-dependent

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Abbreviations used in this paper: *ap*, *Apterous*; APC/C, anaphase-promoting complex/cyclosome; APF, after puparium formation; CD2, cluster of differentiation 2; DP, dimerization partner; Elav, embryonic lethal abnormal vision; Fzr, Fzy related; Fzy, Fizzy; Gem, Geminin; GO, Gene Ontology; MARCM, mosaic analysis with a repressible cell marker; MPM2, mitotic phosphoprotein marker 2; PCNA, proliferating cell nuclear antigen; PIP, PCNA interaction protein motif; Rbf, RB family; Rca1, regulator of CycA; Stg, String; UAS, upstream activating sequence; WT, wild type.

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and -independent manners (Lukas et al., 1997; Mailand and Diffley, 2005; Geng et al., 2007).

Drosophila melanogaster has been a key organism for studies of cell cycle exit in part because its E2F/CycE network is simpler than mammals, consisting of only two RB family (Rbf) members (Rbf and Rbf2), a single Cip/Kip type cyclin-dependent kinase inhibitor, Dacapo (no INK homologues), two E2Fs (one activator, E2F1, and one repressor, E2F2), one DP, and one essential G1-S cyclin, CycE (Duronio and O'Farrell, 1995; de Nooij et al., 1996; Lane et al., 1996; Sawado et al., 1998; Frolov et al., 2001; Stevaux et al., 2002).

Investigations into cell cycle exit in *Drosophila* have demonstrated that RB/E2F repressive activity is not required for permanent exit in vivo (Weng et al., 2003; Frolov et al., 2005). We have suggested that this is caused by independent but parallel repression of E2F and cyclin–Cdk activities, a mechanism we call double-assurance (Buttitta et al., 2007). The restraint of both E2F and CycE activities upon exit partially involve Rbf and Dacapo (Firth and Baker, 2005) but, based on genetic experiments, must also include Rbf- and Dacapo-independent mechanisms (Buttitta et al., 2007).

The finding that E2F activity alone is insufficient to maintain cycling of differentiating cells is surprising because E2F activity itself up-regulates CycE and Cdk2 after exit to levels even higher than those in proliferating cells (Buttitta et al., 2007). High CycE–Cdk2 activity normally promotes increased E2F activity via phosphorylation of RBs and cooperates with E2F-dependent induction of CycE and Cdk2 to establish a positive E2F-CycE feedback loop for cell cycle entry (Yung et al., 2007; Assoian and Yung, 2008), similar to that of Cln1 and -2 in yeast (Skotheim et al., 2008). However, we have found that E2F-CycE positive feedback appears to be limited after terminal differentiation to prevent ectopic cycling (Buttitta et al., 2007). In this study, we examine why both ectopic E2F and CycE activities must be provided to bypass cell cycle exit in *Drosophila* and uncover specific cell cycle control mechanisms that cooperate to limit E2F-CycE positive feedback and maintain a stable postmitotic state.

Results

E2F2 complexes are partially responsible for CycE-resistant E2F repression after exit

CycE induces E2F activity in proliferating and reversibly quiescent cells by preventing the formation of Rbf–E2F repressive complexes. However, our previous results indicated that CycE activity could not prevent repression of E2F target genes after cell cycle exit in terminally differentiated eye and wing cells (Buttitta et al., 2007). Rbf–E2F2-mediated repression is somehow resistant to G1 cyclin–Cdks despite G1 cyclin–Cdk phosphorylation of Rbf (Frolov et al., 2003). This makes E2F2 complexes prime candidates as factors limiting CycE-dependent activation of E2F after cell cycle exit in differentiated cells. Genetic studies of E2F2 have failed to demonstrate a role for E2F2 in cell cycle exit upon differentiation in vivo (Frolov et al., 2001; Weng et al., 2003). *e2f2* mutant cells exit from the cell cycle on time (Frolov et al., 2001), and mitotic tissues of $e2f1^{su89}$,*e2f2* double mutant animals, thought to lack all E2F repressive function, exit the cell cycle normally (Weng et al., 2003). Therefore, we sought to reveal a more subtle role for E2F2 in blocking CycE-E2F feedback specifically after cell cycle exit and influencing exit flexibility in the presence of high CycE–Cdk2 activity.

Clones of cells overexpressing CycE and Cdk2 were generated in an $e2f2^{76Q1/G5.1}$-null background using the *heat shock* (*hs*)–FLP Gal4 method (Pignoni and Zipursky, 1997), where clones are negatively marked by the CD2 (cluster of differentiation 2) antigen and CycE and Cdk2 are expressed under the control of a Gal4 responsive upstream activating sequence (UAS). E2F transcriptional activity was monitored in wings at stages after cell cycle exit, using a reporter containing an E2F-responsive *proliferating cell nuclear antigen* (*PCNA*) promoter fused to GFP (Thacker et al., 2003). In the absence of *e2f2*, CycE–Cdk2 was able to activate the E2F reporter in clones within the wing after terminal differentiation at 36 h after puparium formation (APF; Fig. 1 A), an effect absent in wild-type (WT) wings. However, the CycE–Cdk2-induced E2F activity in *e2f2* mutant wings was temporary, as CycE–Cdk2 was unable to sustain E2F activity beyond 40 h APF in the absence of *e2f2* (Fig. 1 B).

We next examined whether the loss of *e2f2* influenced the flexibility of cell cycle exit in the presence of high CycE–Cdk2 activity. GFP-marked *e2f2*-null mutant clones expressing CycE–Cdk2 were generated using the mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 2001) and examined for ectopic mitoses after normal cell cycle exit in pupal eyes and wings by staining for phosphorylated Ser10 on histone H3 (PH3; Fig. 1, C–E). Ectopic mitoses were evident in $e2f2^{-/-}$ cells expressing CycE–Cdk2 in eyes from 24–44 h APF (Fig. 1 C, 36 h APF shown), indicating a delay of cell cycle exit 8 h beyond the effect of CycE–Cdk2 expression alone in the eye. However, mitoses were no longer observed in $e2f2^{-/-}$ cells expressing CycE–Cdk2 after 44 h APF in eyes (Fig. 1 D) or in wings after 36 h APF (Fig. 1 E and Table I). This reveals a cell cycle exit function for *e2f2* in temporarily limiting CycE activation of E2F but also demonstrates that an *e2f2*-independent mechanism prevents E2F transcriptional activation after prolonged exit in eyes and wings.

Ectopic CycE–Cdk2 activity in terminally differentiated cells leads to E2F1 degradation

Recent work has delineated a Dacapo- and Rbf-independent mechanism limiting E2F1 activity in embryos and proliferating cells. E2F1 is degraded each cell cycle during S phase in a PCNA interaction protein motif (PIP) box–dependent manner via a Cul4 ubiquitin ligase complex (Shibutani et al., 2007; 2008). We considered whether ectopic CycE–Cdk2 activity could inhibit E2F activity after exit by promoting S-phase initiation and thereby E2F1 degradation. This idea was supported by our previous observation that CycE–Cdk2 expression correlated with low E2F1 protein levels in the wing (Buttitta et al., 2007).

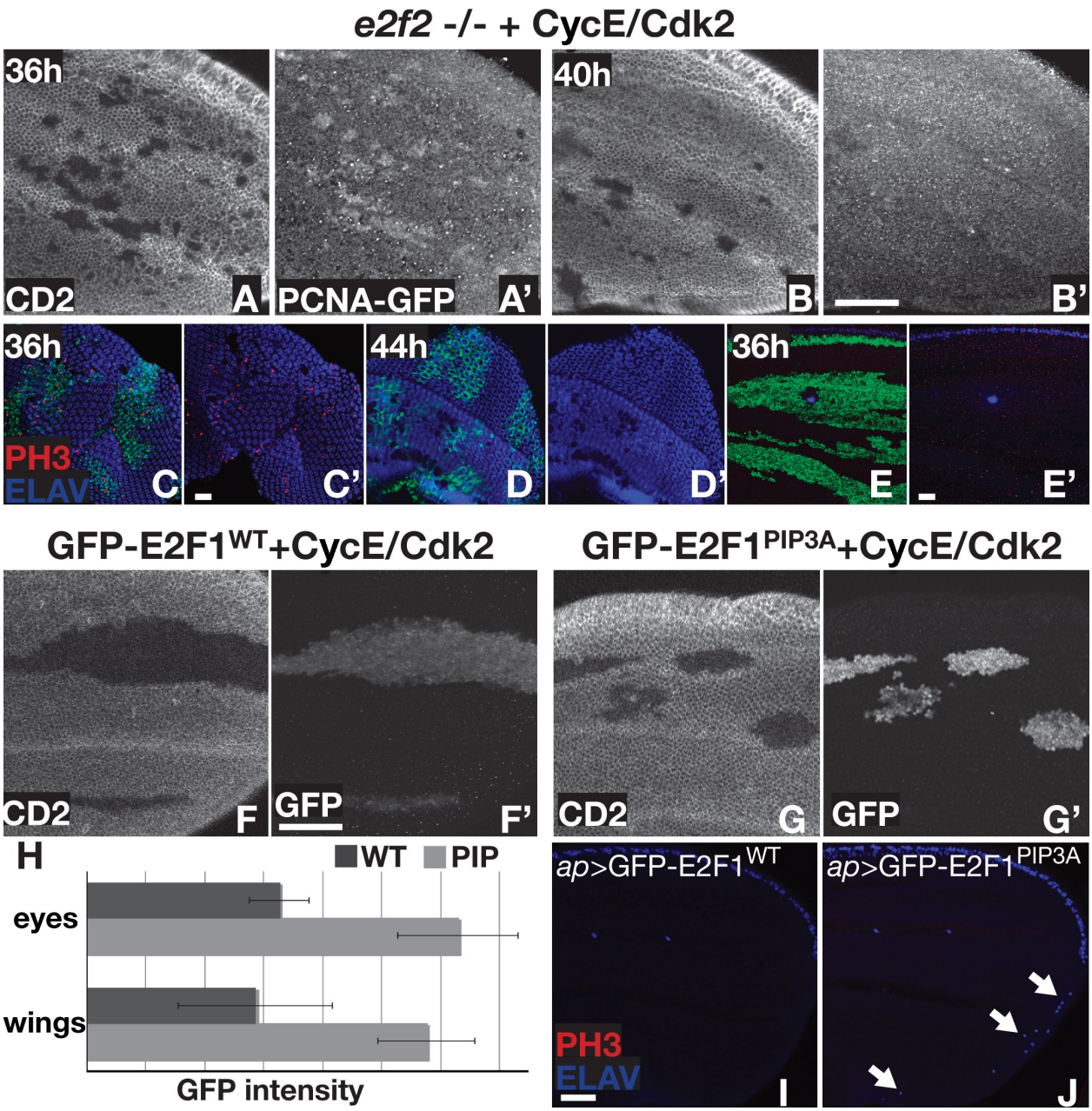

Figure 1. **Degradation of E2F1 activator complexes and a switch to E2F2 repressor complexes contributes to the CycE–Cdk2-resistant repression of E2F activity after exit.** Clones overexpressing CycE–Cdk2 and negatively marked by CD2 (A and B) were generated using *hs*-FLP *act*>Gal4/UAS in $e2f2^{76Q1/G5.1}$ mutant wings. E2F transcriptional activity was monitored at the indicated stages using the PCNA-GFP reporter (A′ and B′). CycE–Cdk2 activity is temporarily able to activate the E2F reporter in the absence of *e2f2* (A) but is unable to sustain E2F activity after 40 h APF (B). (C–E) GFP-marked *e2f2*-null mutant clones expressing CycE–Cdk2 were generated using the MARCM system and examined for ectopic mitoses by staining for PH3. Neurons are marked by expression of Elav. Ectopic mitoses in $e2f2^{-/-}$ cells expressing CycE–Cdk2 are evident in Elav-negative cells in eyes from 24 to 40 h APF (36 h APF shown; C) but are no longer observed by 44 h APF (D). No ectopic mitoses are observed in $e2f2^{-/-}$ cells expressing CycE–Cdk2 in wings after 36 h APF (E). (F–H) Clones expressing either GFP-$E2F1^{WT}$ or GFP-$E2F1^{PIP3A}$ with CycE–Cdk2 were generated using *hs*-FLP *act*>Gal4/UAS in pupal wings (F–H) and eyes (H). Clones were negatively marked by CD2 (F and G), and levels of GFP-tagged E2F1 were compared at 44 h APF. All GFP measurements were acquired at the same gain and at roughly similar tissue sections. Several samples ($n = 7$) across three independent experiments were compared for GFP intensity using ImageJ (National Institutes of Health), and representative examples are shown. Bars in H indicate the mean GFP intensity across all samples measured, and error bars indicate the standard deviation. GFP-$E2F1^{WT}$ is destabilized in the presence of CycE–Cdk2 after exit in eyes and wings (F–H). Wings expressing either GFP-$E2F1^{WT}$ or GFP-$E2F1^{PIP3A}$ in the dorsal domain during pupal stages (under control of *ap*-Gal4/UAS, *tub*-$Gal80^{TS}$) were assayed for PH3. (I and J) No ectopic mitoses were evident in wings expressing either the stabilized or WT forms of E2F1 after 36 h APF (44 h APF shown). However, several ectopic $ELAV^{+}$ neurons were found in the dorsal posterior margin of all GFP-$E2F1^{PIP3A}$–expressing wings (J, arrows). Bars, 50 µm.

To test whether CycE–Cdk2 activity affected degradation of E2F1 after cell cycle exit in a PIP box–dependent manner, we generated clones coexpressing either WT GFP-$E2F1^{WT}$ or E2F with a mutated PIP box, GFP-$E2F1^{PIP3A}$ with CycE–Cdk2 in the wing and eye. Levels of GFP-E2F1 were determined by detection of the GFP tag using an anti-GFP antibody and compared between GFP-$E2F1^{WT}$ and GFP-$E2F1^{PIP3A}$ (Fig. 1, F–H). We found that levels of GFP-$E2F1^{PIP3A}$ in the presence of CycE–Cdk2 after

Table I. **Genetic manipulations of cell cycle regulators and their effects on cell cycle exit**

Genetic backgrounds	Delay exit 24–40 APF?	Delay exit past 44 APF? (mitotic index)	Figure or reference
e2f2$^{-/-}$ + CycE + Cdk2	Yes	No	Fig. 1
GFP-E2F1^{WT}	Yes	No	Fig. 1
GFP-E2F1^{PIP3A}	Yes	No	Fig. 1
GFP-E2F1^{WT} + CycE + Cdk2	Yes	ND	Fig. 1
GFP-E2F1^{PIP3A} + CycE + Cdk2	Yes	ND	Fig. 1
dp$^{-/-}$ + CycE + Cdk2	Yes	No	Fig. 2
dp$^{-/-}$ + CycE + Stg	Yes	Yes (≤3%)	Fig. 2
e2f2$^{-/-}$ + CycE + Stg	Yes	Yes (≤1%)	Fig. 2
CycE + Stg	Yes	Yes (≤1%)	Fig. 2
rbf1$^{-/-}$ + CycE–Cdk2	Yes	No, wing blade Yes, neurons	Buttitta et al., 2007
rbf$^{-/-}$ + CycE + Stg	Yes	Yes (15–20%)	Fig. 3
rbf$^{-/-}$ + Stg	Yes	No	Fig. 3
CycA	Yes	No	Buttitta et al., 2007
Rca1	Yes	No	Fig. 5
E2F1–DP + Rca1	Yes	Yes	Figs. 5 and 6
E2F1–DP + Fzy^{RNAi}	Yes	Yes	Fig. 5
E2F1–DP + Fzr^{RNAi}	Yes	Yes	Fig. 5
E2F1–DP + Cortex^{RNAi}	Yes	No	Fig. 5
E2F1–DP	Yes	No	Fig. 6
CycE + Cdk2	Yes	No	Buttitta et al., 2007
E2F + CycE–Cdk2	Yes	Yes	Buttitta et al., 2007
E2F + CycE + Stg	Yes	Yes	Buttitta et al., 2007

exit were higher in both pupal eyes and wings than GFP-E2F1^{WT} (Fig. 1, F–H). This suggests that if CycE–Cdk2 is aberrantly activated after exit, it could induce S-phase initiation and PIP box–dependent E2F1 degradation to serve as a self-correcting mechanism to limit CycE-E2F positive feedback after exit.

To test whether stabilized E2F1 is sufficient to bypass exit, we expressed GFP-E2F1^{PIP3A} in the dorsal domain of the wing (using *Apterous* [*ap*]-Gal4/UAS) during pupal stages and assayed for ectopic mitoses. No mitoses were evident in wings expressing either the stabilized or WT forms of E2F1 after 36 h APF (Fig. 1, I and J), suggesting that the stabilized E2F1 cannot delay cell cycle exit further than WT E2F1. However, stabilized E2F1 did cause a mild phenotype, as several ectopic neurons were found in the dorsal posterior margin of all GFP-E2F1^{PIP3A}–expressing wings (Fig. 1 J, arrows).

These results suggest that a switch to CycE-resistant E2F2 repressive complexes together with the degradation of E2F1 activator complexes underlie the resistance of terminally differentiated cells to G1 cyclin–Cdk-induced proliferation.

Bypass of cell cycle exit is limited in the absence of all E2F-DP function

Our results suggest that in the absence of *e2f2*, aberrant CycE–Cdk2 activity leads to degradation of E2F1, and the subsequent loss of both E2F activator and repressor complexes results in cell cycle exit, even in the face of high cyclin–Cdk activity. However, because our experiments were performed in the presence of endogenous E2F1, we could not rule out the potential contribution of a novel CycE-resistant E2F1 repressive complex to cell cycle exit. To address this, we directly examined whether CycE–Cdk2 could bypass cell cycle exit in *Dp*-null mutant cells, which are believed to lack all E2F–DP transcriptional function (Frolov et al., 2005).

GFP-labeled *Dp*$^{-/-}$ cells overexpressing CycE–Cdk2 did not bypass exit in wings or eyes after 36 h APF and arrested at the G1-S transition with high levels of CycE–Cdk2 activity, as evident by anti-MPM2 (mitotic phosphoprotein marker 2) antibody staining of CycE–Cdk2-phosphorylated targets at the histone locus body (Fig. 2, A and B, arrows indicate examples; White et al., 2007). The arrest of CycE–Cdk2-expressing *Dp* mutant cells at the G1-S transition is indistinguishable from normal cells expressing CycE–Cdk2 at 36 h APF. (Buttitta et al., 2007).

Because CycE–Cdk2 was insufficient to bypass exit in *Dp* mutant cells, we next examined whether other cell cycle regulators, particularly regulators of the G2-M transition, could bypass exit in *Dp*-null cells. Expression of the G2-M regulators, CycA, Rca1 (regulator of CycA), and the Cdc25c phosphatase, String (Stg), all failed to bypass exit in *Dp*-null cells (Fig. S1). However, some *Dp*$^{-/-}$ cells coexpressing CycE and the G2-M regulator Stg continued cycling at 40–44 h APF, as indicated by PH3 and MPM2, with a mitotic index of 0.9% and 3% in the wing and eye, respectively (Fig. 2, C and D, PH3 shown). Importantly, rare ectopic mitoses were also evident in some control WT wings and eyes expressing CycE and Stg (mitotic index up to 0.3% and 1.0% in some wing and eyes, respectively; Fig. 2, G and H). This suggests that some very low level of cycling, undetected by our previous clonal assays (Buttitta et al., 2007), continues in a small number of cells expressing CycE + Stg. This cycling is moderately increased threefold when all E2F–DP function is lost in *Dp*-null cells. Therefore, we questioned whether the loss of E2F2–DP repressive function was responsible for the increased cycling

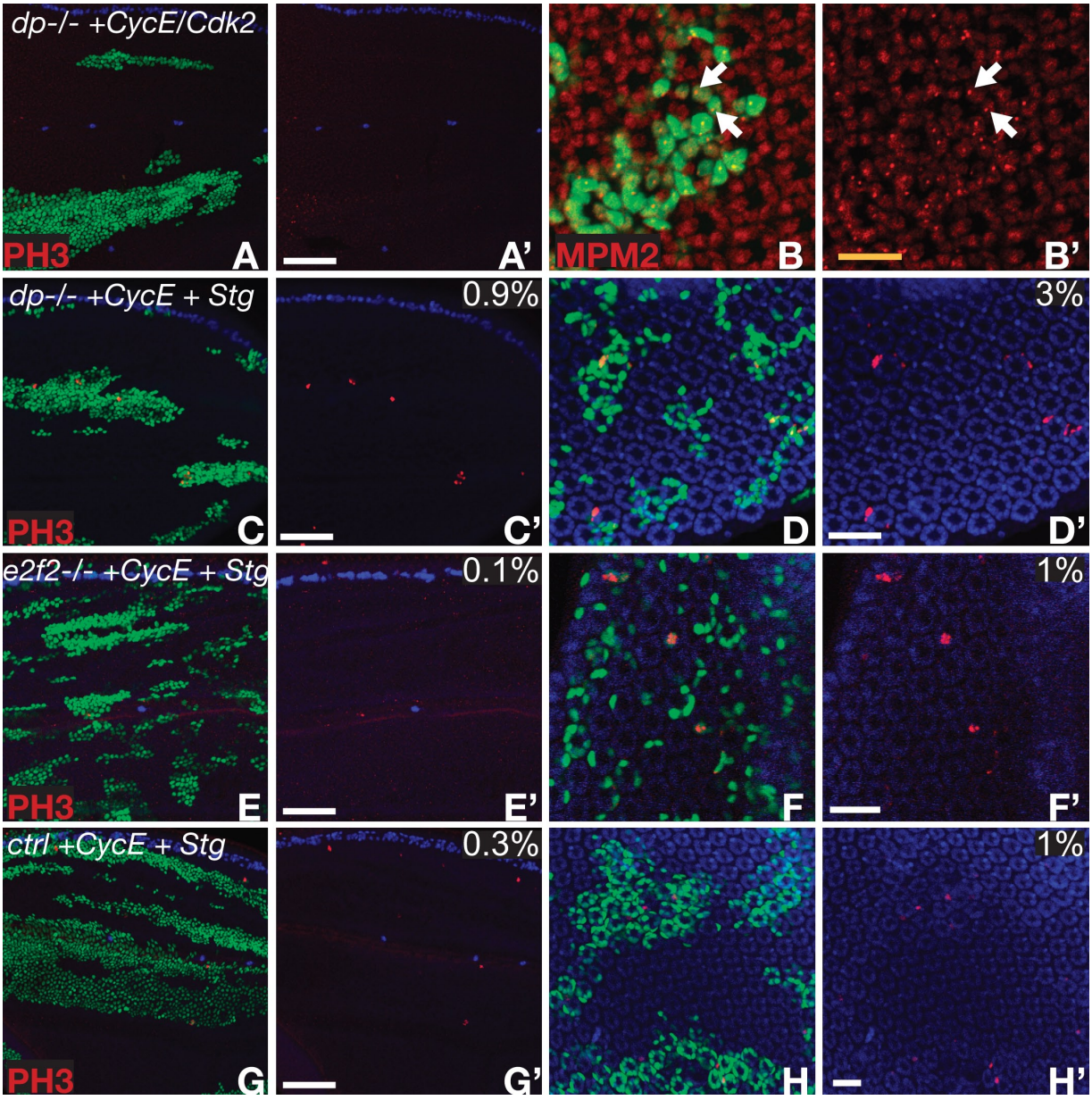

Figure 2. **Bypass of cell cycle exit is limited in the absence of E2F–DP function.** (A–H) GFP-marked control or *e2f2*- or *Dp*-null mutant clones expressing the indicated cell cycle regulators were generated using the MARCM system and examined for ectopic mitoses by staining for PH3 (A and C–H) or ectopic CycE–Cdk2 activity indicated by MPM2 foci (B). Neurons are shown by staining for Elav (blue). $Dp^{-/-}$ cells expressing CycE–Cdk2 do not bypass exit in wings (A) or eyes (B) after 36 h APF (40–44 h shown) and arrest with high levels of CycE–Cdk2 activity, as evident by MPM2-positive nuclear foci (eyes shown; arrows point to examples of foci in B). Some $Dp^{-/-}$ cells expressing CycE and the G2-M regulator Stg continue cycling at 40 h APF, as indicated by PH3 (C and D) with a mitotic index of 0.9% and 3% in the wing and eye, respectively. A few mitoses are also observed in *e2f2*-null mutant cells expressing CycE and Stg at 40–44 h APF (E and F). However, similar levels of ectopic mitoses are evident in some control wings (G; mitotic index up to 0.3%) and eyes (H; up to 1.0%) expressing CycE and Stg (40–44 h APF shown). Bars: (A and C–H) 50 µm; (B) 20 µm.

seen in *Dp* mutant cells expressing CycE + Stg. However, *e2f2*-null mutant cells expressing CycE + Stg in wings and eyes were indistinguishable from control cells expressing CycE + Stg (Fig. 2, E and F).

The failure of most *Dp* mutant cells to significantly bypass cell cycle exit, even in the presence of high CycE and Stg, suggests that E2F–DP activity is somehow required for ectopic proliferation of terminally differentiated cells, even though it is not required for proliferation earlier in development (Frolov et al., 2001, 2005).

Loss of *rbf* together with CycE and Stg prevents cell cycle exit and produces tumor-like overproliferation of terminally differentiated cells

We previously tested whether *rbf* mutant cells expressing CycE–Cdk2 could bypass exit and found continued cycling of differentiated neurons in late-stage pupae but no cycling and an S/G2-phase arrest in the epithelial wing blade (Table I; Buttitta et al., 2007). Based on the low level of mitoses seen in *Dp* mutant cells expressing both CycE and Stg, we reasoned

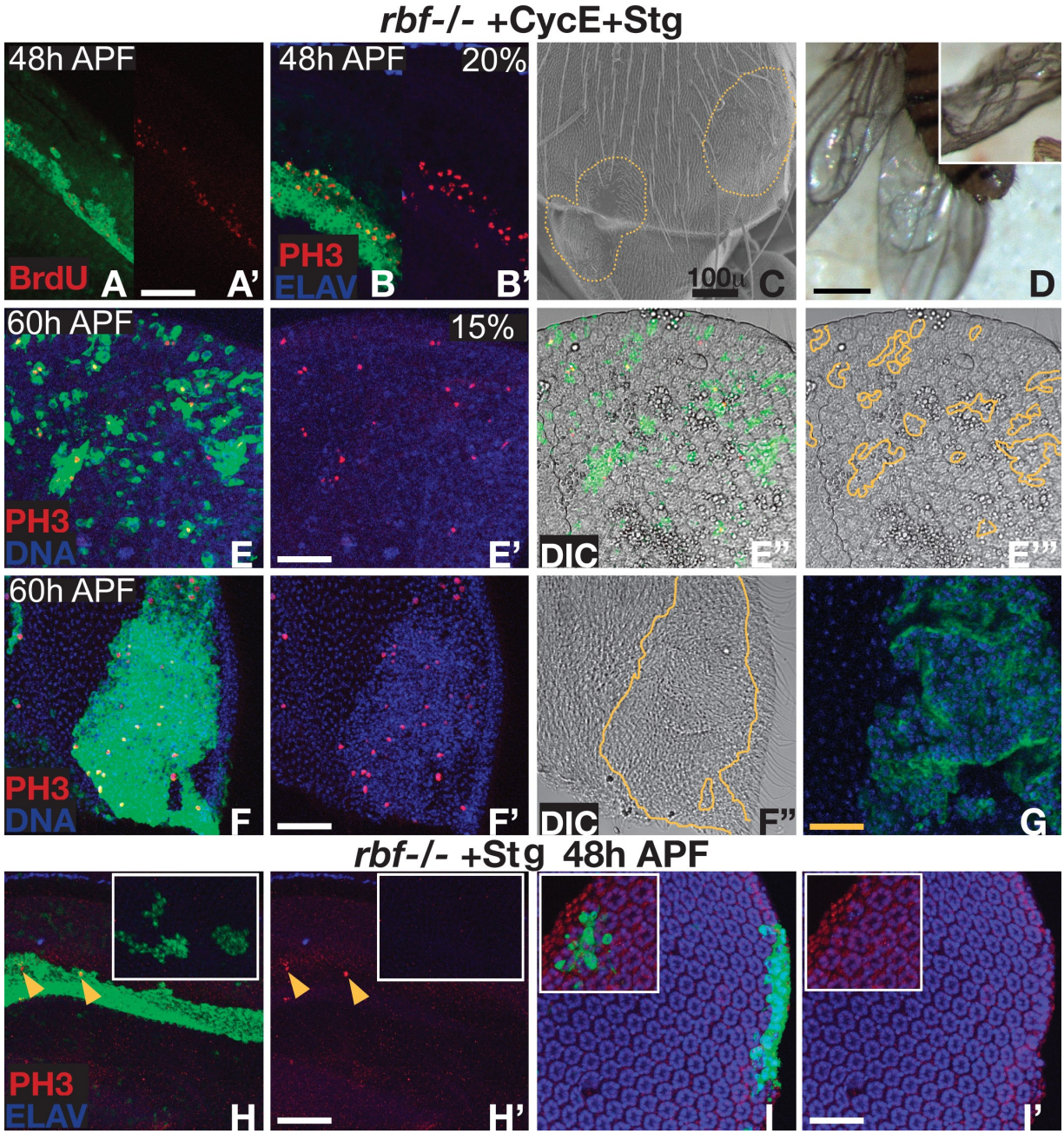

Figure 3. **Loss of *rbf* together with CycE and Stg prevents cell cycle exit and causes overproliferation of terminally differentiated cells.** (A–G) GFP-marked *rbf*-null mutant clones expressing CycE and Stg were generated using the MARCM system and examined for ectopic S phases (BrdU; A) or mitoses (PH3; B, E, and F). Ectopic cell cycles are evident at high levels (mitotic index up to 20%) in wings (A, B, and F) and eyes (E; 15%) at all time points examined, including the latest time point, 60 h APF. Bulges and folding of adult cuticle are observed in regions of mutant clones in the thorax (C) and wing (D and inset). Eye (E) and wing (F) terminal differentiation structures such as lenses, bristles, and wing hairs are evident. A tangential section of a wing clone shows tissue folding caused by overproliferation (G). (H and I) GFP-marked *rbf*-null mutant clones expressing Stg were generated using the MARCM system and examined for ectopic mitoses at late stages up to 56 h APF (PH3; H and I; 48 h APF shown). At stages after 40 h APF, a few mitoses were evident in more proximal regions of some clones (H, arrowheads) but not in most clones in the wing (H, insets) or the eye (I, insets show a second example of a clone in the eye). (C, E‴, and F″) Yellow outlines indicate clone boundaries. DIC, differential interference contrast. Bars: (A, B, E, F, H, and I) 50 µm; (C) 100 µm; (D) 0.5 mm; (G) 20 µm.

that expressing CycE and Stg in $rbf^{-/-}$ cells might completely bypass exit by providing the necessary G2-M activity and E2F activator function. To test this, we generated GFP-marked *rbf*-null mutant clones expressing CycE and Stg using the MARCM system and examined them for ectopic S phases (by BrdU incorporation) or mitoses (PH3). Ectopic cell cycles, including S phases and mitoses, were evident at very high levels (mitotic index up to 20%) in wings and eyes of $rbf^{-/-}$ cells expressing CycE + Stg at all time points examined, including the latest time point at which we could perform antibody staining, 60 h APF (Fig. 3, A, B, and E–G). Overproliferation continued at 60 h APF when ommatidia differentiation, wing adult cuticle, and trichome differentiation were evident (Fig. 3, E and F). Proliferation of differentiated cells in the late wing generated

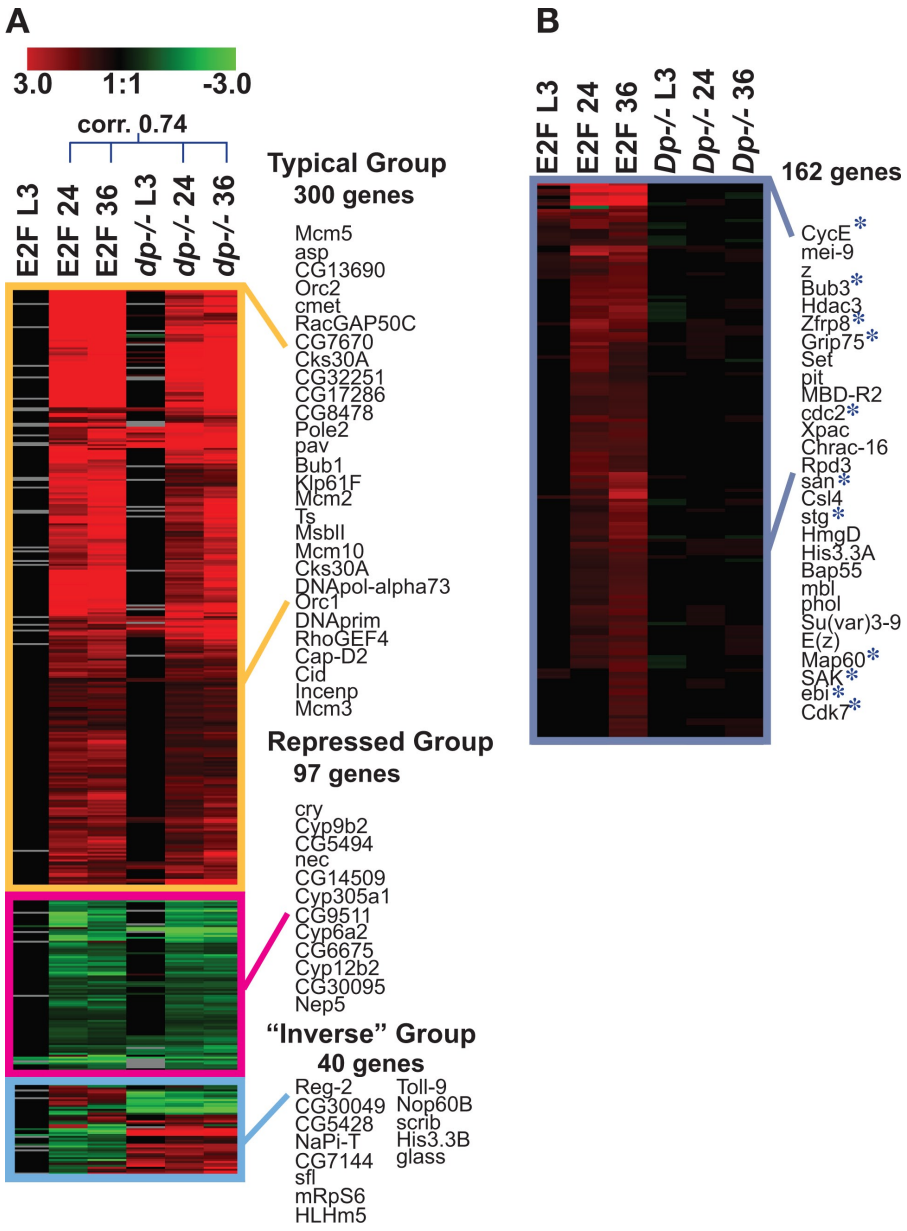

Figure 4. **E2F transcriptional activation and *Dp* loss are highly similar, except for specific genes that require E2F activity for expression after exit.** Using microarrays, we compared gene expression in E2F1–DP-expressing wings (*ap*-Gal4/UAS-E2F1, UAS-DP, *tub*-Gal80^{TS}) with controls (*ap*-Gal4/UAS, *tub*-Gal80^{TS}) and *Dp* mutant wings ($w;Dp^{a1/a2}$) with controls (w) at three time points. (A) Heat map of transcript changes (color range indicates the $\log_2$ ratio of expression compared with controls). All transcripts with a fold change of 1.5 or more ($>\log_2 \pm 0.6$) at one or more time points are shown. Gray bars indicate transcripts that were removed from analysis because of high variability among independently replicated experiments ($n = 4$ for E2F; $n = 3$ for $Dp^{-/-}$). Transcripts were clustered using Genesis software for hierarchical clustering. Representative genes from each major cluster are listed on the right. Typical group transcripts are up-regulated in E2F-expressing and $Dp^{-/-}$ wings at 24 and 36 h APF, whereas other transcripts are repressed or inversely regulated. Arrays for E2F and $Dp^{-/-}$ at 24 and 36 h were highly similar with a correlation coefficient of 0.74. (B) A group of 162 genes was up-regulated >1.75-fold ($>\log_2$ 0.82) by E2F activity at one or both time points APF but was not significantly increased in $Dp^{-/-}$ (change <1.3-fold; $<\log_2$ 0.4). Asterisks indicate examples of genes that regulate cyclin–Cdk activity or mitosis.

clusters of small cells, resulting in folding of the epithelium, reminiscent of tumor-like overgrowth (Fig. 3 G). We also observed outgrowths on adult nota and folding of the adult wing indicative of overproliferation with relatively normal terminal differentiation (Fig. 3, C and D). Importantly, the dramatic bypass of exit observed in $rbf^{-/-}$ CycE/Stg-expressing cells was not recapitulated in *rbf*-null mutant clones expressing only Stg. Cells mutant for *rbf* expressing Stg exhibited few mitoses restricted to the proximal regions of some clones in the wing at 48 h APF, and no mitoses were observed in eyes after 48 h APF (Fig. 3, H and I).

Our results indicate that the E2F activity provided by the loss of $rbf^{-/-}$ plays an essential role in bypassing exit. We confirmed that $rbf^{-/-}$ cells ectopically activate the E2F-responsive PCNA-GFP reporter after exit, demonstrating aberrantly high E2F1 transcriptional activity (Fig. S2 G). Yet despite the E2F activity, the threshold for both CycE and Stg activities somehow remains high in $rbf^{-/-}$ cells, and independent activation of both is required to bypass cell cycle exit.

Gene expression changes caused by E2F transcriptional activation and DP loss are highly similar after cell cycle exit

Our results with $rbf^{-/-}$ cells indicate that some level of E2F activator, not simply E2F derepression, is required to bypass cell cycle exit upon terminal differentiation. This requirement is specific to the forced proliferation of terminally differentiating cells, as cells lacking all E2F–DP activity can proliferate during normal development (Frolov et al., 2001, 2003, 2005). This suggests that certain critical E2F targets may require the activator function of E2F for expression in differentiating cells. Therefore, we chose to compare gene expression changes caused by high E2F activity to those caused by the loss of all E2F–DP function in terminally differentiating cells.

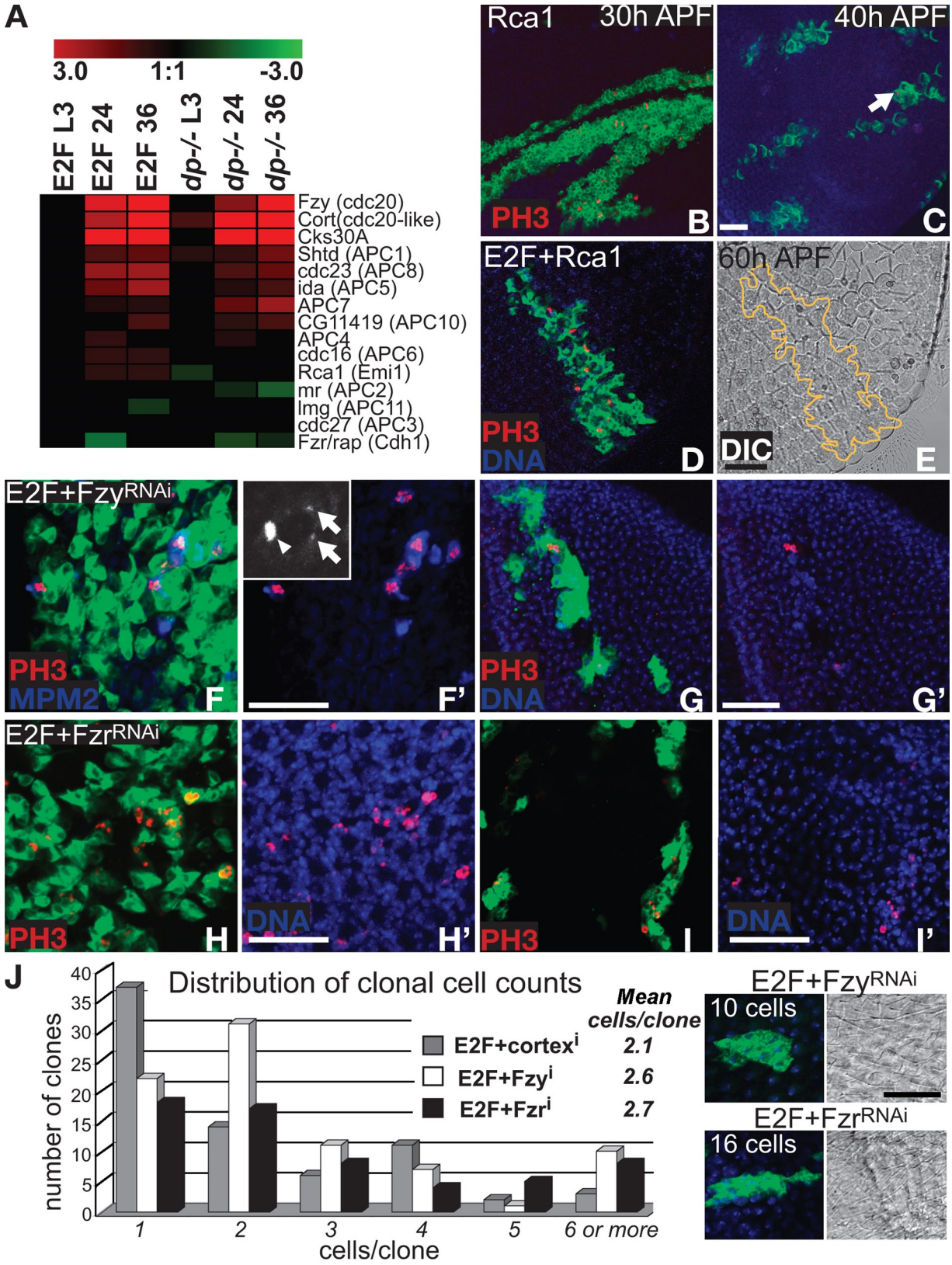

Figure 5. **APC/C activity limits E2F-induced bypass of exit.** (A) Several APC/C components (*APC1*, *-4*, *-5*, *-6*, *-7*, *-8*, and *-10*) and regulators (*cdc20*, *cdc20-like*, and *Cks30A*) are transcriptionally increased (1.5–40-fold) when E2F activity is high or de-repressed in *Dp* mutant wings. (B–I) GFP-marked clones expressing the indicated cell cycle regulators were generated using *hs*-FLP *tub*>Gal4/UAS, *tub*-Gal80^{TS} and examined for PH3 (B–I) or MPM2 (F). Nuclei were stained with Hoechst (blue; G–I). PH3 is seen in clones expressing the APC/C inhibitor Rca1 at 24–36 h APF in the eye and wing (wing shown; B) but not the late wing and was only observed once in the eye at 40–44 h APF (C, arrow). When E2F1–DP and Rca1 are coexpressed, bypass of exit is observed as late as 60 h APF (D) without disrupting terminal differentiation of ommatidia or bristles (E). Yellow outline indicates clone boundary. Coexpression of E2F1–DP with Fzy RNAi or Fzr RNAi also led to ectopic mitoses in eyes (F and H) and wings (G and I) at 48 h APF, as shown by PH3

We took advantage of the relatively synchronous cell cycle exit in the wing to examine gene expression by microarray in E2F–DP-expressing wings, which temporarily delay exit because of E2F activity, and *Dp* mutant wings, which lack E2F–DP function but exit on time. We directly compared E2F1–DP-expressing wings (*ap*-Gal4/UAS-E2F1, UAS-DP, *tub*-Gal80^{TS}) with controls (*ap*-Gal4/UAS, *tub*-Gal80^{TS}) and *Dp* mutants (*w;Dp*$^{a1/a2}$) with controls (*w*) during proliferation (third larval instar [L3]), at normal exit (24 h APF), and after prolonged exit when E2F no longer promotes cycling (36 h APF). Fig. 4 shows the hierarchically clustered heat maps of transcript changes ($\log_2$ ratio of expression compared with controls) for all transcripts with a fold change of 1.5 or more ($>\log_2 \pm 0.6$) at one or more time points. Gray bars indicate transcripts that were removed from the analysis because of high variability among replicates (Fig. 4 A). The complete dataset is available as tab-delimited text in the online supplemental material as a txt file, additional array data descriptions can be found in Materials and methods, and complete clusters can be viewed in high resolution on the JCB DataViewer.

Overall, transcript changes caused by E2F1–DP (E2F) overexpression and *Dp* loss were strikingly similar in terminally differentiating cells. When arrays were compared, a correlation coefficient of 0.74 was found for E2F and *Dp*$^{-/-}$ arrays at 24 and 36 h APF. Representative genes from each major cluster are listed in Fig. 4. The largest cluster consists of what we call typical transcripts, which represent many known E2F target genes, that are up-regulated in both E2F-expressing and *Dp*$^{-/-}$ wings at 24 and 36 h APF. This group contains most of the 157 genes identified as *Drosophila* E2F targets in proliferating S2 cells by Dimova et al. (2003). This even includes genes in groups B and C from that study, which were unaffected by *Dp* loss in S2 cells (Dimova et al., 2003) but we find to be affected by *Dp* loss in wings in vivo. We also identified many additional E2F targets corroborated by other model system studies (for review see Bracken et al., 2004). Typical group transcripts were enriched for Gene Ontology (GO) terms involved in cell cycle ($P < 6.84^{-9}$), DNA replication and repair ($P < 2.56^{-8}$), microtubule cytoskeleton organization ($P < 2.14^{-7}$), and DNA packaging ($P < 2.84^{-4}$).

The second largest cluster of coregulated genes in E2F-overexpressing and *Dp* mutant wings consisted of genes repressed in differentiating cells. Because E2F1–DP is thought to act as a transcriptional activator, this group of repressed genes is somewhat unexpected. These genes are not repressed indirectly by a delay in cell cycle exit, as they are similarly repressed in *Dp* mutants, which exit normally. We also don't believe this group is caused by E2F1–DP repressive complexes blocking transcription, as such targets would be expected to increase rather than decrease in *Dp* mutants.

The third group of transcripts altered by E2F expression and *Dp* loss are inversely regulated between the two genotypes. These include transcripts increased by E2F expression but decreased in *Dp* mutants, and vice versa. Genes up-regulated by high E2F but decreased in *Dp* mutants could be genes that require E2F1–DP complexes for basal expression as well as activation, and therefore, expression is lost in the absence of *Dp*. In contrast, genes decreased by E2F but increased in *Dp* mutants could be genes somehow actively repressed by E2F1–DP complexes, repression which is lost upon the loss of *Dp*. Neither subgroup of conversely regulated transcripts exhibited any statistically significant enrichment in GO terms, although several of these genes are known to be important in developmental signaling, differentiation, and proliferation (i.e., *HLHm5*, *scribbled*, *Toll-9*, and *glass*).

Specific cell cycle genes require E2F activity for expression after exit

A group of 162 genes was up-regulated >1.75-fold ($>\log_2$ 0.82) by increased E2F activity at one or both time points after exit but was not significantly increased in *Dp* mutants at any time point (change <1.3-fold; $<\log_2$ 0.4; Fig. 4 B). This group includes genes that may become limiting for bypass of exit in *Dp* mutants, which is consistent with the finding that this group is significantly enriched for genes regulating the cell cycle and mitosis ($P < 8.07^{-4}$) but is also enriched for genes involved in RNA processing ($P < 7.15^{-5}$) and chromatin regulation ($P < 3.4^{-4}$). We noted that *cycE* and *stg* were among this group, as well as the cyclin–Cdk-activating kinase component, *cdk7*, which is important for maximal CycB–Cdk1 activity (Larochelle et al., 1998), and the Cdk target of Stg phosphatase activity, Cdk1 (*cdc2*). Asterisks in Fig. 4 B indicate several examples of genes in this group that regulate cyclin–Cdk activity or mitosis.

Based on our array analysis, E2F activation and *Dp* loss result in highly similar changes in gene expression upon terminal differentiation. This is because a major role for DP after cell cycle exit is in forming repressive E2F–DP complexes, and therefore, loss of DP after exit more closely resembles E2F derepression. However, we found a set of genes important for mitosis, cell cycle progression, and chromatin regulation that specifically require E2F activator function for expression in terminally differentiating cells. We propose that it is the E2F-dependent transcriptional activation of these genes, together with high CycE and Stg activity, that is required to abrogate cell cycle exit upon terminal differentiation.

Anaphase-promoting complex/cyclosome (APC/C) activity limits E2F-induced bypass of exit

Although E2F activity induces robust expression of many cell cycle genes after exit, including *cycE* and *stg*, the threshold of CycE and Stg activity required to bypass exit is increased in terminally differentiating cells. The increased threshold for CycE is not simply a result of an up-regulation of Dacapo because cell cycle exit still occurs in wings and certain cell types in the eye

staining (F–I) and MPM2 cytoplasmic mitotic staining (F; white in F′ inset; arrowhead indicates cytoplasmic MPM2 staining). G1-S progression was also observed in clones expressing E2F1–DP with Fzy RNAi in eyes at 48 h APF, as indicated by MPM2 nuclear foci (F; white in F′ inset; arrows point to foci). Sparse clones overexpressing E2F + RNAis for the three APC/C activators were induced at 0 h white prepupae, and cells per clone for at least 50 clones/genotype were quantified at 54–56 h APF. The distributions of clonal cell counts and mean cells/clone are indicated in J. (J, right) Examples of large clones and overlying wing cuticle and hairs at 56 h APF. DIC, differential interference contrast. Bars, 50 μm.

in *dacapo*-null mutant cells overexpressing E2F1–DP (Buttitta et al., 2007). In our array analysis, we noted that E2F activity (or *Dp* loss) induced expression of several components of the APC/C and certain APC/C activators (Fig. 5 A). Therefore, we wondered whether high APC/C activity could limit E2F-induced cell cycle reentry after differentiation.

The fly orthologues of at least one APC/C activator (Cdh1 or Fizzy [Fzy]-related [Fzr] in flies) and one core APC/C component (APC1 or Shattered in flies) normally increase after cell cycle exit in eyes (Pimentel and Venkatesh, 2005; Tanaka-Matakatsu et al., 2007; Zielke, 2007). We have confirmed that both Fzr transcript and protein also increase in wings after normal exit (Fig. S2 C). We speculated that an increase in APC/C activity after exit could limit E2F-induced bypass of exit by degrading the G2-M cyclin–Cdks and Stg, which are known APC/C targets. This could also raise the threshold for CycE after exit by requiring increased CycE–Cdk2 activity to inhibit the high levels of APC/C (Sigrist et al., 1995; Sigrist and Lehner, 1997; Reber et al., 2006). This idea is consistent with the finding that the loss of *fzr* or *shattered* delays cell cycle exit by one cycle in the embryo and larval eye (Sigrist and Lehner, 1997; Pimentel and Venkatesh, 2005; Tanaka-Matakatsu et al., 2007). Therefore, we examined whether APC/C activity contributes to cell cycle exit in both the pupal eye and wing and to what extent inhibition of the APC/C could delay exit.

To inhibit the APC/C upon cell cycle exit without disturbing mitotic cycles, we overexpressed Rca1, an S and G2 inhibitor of the APC/C which leads to accumulation of CycA but does not disrupt mitosis when overexpressed (Grosskortenhaus and Sprenger, 2002). Clones of cells expressing Rca1 temporarily delayed cell cycle exit (Fig. 5 B) but did not continue significant cycling after 40 h APF in the eye or wing (Fig. 5 C, eye shown exhibiting the sole mitosis observed). However, when E2F and Rca1 were coexpressed, continued mitoses were observed very late in the eye and wing, including the latest time point tested, 60 h APF, without disrupting terminal differentiation of ommatidia (Fig. 5, D and E; and Fig. S2 A).

Rca1 increases CycA by inhibition of the APC/C. However, the effect of Rca1 + E2F on cell cycle exit was not simply caused by an increase in CycA levels, as coexpression of CycA with E2F1–DP could not continue to bypass exit after 40–44 h APF in wings or eyes (Fig. S2 B). APC/C activity is dependent on sufficient levels of core components and the presence of the activators Cdc20 (Fzy in *Drosophila*) and Fzr. To directly reduce APC/C activity in cells overexpressing E2F, we coexpressed E2F with RNAis to core APC/C components (APC2 and -8) or the activators Fzy or Fzr in clones and examined late pupal eyes and wings after 48 h APF for ectopic cycling. We observed frequent mitoses, as assessed by both PH3 and MPM2 cytoplasmic staining in pupal eyes and wings at 56 h APF, when E2F was coexpressed with each RNAi (Fig. 5, F–I; and Fig. S2, E and F). We also observed MPM2 nuclear foci indicative of G1-S progression in cells expressing E2F + Fzy^{RNAi} (Fig. 5 F, inset), suggesting complete additional cell cycles occur.

To confirm that additional cell cycles occurred, we performed a clonal analysis on cells coexpressing E2F with Fzy or Fzr RNAi, as well as cells expressing E2F and an RNAi to Cortex, a germline cdc20-like protein which is up-regulated by E2F after exit in wings (Fig. 5 A) but had no effect on cycling or E2F delay of cell cycle exit in eyes or wings. When clones expressing E2F + $Cortex^{RNAi}$ were induced by a limited heat shock at 0 h APF, very few clones containing more than four cells were observed at 48–56 h APF. However, we observed an increase in the mean cells/clone when Fzy or Fzr RNAis were coexpressed with E2F, as well as an increase in the number of clones containing six or more cells (Fig. 5 J). Some of the E2F + Fzy^{RNAi} and E2F + Fzr^{RNAi} clones contained as many as 10–16 cells/clone (Fig. 5 J, right), indicating that multiple rounds of complete extra cell cycles occurred with normal adult wing cuticle differentiation. This finding was consistent with our observation that E2F + Rca1 expression also increased the frequency of larger clones in the wing (Fig. S2 D).

We hypothesized that high APC/C activity after exit could limit E2F-induced bypass of cell cycle exit by degrading key E2F targets. This is consistent with our finding that E2F1–DP-overexpressing cells do not accumulate the known APC/C targets CycA, Geminin (Gem; Zielke et al., 2008), CycB, or Stg (based on a Stg-GFP fusion protein) after prolonged exit in wings or eyes (40–44 h APF; Fig. 6, A, C, E, and G; and Fig. S3), despite expressing high levels of *cycA*, *gem*, *stg*, and *cycB* transcripts by microarray (see supplemental dataset and JCB DataViewer). In contrast, cells expressing E2F + Rca1 together accumulated CycA in wings (Fig. 6 B), Stg-GFP in eyes (Fig. 6 D), Gem in eyes (Fig. 6 H), and very low levels of CycB in wings and eyes (Fig. 6 F and Fig. S3).

In contrast to other cyclins, E2F activity is able to induce CycE protein accumulation after prolonged exit (Buttitta et al., 2007) because CycE is not a target for degradation by the APC/C. CycE activity is thought to inhibit the APC/C (Sigrist and Lehner, 1997; Reber et al., 2006). Therefore, an increase in APC/C levels could raise the CycE threshold after exit by requiring increased CycE for complete APC/C inhibition. Consistent with this, we found that cells overexpressing high levels of CycE–Cdk2 accumulated APC/C targets such as Gem and CycA after exit (Fig. 6, I and J).

We suggest that high APC/C activity after exit raises the threshold of CycE and Stg required for cell cycle reentry of differentiated cells. This appears to be increased even further by aberrant E2F activity in differentiated cells by increasing the expression of several core components as well as inducing expression of the activator *fzy*. This could explain why the increased CycE expression provided by high E2F activity alone is insufficient to bypass cell cycle exit after terminal differentiation.

Discussion

Studies of terminal differentiation and cell cycle exit have largely focused on events initiating the inhibition of CycE and E2F activity (for reviews see Myster and Duronio, 2000; Buttitta and Edgar, 2007; Miller et al., 2007; Pajalunga et al., 2008). Several genes have been identified that can temporarily delay exit either by increasing cyclin–Cdk activity or by preventing E2F inhibition in *Drosophila*. Relatively little attention, though, has focused on the mechanisms that ultimately act to establish

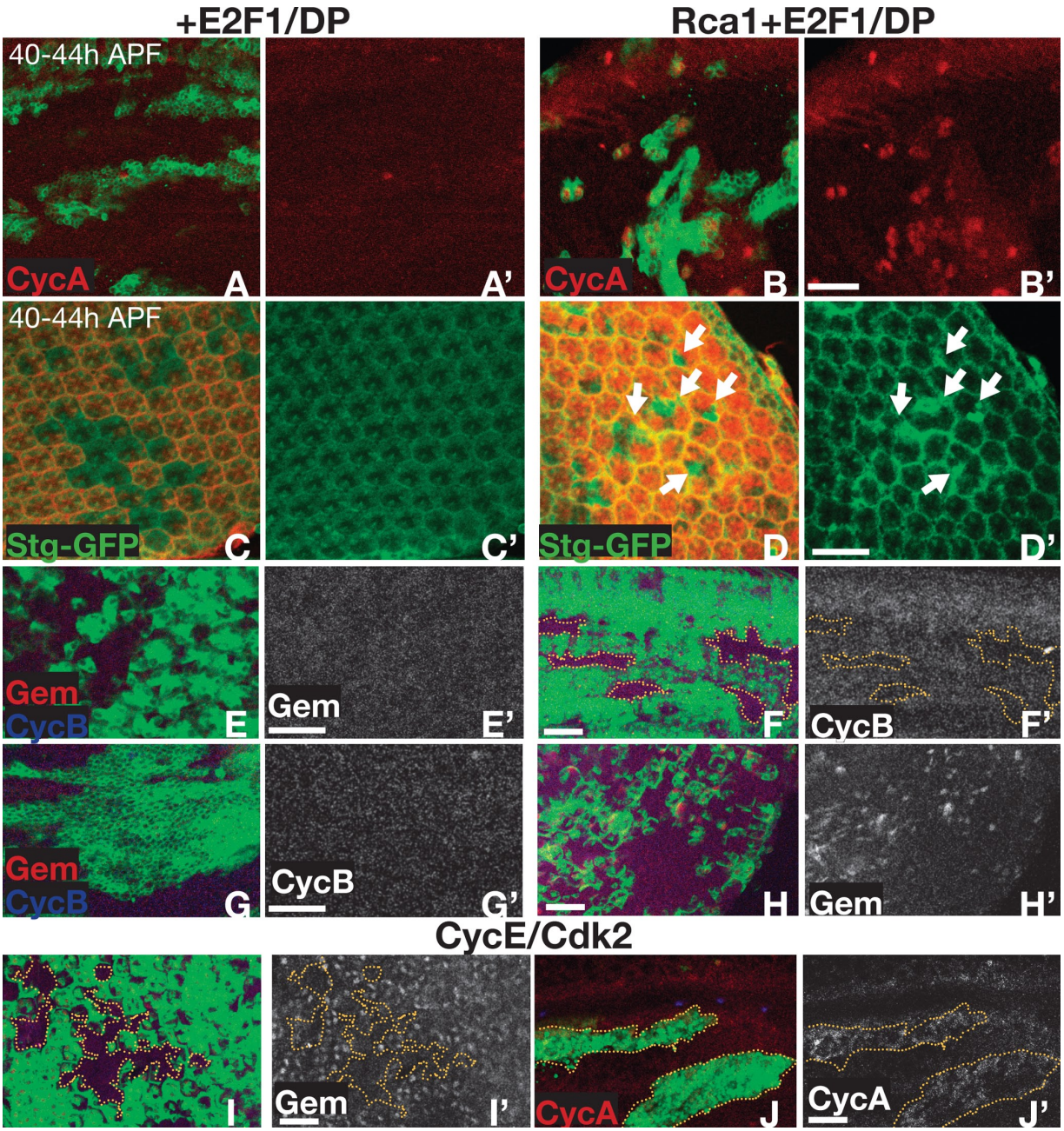

Figure 6. **APC/C activity limits accumulation of E2F-induced cell cycle regulators.** GFP-marked clones expressing the indicated cell cycle regulators were generated using *hs*-FLP *tub*>Gal4/UAS, *tub*-Gal80^{TS} and examined for CycA, CycB, or Gem (A, B, and E–J). Clones negatively marked by CD2, expressing the indicated regulators (C and D) were generated using *hs*-FLP *act*>Gal4/UAS and examined for Stg-GFP fusion protein. Cells expressing E2F1–DP in the wing and eye fail to accumulate CycA (A), Stg-GFP (C), Gem (E), and CycB (G) at 40–48 h APF. Cells coexpressing E2F1–DP + Rca1 accumulate CycA (B) and low levels of CycB in the wing (F), as well as Stg-GFP and Gem in the eye (D and H). Cells expressing CycE–Cdk2 accumulate Gem in the eye (I) and CycA in wing (J). (F, I, and J) Yellow outlines indicate clone boundaries. (D) Arrows indicate examples of clones with increased Stg-GFP expression. Bars, 50 µm.

and maintain robust cell cycle exit in these situations. The data we present in this study suggest a model for robust maintenance of cell cycle exit in terminally differentiated cells, even in the face of aberrant CycE or E2F activity.

Robust cell cycle exit mechanisms

Previous studies in *Drosophila* have suggested that cell cycle exit is normally triggered by a decrease in CycE–Cdk2 activity, through the cooperation of multiple CycE inhibitors such as Dacapo, the CycE ubiquitin ligase Fbw7 (Archipelago in *Drosophila*), Hippo signaling, and *cycE* transcriptional down-regulation (Li and Vaessin, 2000; Moberg et al., 2001; Firth and Baker, 2005; Choksi et al., 2006; Sukhanova et al., 2007; Nicolay and Frolov, 2008). This leads to hypophosphorylation of Rbf, resulting in E2F–DP target gene repression and stable cell cycle arrest. Yet, cell cycle exit still occurs in mutants lacking these CycE inhibitors and in the presence of high CycE–Cdk2 activity, demonstrating that additional mechanisms can trigger exit (Buttitta et al., 2007).

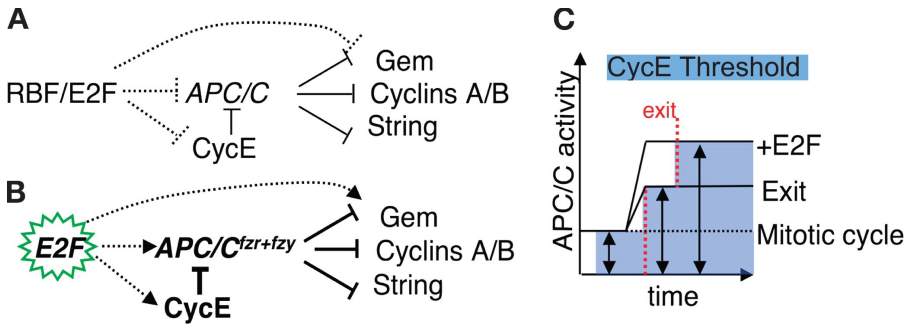

Figure 7. **A robust cell cycle control mechanism limits E2F-induced proliferation after cell cycle exit.** (A) E2F-dependent transcriptional repression (dotted lines) of targets, including many APC/C components, is established at exit. (B) If aberrant E2F activity occurs, transcriptional activation (dotted arrows) of APC/C components and activators limits the accumulation of key cell cycle targets such as Stg. (C) The resulting high APC/C activity in differentiating cells requires increased CycE to bypass exit, thereby raising the CycE threshold.

APC/C^{Fzr} activity normally increases at G1 arrest, degrading G2 and mitotic cell cycle targets such as CycA, CycB, and Stg (Fig. S2; Sigrist and Lehner, 1997; Pimentel and Venkatesh, 2005; Tanaka-Matakatsu et al., 2007; Zielke, 2007). This function of the APC/C is essential for timely exit, as loss of the APC/C is sufficient to cause an additional cell cycle, likely via unrestrained CycA activity (Table I; Sigrist and Lehner, 1997; Jacobs et al., 2001; Buttitta et al., 2007; Tanaka-Matakatsu et al., 2007). The mammalian APC/$C^{Fzr/Cdh1}$ has been shown to interact with RB to promote cell cycle exit via degradation of Skp2 and subsequent stabilization of $p27^{Kip1}$ (Binné et al., 2007). A similar mechanism may also act to promote exit in *Drosophila* via APC/C^{Fzr}, Rbf, and Dacapo, but functions for a *Drosophila* Skp2 homologue in cell cycle control have not yet been examined.

When E2F is aberrantly activated in differentiating cells, expression of several APC/C^{Fzy} components increases and may cooperate with high APC/C^{Fzr} to limit the accumulation of mitotic cyclins and other essential E2F transcriptional targets for cycling such as Stg (Figs. 5 and 6). CycE–Cdk2 activity has been shown to inhibit APC/C (Sigrist and Lehner, 1997; Reber et al., 2006), and thus, the increased APC/C activity present in differentiating cells may raise the threshold levels of CycE required for APC/C suppression. Our data suggest that this may explain, at least in part, why supraphysiological levels of ectopic CycE are required to bypass cell cycle exit in differentiating wings and eyes (Fig. 7).

If CycE–Cdk2 is aberrantly activated in differentiating cells, cell cycle exit is temporarily delayed with cells arresting at the G1-S transition or in early S phase with phosphorylated histone locus bodies and some partially replicated DNA (Buttitta et al., 2007). In such cells, the S phase–dependent degradation of E2F1 protein by the Cul4 ubiquitin E3 ligase appears to remain on, leading to an increased ratio of repressive E2F2 to E2F1. This ultimately results in stable CycE–Cdk2-resistant repression of E2F target genes and cell cycle exit (Fig. 1). If E2F2 is absent, aberrant CycE–Cdk2 leads to S-phase initiation and PIP box–dependent Cul4-mediated destruction of E2F1, resulting in loss of both E2Fs and thereby loss of all E2F–DP function. This situation is similar to that in *Dp* mutants, which lack all E2F-dependent transcriptional function. In these mutants, critical cell cycle genes, including Stg and other cyclin–Cdk regulators, are protected from derepression (Fig. 4 B), and their expression remains low, ensuring a noncycling state.

The S phase–dependent degradation of E2F1 occurs normally during proliferation (Shibutani et al., 2008), so how does the normally transient degradation of E2F1 have such a profound effect on the cell cycle after terminal differentiation? During proliferative stages, overexpression of CycE–Cdk2 does not arrest cells, and E2F1 is able to reaccumulate after S phase. In contrast, cells with high CycE–Cdk2 after differentiation fail to complete their final G1-S transition or arrest in S phase with low E2F1 protein levels (Buttitta et al., 2007), suggesting that the E2F1 degradation machinery remains active. This arrest may be partially caused by the depletion of E2F target genes critical for S-phase completion. But the arrest of $rbf^{-/-}$ cells overexpressing CycE in the wing blade, which have high levels of E2F target gene expression from previous cell cycles, suggests that there may also be an activation of a checkpoint or inhibition of a key activity for S-phase completion independent of E2F transcriptional regulation after terminal differentiation. Although we do not know the specific mechanism for this arrest, the addition of Stg is able to restore cycling in $rbf^{-/-}$ cells overexpressing CycE (Fig. 3 and Table I). This demonstrates that Stg or a target of Cdk1 activity is able to bypass the arrest of cells with high CycE–Cdk2 after terminal differentiation to allow E2F1 reaccumulation.

Importantly, the cell cycle control mechanisms we describe in this study do not act alone at exit. They likely cooperate with other cell cycle inhibitors known to promote exit such as Dacapo, Fbw7, and Hippo signaling. These cell cycle controls together limit cell cycle reentry to ensure robust cell cycle exit upon terminal differentiation.

APC/C functions in terminally differentiated cells

The data presented in this study demonstrate an important role for the APC/C in maintaining cell cycle exit in the face of aberrant E2F activity in terminally differentiated postmitotic tissues (Fig. 5). Interestingly, work in mammalian neurons has shown additional postmitotic functions for the APC/C in mature cells (Puram et al., 2010). Both the APC/$C^{Fzr/Cdh1}$ and APC/C^{Cdc20} complexes carry out important nonmitotic functions in the control of axon and dendrite growth and patterning, synapse development, and neuron survival (Konishi et al., 2004; Li et al., 2008; Huynh et al., 2009; Kim et al., 2009; Yang et al., 2009). This suggests that the APC/C complexes in many terminally differentiated cells carry out previously unappreciated dual roles in promoting postmitotic cell maturation and protecting against aberrant cell cycle reentry. This is supported by the recent finding that Cdh1 is a haplo-insufficient tumor suppressor in mice (García-Higuera et al., 2008), although further studies will be required to determine whether the tumors in Cdh1

heterozygotes arise from defects in cell cycle exit, cell cycle reentry, or genetic instability in proliferating cells.

Cell cycle exit, terminal differentiation, and cancer

It is becoming increasingly clear that certain highly differentiated cells retain the ability to enter the cell cycle. In some cases, this serves a beneficial regenerative function, as recently shown for terminally differentiated cardiomyocytes in the zebrafish heart (Jopling et al., 2010; Kikuchi et al., 2010). But in other cases, this ability seems to be a part of the normal tissue development, as shown for differentiated horizontal cells that reenter the cell cycle for their final division in the zebrafish retina before full maturation (Godinho et al., 2007). Proliferation of terminally differentiated mammalian cells has also been observed in monocytes and macrophages lacking the c-Maf and MafB transcription factors (Aziz et al., 2009). Strikingly, Maf double knockout macrophages appear to fully retain their differentiation-associated functions in vivo despite exhibiting continued cycling for months, demonstrating a clear independence of differentiation and cell cycle exit in these cells (Aziz et al., 2009).

We have shown that cell cycle exit is not essential for terminal differentiation in *Drosophila* eyes and wings. Evasion of the robust cell cycle exit mechanism by loss of the tumor suppressor *rbf* together with deregulation of CycE and Stg can lead to tumor-like overproliferation of differentiated tissues (Fig. 3). Our results may be relevant to specific cancers such as retinoblastoma, where aberrantly dividing differentiated cells have been proposed to be the cancer cell of origin (Chen et al., 2004; Ajioka et al., 2007; Xu et al., 2009). Aberrant division of differentiated cells could lead to genetic instability and acquisition of secondary mutations, leading to dedifferentiation and ultimately to metastatic cancer. Our data show that the robust exit mechanism is essential for proper tissue development and suggest that even terminally differentiated cells should not be excluded as a potential source of cancer.

Materials and methods

Fly strains

The following fly strains were used: w^{1118}; y w $hsflp^{122}$;UAS-CycE (Neufeld et al., 1998); y w $hsflp^{122}$;+;UAS-CycE,UAS-Stg (Neufeld et al., 1998); y w $hsflp^{122}$;+;UAS-CycE,UAS-Cdk2 (Meyer et al., 2000); y w $hsflp^{122}$;UAS-E2F,UAS-DP/Cyo-GFP (UAS-DP from N. Dyson, Massachusetts General Hospital, Boston, MA; Neufeld et al., 1998); y w $hsflp^{122}$;UASp-GFP-$E2F1^{WT}$;UAS-CycE,UAS-Cdk2 and y w $hsflp^{122}$;UASp-GFP-$E2F1^{PIP3A}$; UAS-CycE,UAS-Cdk2 (UASp-GFP-E2F1 from Shibutani et al. [2008]); w; UAS_T-GFP-$E2F1^{WT}$ and w;UAS_T-GFP-$E2F1^{WT\ PIP3A}$ (provided by J. Davidson and R. Duronio, University of North Carolina, Chapel Hill, NC); y w $hsflp^{122}$;UAS-CycA;+ (Jacobs et al., 2001); w;act>CD2>Gal4,UAS-GFP (Pignoni and Zipursky, 1997); w;tub>CD2>Gal4,UAS-GFP;tub-$Gal80^{TS}$ (tub>CD2>Gal4 from F. Pignoni, Harvard Medical School, Boston, MA; $Gal80^{TS}$ described in McGuire et al. [2004]); w;tub>CD2>Gal4,UAS-GFP;tub-$Gal80^{TS}$,UAS-Diap (UAS-Diap from Lohmann et al. [2002]); w;*ap*-Gal4,UAS-GFP/CyO;tub-$Gal80^{TS}$ (*ap*-Gal4 described in Calleja et al. [1996]); FRT19A,w,rbf^{14}/FM7 (from W. Du, University of Chicago, Chicago, IL; Du and Dyson, 1999); y w hsflp122;+;UAS-Rca1 (from F. Sprenger, University of Regensburg, Regensburg, Germany; Zielke et al., 2008); and FRT19A,w,rbf^{14}/FM7;+; UAS-CycE,UAS-Stg.

FRT19A,ywhsflp^{122}, tub-Gal80; tub-Gal4,UAS-GFP/MKRS (Lee and Luo, 2001); y w $hsflp^{122}$,tub-Gal4,UAS-GFP;FRT42D tub-Gal80 (Jiang et al., 2009); w;+; act>CD2>Gal4, PCNA-GFP (PCNA-GFP from Thacker et al. [2003]); FRT42D,Dp^{a3}/CyO-GFP;+ (from Frolov et al. [2005]); w;Dp^{a1}/CyO-GFP and w;Dp^{a2}/Cyo-GFP (Royzman et al., 1997); w; de2f2^{c03344} FRT40A (from M. Frolov, University of Illinois, Chicago, IL; Ambrus et al., 2007); de2f2$^{G5.1}$ and de2f2^{76Q1} (both from Frolov et al. [2001]); and w; Stg-GFP Yale Trap insertions YD0685 and YD0246 were also used. w; UAS-Fzr^{RNAi}, w; UAS-Fzy^{RNAi}, w; UAS-$Cortex^{RNAi}$, w; UAS-$APC2^{RNAi}$, and w; UAS-$APC8^{RNAi}$ are all from the Vienna *Drosophila* RNAi Center (#25550, #44834, #41472, #2993, and #52279, respectively).

Antibodies

Antibodies used were rabbit α–phospho-Ser10–histone H3 (PH3; 1:4,000; Millipore), mouse α–phospho-Ser10–histone H3 (PH3; 1:1,000; Millipore), mouse α-MPM2 (1:500; Millipore), mouse α-BrdU (1:100; BD), rat α-ELAV (embryonic lethal abnormal vision; 1:200; Developmental Studies Hybridoma Bank), rabbit α-GFP (1:1,000; Invitrogen), mouse α–rat CD2 (1:800; AbD Serotec), mouse α-CycA (1:100; Developmental Studies Hybridoma Bank), mouse α-CycB (1:50; Developmental Studies Hybridoma Bank), rabbit α-Gem (1:100; provided by B. Calvi, Indiana University, Bloomington, IN), and rabbit α-Fzr (1:3,000; provided by C. Lehner, University of Zurich, Zurich, Switzerland). Appropriate secondary antibodies were Alexa Fluor 488, 568, or 633 conjugated (Invitrogen) and used at 1:4,000.

Histology

Pupae, staged from white prepupae (0 h) at 25°C, were dissected and fixed with 4% paraformaldehyde in 1× PBS for 30 min at room temperature. Pupal cuticle was removed from wings after fixation. Tissues 24–36 h APF were blocked in PBS + 0.1% Triton X-100 + 1% BSA for 1 h. Tissues 36–44 h APF were blocked in PBS + 0.3% Triton X-100 + 1% BSA (PAT) overnight. For stages after 44 h APF, we incorporated a methanol dehydration series after fixation (25, 50, 75, and 100% methanol/1× PBS, 10 min each) that allowed improved antibody penetration, increased sensitivity, and BrdU detection for tissues 44–60 h APF. After step-wise rehydration, tissues were blocked in PAT overnight, and antibody staining or BrdU incorporation was performed as described previously (Buttitta et al., 2007; Zielke et al., 2008) using Alexa Fluor 488–, Alexa Fluor 568– or Alexa Fluor 633–conjugated secondary antibodies and Vectashield mounting medium (Vector Laboratories). Wing hinge and notum as well as head capsule and antenna were excluded from our analyses. 1 µg/ml Hoechst 33258 (Invitrogen) was used to label nuclei. Confocal sections and differential interference contrast images were collected on a microscope (LSM 510; Carl Zeiss, Inc.) using a 25× objective. Single representative sections (2.2 µm) are shown, except for Fig. 3 (E and F), which are projections of eight sections at a 1-µm interval. Images were cropped using Photoshop (Adobe). Mitotic index was calculated as the mean number of PH3-positive cells/100 GFP-positive cells among multiple clones in several samples, counted blind, from at least two independent experiments.

Clonal overexpression

We used the *hs*-Flp/FRT *tub*>CD2>Gal4 method for clonal overexpression, with *tub* temperature-sensitive Gal80 ($Gal80^{TS}$; McGuire et al., 2004). $Gal80^{TS}$ was inactivated at 29°C. Hours APF are presented as equivalent time at 25°C for simplicity. All incubation times were adjusted appropriately as described previously (Buttitta et al., 2007). Loss of function clones (or appropriate controls) were generated using MARCM (Lee and Luo, 2001). Larvae were heat shocked for 45 min at 37°C at 60–72 h after egg deposition, collected for staging at 0 h APF, aged at 25°C, and dissected at the indicated times.

Clonal cell counts

Nonoverlapping clones labeled with membrane-bound GFP, expressing the indicated regulators, were induced at 0 h APF (white prepupae) and fixed 54–56 h later, nuclei were labeled with 1 µg/ml Hoechst 33258, and cells/clone were counted. We excluded wings with >50 clones/wing (to reduce the possibility of independent clones merging). We also excluded clones in the wing margin, hinge, notum area, and hemocytes in the veins. No apoptosis inhibitor was used in the clonal cell count experiments.

Microarray

For microarray experiments, 10 pupal wings from animals expressing E2F1 and DP (*ap*-Gal4/UAS-E2F1, UAS-DP, *tub*-$Gal80^{TS}$) and controls (*ap*-Gal4/UAS, *tub*-$Gal80^{TS}$) or *Dp* mutants (*w*;$Dp^{a1/a2}$) and controls (*w*) were dissected at the appropriate time points. For E2F1–DP samples, temperature shifts limited the overexpression to pupal stages. For L3 discs, E2F1–DP was expressed for the equivalent of 24 h before dissection of wandering larvae. RNA was isolated using standard techniques (TRIZOL), and cDNA synthesis was performed with one subsequent round of T7-dependent linear

RNA amplification using the commercially available Message AmpTM kit (Applied Biosystems) as described previously (Reeves and Posakony, 2005). Amplified RNA was labeled in a subsequent cDNA synthesis reaction according to NimbleGen protocols and hybridized to NimbleGen 4-plex 60-mer *Drosophila* expression arrays (http://www.nimblegen.com). Hybridizations were repeated four times for E2F expression and three times for *Dp* mutants with independently obtained biological replicates to ensure maximal confidence in data reproducibility. Statistically significant changes with a 1.5-fold cutoff were determined using analysis of variance (Tusher et al., 2001). Hierarchical clustering was performed using the Genesis program (Sturn et al., 2002). GO enrichment was examined using GOrilla (http://cbl-gorilla.cs.technion.ac.il) and FatiGO (http://www.babelomics.org) programs.

Additional description of microarray data. In addition to the analysis provided in the results section, we also examined the other clusters of transcripts affected by E2F activity in the wing. The second largest cluster of coregulated genes in E2F-overexpressing and *Dp* mutant wings was repressed in differentiating cells. Because E2F1–DP is thought to act as a transcriptional activator, this group of repressed genes is somewhat unexpected. These genes are not repressed indirectly by a delay in cell cycle exit, as they are similarly repressed in *Dp* mutants, which exit normally. We also do not believe that this group is caused by E2F1–DP repressive complexes blocking transcription, as such targets would be expected to increase rather than decrease in *Dp* mutants. One possibility suggested by Bracken et al. (2004) is that the extensive transcriptional up-regulation caused by E2F (or in *Dp* mutants) could cause some spurious antisense regulation of genes. Although we have not investigated this possibility, >0% of the transcriptional changes we observed in both genotypes are indeed increases in transcript levels. Overall, this group of repressed transcripts is significantly enriched for genes involved in monooxygenase activity (caused by six cytochromeP450 genes; $P < 8.08^{-4}$) and genes with catalytic activity ($P < 5.85^{-4}$), suggesting some functional significance for this group.

We were surprised to find that most transcripts were not affected by E2F overexpression or *Dp* loss at the proliferative L3 time point. This may be because E2F target gene expression is already high in controls during proliferation, as evident by the high activity of the E2F reporter construct (PCNA-GFP) and high levels of E2F target gene expression at this stage (Thacker et al., 2003). This is also supported by our finding that the few transcripts up-regulated by E2F and/or *Dp* loss at L3 are normally at low abundance in wings and were significantly ($P < 0.009$) enriched for genes with expression one standard deviation lower than the mean gene expression. These transcripts were also enriched for genes identified as group D or E germline/differentiation genes specifically regulated by E2F2 in Dimova et al. (2003), which we find to be ectopically induced in nongermline tissues by E2F overexpression and *Dp* loss (examples include *spn-E*, *nanos*, *BthD*, CG6790, CG8316, and CG7628). Thus, the transcriptional activation of E2F targets at proliferative stages seems to be masked by the high levels of E2F activity already present in the controls. Interestingly, this also suggests that normal levels of E2F targets are somehow maintained in an E2F–DP-independent manner in proliferating *Dp* mutant cells.

Western blotting

Western blots were performed as described previously (Prober and Edgar, 2000) with the following modifications. Protein was isolated from 20 pupal or larval wings (with notum removed) by boiling for 5 min in 40 µl of 1× SDS sample buffer containing β-mercaptoethanol. 20 µl of sample was then used for SDS-PAGE. Rabbit anti-Fzr (provided by C. Lehner) was used at 1:3,000 with the appropriate HRP-conjugated secondary antibody.

Online supplemental material

Fig. S1 shows that bypass of cell cycle exit is limited, even in the absence of all E2F–DP function. Fig. S2 shows GFP-labeled clones expressing the indicated cell cycle regulators generated using *hs*-FLP *tub*>Gal4/UAS, *tub*-Gal80^{TS}. Fig. S3 shows GFP-marked clones expressing the indicated cell cycle regulators generated using *hs*-FLP *tub*>Gal4/UAS, *tub*-Gal80^{TS} and examined for CycB or Gem. The complete E2F and $Dp^{-/-}$ array dataset (provided as tab-delimited text) is included as a txt file. Complete microarray clusters are also available for viewing on the JCB DataViewer. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200910006/DC1.

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