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Intrinsic negative cell cycle regulation provided by PIP box- and Cul4^{Cdt2}-mediated destruction of E2f1 during S phase

Shusaku T. Shibutani[1], **Aida Flor A de la Cruz**[4], **Vuong Tran**[4], **William J. Turbyfill III**[1], **Tânia Reis**[4,5], **Bruce A. Edgar**[4], and **Robert J. Duronio**[1,2,3,*]

1 *Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599*

2 *Program in Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, North Carolina 27599*

3 *Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599*

4 *Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, Washington 98109*

Summary

E2F transcription factors are key regulators of cell proliferation that are inhibited by pRb family tumor suppressors. pRb-independent modes of E2F inhibition have also been described, but their contribution to animal development and tumor suppression is unclear. Here we show that S phase-specific destruction of *Drosophila* E2f1 provides a novel mechanism for cell cycle regulation. E2f1 destruction is mediated by a PCNA-interacting-protein (PIP) motif in E2f1 and the Cul4^{Cdt2} E3 ubiquitin ligase, and requires the Dp dimerization partner but not direct Cdk phosphorylation or Rbf1 binding. E2f1 lacking a functional PIP motif accumulates inappropriately during S phase and is more potent than wild type E2f1 at accelerating cell cycle progression and inducing apoptosis. Thus, S phase-coupled destruction is a key negative regulator of E2f1 activity. We propose that pRb-independent inhibition of E2F during S phase is an evolutionarily conserved feature of the metazoan cell cycle that is necessary for development.

Keywords

Drosophila; E2F; PCNA; Cul4; Cdt2; Ddb1; cell cycle; ubiquitin; apoptosis

Introduction

The E2F family of transcription factors regulates the expression of genes involved in DNA synthesis, mitosis, apoptosis, DNA repair, and differentiation, and consequently plays a key role during normal animal development and disease, particularly cancer (Burkhart and Sage, 2008; van den Heuvel and Dyson, 2008). Multiple E2F genes in diverse species encode proteins that can be generally categorized as activators or repressors. Activator E2F over-expression induces S phase entry followed by apoptosis, while depletion causes cell cycle arrest. Since

*To whom correspondence should be addressed: Robert J. Duronio, Department of Biology, CB#3280, University of North Carolina, Chapel Hill, NC 27599, Voice: (919) 962-7749, FAX: (919) 962-8472, email: E-mail: duronio@med.unc.edu.

[5]Present address: Department of Molecular and Cell Biology, UC Berkeley, 142 Life Science Addition, Berkeley, California 94720-3200

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activator E2Fs are strong positive regulators of cell proliferation, their activity is tightly controlled. A significant number of studies have identified multiple mechanisms that restrain activator E2F function. The best studied mechanism is inhibition by members of the retinoblastoma (pRb) tumor suppressor protein family, which bind to and block transcriptional activation by E2Fs and recruit chromatin modifying enzymes to pRb/E2F complexes that contribute to transcriptional repression (Blais and Dynlacht, 2007). The importance of pRb-mediated repression of activator E2Fs is inferred by the observations that loss of pRb function in vivo leads to failure to maintain cell cycle arrest and that reduction of E2F activity suppresses tumor formation in $Rb^{+/-}$ heterozygous mice (Burkhart and Sage, 2008).

Several modes of pRb-independent inhibition of activator E2Fs have also been described, and these may be important during S/G2 when hyper-phosphorylated pRb is not associated with E2F. For instance, homo- and heterodimers of the recently identified E2F7 and E2F8 proteins, which lack obvious pRb family member binding sites (Christensen et al., 2005; Logan et al., 2005; Maiti et al., 2005), bind to the promoter of the *E2f1* gene and repress the transcription of *E2f1* and its targets in S/G2 (Li et al., 2008; Zalmas et al., 2008). Activator E2Fs bind DNA via dimerization with a DP protein, and during S phase Cyclin A/Cdk2 binds the E2F N-terminus and phosphorylates E2F-bound DP, leading to the dissociation of E2F/DP from DNA (Dynlacht et al., 1994b; Krek et al., 1994; Krek et al., 1995). Finally, activator E2Fs are degraded via the ubiquitin-proteasome pathway (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996; Marti et al., 1999). However, the relative contribution of each of these regulatory mechanisms to growth, development, and cancer, and how they interface with pRb-regulation of E2F, is only beginning to be explored.

In particular, it is not known whether ubiquitin-mediated proteolysis of activator E2Fs is important during normal development. We are studying this question in *Drosophila*, which contain one activator E2F (E2f1) and one repressor E2F (E2f2), each of which bind a single Dp. *Drosophila* E2f1 shares characteristics with mammalian activator E2Fs, and functions as a regulator of the G1-to-S transition and apoptosis (van den Heuvel and Dyson, 2008). E2f1 is required for the expression of genes involved in DNA synthesis, such as *Cyclin E*, *RnrS*, *Pcna*, and *DNA polymerase* α (Dimova et al., 2003; Duronio et al., 1995; Royzman et al., 1997), and regulates expression of pro-apoptotic genes such as *reaper* (Asano et al., 1996), *hid*, and *Dcp-1* (Moon et al., 2005). *E2f1* is essential for fly development, and *E2f1* mutant cells proliferate poorly or arrest (Brook et al., 1996; Duronio et al., 1995; Neufeld et al., 1998). Conversely, E2f1 over-expression can induce ectopic S phase entry, accelerate cell cycle progression, and trigger apoptosis (Asano et al., 1996; Du et al., 1996b; Duronio et al., 1996; Morris et al., 2006; Neufeld et al., 1998). E2f1 is inhibited by binding to Rbf1, one of two pRb-related *Drosophila* proteins (Du et al., 1996a; Xin et al., 2002), and the E2f2 repressor antagonizes the function of E2f1 (Frolov et al., 2001; Rasheva et al., 2006; Weng et al., 2003), similar to mammalian repressor E2Fs that antagonize activator E2Fs by replacing the activators on promotors during G0/G1 and turning off E2F-responsive genes (Trimarchi and Lees, 2002). However, flies containing a missense mutation in the COOH-terminus of E2f1 that blocks interaction with Rbf1 are viable, as are *E2f2* null mutants, indicating that inhibition of E2f1 activity by Rbf1 or E2f2 is not required for normal *Drosophila* development (Cayirlioglu et al., 2001; Frolov et al., 2001; Weng et al., 2003). Thus, as in mammals, Rbf1-independent mechanisms of E2f1 regulation are likely to be important.

E2f1 protein abundance is tightly cell cycle regulated in *Drosophila*. In embryos and growing larvae, E2f1 is destroyed specifically during early S phase (Asano et al., 1996; Heriche et al., 2003; Reis and Edgar, 2004; Shibutani et al., 2007; Zielke et al., 2008). We therefore hypothesize that regulated proteolysis provides an important, Rbf1- and E2f2-independent mechanism for inhibiting E2f1 activity during the cell cycle. Previously, the S phase-specific destruction of E2f1 was linked to the $Cul1^{Slmb}$ E3 ubiquitin ligase (Heriche et al., 2003).

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However, the stabilization of E2f1 caused by the inhibition of Cul1 or Slmb was incomplete, suggesting the possibility that other E3 ligases are involved. Here we identify a motif in *Drosophila* E2f1 that is necessary for destruction during S phase. We show that S phase-specific destruction of E2f1 requires the $Cul4^{Cdt2}$ E3 ubiquitin ligase and is necessary to restrain cell cycle progression during development.

Results

An assay for S phase-specific E2f1 destruction in cultured S2 cells

To determine the mechanism of S phase-specific destruction of E2f1, we established a fluorescence activated cell sorting (FACS)-based experimental system that allowed us to rapidly detect accumulation of E2f1 in any phase of the cell cycle. S2 cells were either stably (Fig. 1A) or transiently (Fig. 1C) transfected with plasmids that express either GFP or GFP fused to the NH_2-terminus of full length E2f1 under the control of the *Actin5C* promoter. The transfected cells were fixed, stained for DNA, and analyzed by FACS. Fig. 1 shows histograms of the DNA content of GFP-positive (gray area) cells overlaid on the profile of all cells in the entire population (open area). In stably transfected lines, GFP was expressed in all cells of the population regardless of cell cycle phase (Fig. 1A, top panel), whereas few S phase cells accumulate GFP-E2f1 (Fig. 1A, bottom panel). Because the *Actin5C* promoter is expressed constitutively in S2 cells (Angelichio et al., 1991), we conclude from these results that GFP-E2f1 is destroyed during S phase. Transiently transfected cells produced similar results (Fig. 1C, GFP versus WT (1–805); we also often observed fewer cells with G1 DNA content that were GFP-E2f1 positive, in addition to GFP-E2f1 destruction in S phase, perhaps because the level of ectopic GFP-E2f1 achieved from transient transfection promoted S phase entry). We used this FACS-based assay to explore the requirements for E2f1 destruction during S phase.

E2f1 destruction during S phase does not require direct Cdk phosphorylation or Rbf1 binding

Previous reports indicated that increased Cdk activity reduced E2f1 protein in wing imaginal discs (Reis and Edgar, 2004) and that Slimb/β-TRCP, a substrate receptor for a Cul1-based E3 ubiquitin ligase, binds to E2f1 in a phosphorylation-dependent manner (Heriche et al., 2003). If the phosphorylation of E2f1 by Cdk promotes E2f1 destruction via Cul1-mediated ubiquitylation, then mutations in the Cdk phosphorylation sites of E2f1 should stabilize the protein. To test this, we mutated all seven putative Cdk phosphorylation sites (TP or SP) in E2f1 to alanine (Fig. 1B, $E2f1^{\Delta Cdk}$). GFP-$E2f1^{\Delta Cdk}$ accumulation remained low in S phase cells (Fig. 1C), indicating that direct Cdk phosphorylation is not required for E2f1 destruction during S phase.

We next tested the contribution of Rbf1 binding to E2f1 destruction. A L786Q missense mutation in the COOH-terminus of E2f1 (Fig. 1B) renders E2f1 insensitive to repression by over-expressed Rbf1, and prevents Rbf1 from binding E2f1 (Weng et al., 2003). If Rbf1 binding protects E2f1 from destruction, then the L786Q mutation should destabilize E2f1 throughout the cell cycle. However, GFP-$E2f1^{L786Q}$ accumulation during the cell cycle was indistinguishable from GFP-E2f1 (Fig. 1C). In addition, *Rbf1* RNAi did not affect GFP-E2f1 accumulation (Fig. 3A). These data suggest that the destruction of wild type E2f1 during S phase is not regulated via interaction with Rbf1.

E2f1 contains a motif required for S phase-specific destruction

We hypothesized that E2f1 contains a motif that is sufficient to confer destruction during S phase. To test this, S2 cells were transiently transfected with plasmids expressing fragments of E2f1 with NH_2-terminal GFP tags under the control of the *Actin5C* promoter (Fig. 1B). We first divided E2f1 into thirds and found that E2f1 amino acids 1–230, in which no conserved motifs have been previously reported, was sufficient to induce S phase-specific destruction,

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while GFP-E2f1$^{231–528}$ and GFP-E2f1$^{529–805}$ accumulated in S phase (Fig. 1C). We then created four smaller, overlapping fragments of region 1–230 (Fig. 1B). Amino acids 93–184 and 139–230 conferred destruction during S phase, whereas amino acids 1–92 and 47–138 did not (Fig. 1C). These results suggest the presence of an S phase-specific destruction motif between amino acids 139–184 of E2f1 (Fig. 1B).

The region containing amino acids 139–184 is highly conserved in the E2f1 ortholog from other *Drosophilid* species, and in this region we identified a short sequence resembling a PCNA-interacting peptide or "PIP box" (Fig. 2A). This was a good candidate for the E2f1 destruction motif, since a PIP box is necessary for S phase-specific destruction of Cdt1, a member of the pre-replication complex. During S phase, chromatin-bound PCNA binds to Cdt1 via the PIP box, and this interaction triggers the ubiquitylation of Cdt1 by a Cul4 ubiquitin ligase complex containing the substrate receptor Cdt2, which binds and recruits Cdt1 to Cul4 via the Ddb1 adaptor (Fig. 3D)(Arias and Walter, 2006;Higa et al., 2006;Hu and Xiong, 2006;Jin et al., 2006;Senga et al., 2006). *Drosophila* E2f1 does not contain the glutamine residue at position 1 that is conserved in Cdt1's PIP box, but it does contain a hydrophobic residue at position 4 and aromatic residues at positions 7 and 8 that have been shown to be required for Cul4$^{Ddb1-Cdt2}$-mediated Cdt1 destruction (Fig. 2A)(Arias and Walter, 2006;Senga et al., 2006).

In order to test whether these amino acids are required for S phase-specific destruction, we mutated the conserved I153, Y156 and Y157 residues to alanine (E2f1^{PIP-3A}) and changed to alanine (E2f1^{PIP-7A}) or deleted (E2f1$^{PIP-7del}$) the entire putative PIP box. We first attempted to generate stable S2 cell lines that express GFP-E2f1^{PIP-3A}. Whereas hygromycin-resistant, stable transfectants expressing GFP or GFP-E2f1 from the *Actin5C* promoter were readily obtained, no selection-resistant cells of GFP-E2f1^{PIP-3A} were recovered, suggesting that the constitutive expression of E2f1^{PIP-3A} is poorly tolerated by cells. To circumvent this problem, we developed an assay for S phase-specific E2f1 destruction using a stably transfected cell line containing GFP-E2f1 under the control of the heat shock-inducible *Hsp70* promoter. Under normal 25–28°C growth conditions, these cells express little GFP-E2f1 (Fig. 2B). 45 min after a 30 min, 37°C heat shock, GFP-E2f1 accumulated in all cell cycle phases (Fig. 2B,C). At later time points after heat shock we observed a progressive decrease in the percentage of S phase cells expressing GFP-E2f1, such that by 225 min only 7% of the S phase cells were in the GFP-positive population (Fig. 2B). In addition, by 285 min after heat shock we observed an increase in the S phase population relative to the starting condition (29% vs. 42%), suggesting that GFP-E2f1 promoted the G1-to-S transition.

Based on these results, we stably transfected S2 cells with *Hsp70*-controlled GFP-E2f1 variants (WT, ΔCdk, L786Q, PIP-3A, PIP-7A and PIP-7del), and analyzed them at 225 min post heat shock. S phase-specific destruction was observed with the E2f1$^{\Delta Cdk}$ and E2f1^{L786Q} mutants (Fig. 2D). In marked contrast, all three PIP mutant E2f1s were not degraded during S phase (Fig. 2D). A quantification of three independent heat shock/FACS experiments is shown in Fig. 2E. Furthermore, GFP/BrdU double positive nuclei were observed specifically in the GFP-E2f1^{PIP}-expressing cultures (Fig. S1). These data demonstrate that a PIP box-like sequence is an essential component of a motif that mediates S phase-specific destruction of E2f1 in S2 cells.

E2f1 destruction requires a Cul4 E3 ubiquitin ligase

Our discovery of a putative PIP box in E2f1 led us to hypothesize that *Drosophila* E2f1 is degraded by the same pathway used for Cdt1. To test this hypothesis, S2 cells stably transfected with *Hsp70*-GFP-E2f1 were incubated for 2 days with dsRNAs targeting Cul4, Ddb1, Cdt2 or Pcna and tested for S phase-specific GFP-E2f1 destruction using the assay described above (Fig. 3A). The degree of knockdown of RNAi-targets was assessed by RT-PCR (Figs. 3B, S2)

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or western blot (Figs. 3C, S3B). sdRNA targeting Pcna and two, independent sdRNA targeting Cul4 and Cdt2 stabilized GFP-E2f1 during S phase (Figs. 3A, S3A). Cul4 and Cdt2 RNAi depletion also stabilized endogenous E2f1 (Fig. S3B). Surprisingly, the knockdown of Ddb1, which encodes the only known Cul4-associated adapter protein (Fig. 3D), did not result in E2f1 stabilization (Figs. 3A, S3A) even though two, independent sdRNAs successfully reduced *Ddb1* transcript and Ddb1 protein (Figs. 3B,C; S3A,B). We suspect that residual Ddb1 present after RNAi is still sufficient for E2f1 destruction. While we favor this interpretation, we cannot exclude the possibility that a different adaptor protein forms a functional ubiquitin ligase complex with Cul4 and Cdt2 to target E2f1.

To test whether E2f1 can associate with a Cul4 E3 ligase, myc-tagged E2f1 was co-expressed with HA-tagged Cul4, Cdt2, or Dp in S2 cells, and lysates from transfected cells were subjected to immunoprecipitation analysis. Although we could detect interaction between E2f1 and Dp, we were unable to detect a specific interaction between E2f1 and PCNA, Cul4, or Cdt2 (not shown). Since in vitro experiments indicate that Cdt1 ubiquitylation by Cul4 depends on association with chromatin-bound PCNA (Arias and Walter, 2006), the over-expression of exogenous proteins unlikely recapitulates all of the physiological conditions necessary to detect interactions associated with the PIP-box Cul4 destruction mechanism. Interestingly, the accumulation of myc-E2f1 and HA-Dp was reduced when these two constructs were co-transfected (not shown). We hypothesized that the stability of E2f1 is affected by Dp. Consistent with this possibility, RNAi depletion of Dp stabilized E2f1 during S phase in S2 cells (Fig. 3A-C). We confirmed this in vivo by depleting Dp from larval salivary glands using a UAS-hairpin RNAi (Fig. S4A-C). These data indicate that a $Cul4^{Cdt2}$ E3 ligase can bind E2f1, and raise the possibility that the E2f1/Dp heterodimer is the relevant Cul4 E3 ligase substrate in vivo.

$Cul1^{Slmb}$ and $Cul1^{Skp2}$ were previously suggested to be involved in the destruction of *Drosophila* E2f1 and human E2f1, respectively (Heriche et al., 2003; Marti et al., 1999). However, RNAi knockdown of components of Cul1 E3 ubiquitin ligases (Cul1, the adaptor Skp1, and the F-box proteins Slimb, Skp2 or Ago; Fig. S2) did not cause strong E2f1 stabilization during S phase (Fig. 3A). With Cul1 or Slimb RNAi there was a small increase in the number of GFP-E2f1-positive S phase cells relative to controls, consistent with observations made in wing disc cells after genetic inhibition of Cul1 function using a dominant negative transgene (Heriche et al., 2003). However, clones of *Cul1*, *Ago*, or *Slmb* mutant wing disc cells do not obviously alter the periodic depletion of E2f1 or the overall level of E2f1 protein accumulation (Fig. S5). These data suggest that $Cul4^{Cdt2}$ and PCNA play a more significant role in E2f1 destruction during S phase than a Cul1 E3 ligase.

PIP mutations stabilize E2f1 during S phase in vivo

We next created transgenic flies carrying UAS-GFP-E2f1, -$E2f1^{PIP\text{-}3A}$, -$E2f1^{PIP\text{-}7A}$ or -$E2f1^{PIP\text{-}7del}$ constructs to test whether PIP-box-dependent destruction of E2f1 during S phase occurs in vivo. The *engrailed* (*en*)-Gal4 driver was used to induce expression of these proteins in the embryonic epidermis at a time when a group of cells in the first thoracic segment are replicating while neighboring epidermal cells have entered G1 arrest (Fig. 4A). GFP-E2f1 was degraded in 94% (n=185) of these epidermal S phase cells (Fig. 4A, top bracket) while GFP-$E2f1^{PIP\text{-}3A}$ (Fig. 4A, bottom bracket), GFP-$E2f1^{PIP\text{-}7A}$ and GFP-$E2f1^{PIP\text{-}7del}$ (data not shown) were not. GFP-$E2f1^{PIP\text{-}3A}$ accumulated in a majority of (69%, n=176), but not all, BrdU positive cells, suggesting that the stabilization was not entirely complete. We performed a similar analysis in wing imaginal discs of third instar larvae. When we expressed GFP-E2f1 in the posterior compartment with *en*-Gal4, GFP-E2f1 was not detected in BrdU-positive S phase cells (Fig. 4B, red and green arrows, respectively). In contrast, GFP-$E2f1^{PIP\text{-}3A}$ did

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accumulate in S phase cells (Fig. 4B, yellow arrows). These data indicate that the PIP box-containing motif is necessary for E2f1 destruction in vivo.

PIP box mutations enhance the phenotypes caused by ectopic E2f1 expression

Wild type E2f1 can induce ectopic expression of replication factor genes and S phase when over-expressed, consistent with its role as a transcriptional activator that stimulates the G1-to-S transition (Asano et al., 1996; Du et al., 1996b; Duronio et al., 1996). To test whether PIP box mutations alter these activities, we expressed GFP-E2f1 or GFP-E2f1^{PIP} mutants in embryos and monitored BrdU incorporation and expression of *RnrS*, a well-characterized E2f1-target gene. Expression of GFP-E2f1 or GFP-E2f1$^{PIP\text{-}3A}$ in stripes in the epidermis using the *paired* (*prd*)-Gal4 driver resulted in ectopic accumulation of *RnrS* transcripts, with slightly higher levels induced by GFP-E2f1$^{PIP\text{-}3A}$ (Fig. 5A, top row). This difference in *RnrS* expression was more obvious when GFP-E2f1 or GFP-E2f1$^{PIP\text{-}3A}$ were expressed in endocycling cells (Fig. 5A, middle and bottom rows), which are particularly sensitive to ectopic E2f1 activity (Duronio et al., 1996). In the midgut of a stage 14 wild type embryo, *RnrS* expression is high in the central midgut, where cells are undergoing their second endoreduplication S phase, and low in cells of the anterior and posterior midgut, where cells are in the gap phase between their first and second endoreduplication S phase (Fig. 5A and 5B, wild type, arrows). This stereotyped pattern of *RnrS* expression and endocycle S phase is disrupted by *armadillo* (*arm*)-Gal4 driven expression of GFP-E2f1 or GFP-E2f1$^{PIP\text{-}3A}$ such that ectopic *RnrS* expression and BrdU incorporation occur inappropriately in the anterior and posterior midgut (Fig. 5A, B, arrows). This altered pattern of BrdU/*RnrS* indicates precocious entry into the second endoreduplication S phase as a result of activation of E2f1 transcriptional targets from excess E2f1 protein (Duronio et al., 1996). Ectopic *RnrS* expression was higher and more widespread in the endocycling tissues of GFP-E2f1$^{PIP\text{-}3A}$-expressing embryos compared to GFP-E2f1-expressing embryos (Fig. 5A, arrows and arrowheads). Similar results were observed in each of four independent lines of UAS-GFP-E2f1 and UAS-GFP-E2f1$^{PIP\text{-}3A}$, as well as one UAS-GFP-E2f1$^{PIP\text{-}7A}$ and one UAS-GFP-E2f1$^{PIP\text{-}7del}$ line (data not shown). Thus, the PIP box mutation does not abrogate key functional properties of E2f1, and in fact enhances the ability of E2f1 to induce ectopic target gene expression.

GFP-E2f1^{PIP} disrupts normal cell cycle progression and induces apoptosis

We next tested whether destruction of E2f1 during S phase is important for cell cycle regulation by analyzing wing imaginal discs, which during larval stages of development grow from a precursor population of ~50 cells to ~50,000 cells. We expressed GFP-E2f1 and GFP-E2f1$^{PIP\text{-}3A}$ to similar levels specifically in posterior compartment cells using *en*-Gal4 (Fig. 6B, C). Discs from *en*-Gal4>UAS-GFP-E2f1$^{PIP\text{-}3A}$ larvae grew to a normal size and contained both S phase and mitotic cells in the posterior compartment, as indicated by BrdU labeling (Fig. 4B) and phospho-histone H3 staining (Fig. S6), respectively. This indicates that ectopic GFP-E2f1$^{PIP\text{-}3A}$ expression does not cause cell cycle arrest in the wing disc. We therefore tested whether E2f1$^{PIP\text{-}3A}$ expression would instead accelerate the cell cycle, as E2f1 over-expression does (Neufeld et al., 1998). Both GFP-E2f1 and GFP-E2f1$^{PIP\text{-}3A}$ expressing cells had reduced doubling times compared to control GFP-expressing cells (9.3 and 10.3 hrs versus 12 hrs, respectively), indicating that E2f1$^{PIP\text{-}3A}$ expression increases cell cycle speed. Consistent with this, there was a statistically significant increase in the number of PH3 positive cells in the posterior compartment of GFP-E2f1- and GFP-E2f1$^{PIP\text{-}3A}$-expressing discs compared to the non-expressing anterior compartment (Fig. S6). In addition, the mitotic index was higher after GFP-E2f1$^{PIP\text{-}3A}$ expression compared to GFP-E2f1 (Fig. S6).

To further assess effects on cell cycle progression, 3rd instar imaginal discs expressing GFP, GFP-E2f1 or GFP-E2f1$^{PIP\text{-}3A}$ with en-Gal4 were dissociated to single cells with trypsin and analyzed by FACS (Fig. 6A). GFP-E2f1$^{PIP\text{-}3A}$-expressing cells were smaller than control

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anterior compartment cells, and this difference was greater than that seen with GFP-E2f1 (Fig. 6A, right panels). GFP-E2f1^{PIP-3A} expression caused an increase in the number of S phase cells and fewer G1 cells relative to GFP-E2f1 or GFP expression (Fig. 6A, left panels). These data suggest that preventing S phase destruction of E2f1 accelerates the cell cycle by reducing the duration of G1. These cell cycle changes appear to be poorly tolerated by cells, since under our conditions GFP-E2f1^{PIP-3A} induced extensive apoptosis whereas GFP-E2f1 did not (Fig. 7A). This may explain why the cell doubling time (CDT) of GFP-E2f1^{PIP-3A} expressing cells was calculated to be longer than GFP-E2F1 expressing cells, since apoptosis would reduce the number of PIP-3A expressing cells/time, which is used to derive CDT (see Experimental Procedures). This apoptosis is unlikely to be a consequence of damaged DNA, as we did not observe an increase in staining with an antibody that recognizes the DNA damage-induced phosphorylation of histone H2aV in E2f1^{PIP-3A} expressing cells (data not shown).

In spite of the obvious cell cycle defects and extensive apoptosis, *en*-Gal4>UAS-E2f1^{PIP-3A} flies could develop to adulthood, although only ~50% as many adults eclosed as compared to *en*-Gal4>GFP or *en*-Gal4>GFP-E2f1 cultures. With the *arm*-Gal4 driver, this was reduced to ~30%. However, the morphology of the adult wing of *en*-Gal4>UAS-E2f1^{PIP-3A} flies was grossly abnormal, in contrast to the normal morphology of GFP-E2f1-expressing wings (Fig. 7B). Similar results were obtained with ap-Gal4, which drives expression in the dorsal compartment of the wing (data not shown). The en-Gal4>GFP-E2f1^{PIP-3A}-induced wing phenotype varied from mild (59% of wings, Fig. 7B, middle panel) to severe (36% of wings, Fig. 7B, right panel). Since even higher levels of wild type E2f1 over-expression than we achieved in our experiments can cause similar phenotypes (not shown)(Morris et al., 2006), our findings suggest that E2f1^{PIP-3A} is a hyperactive variant of E2f1.

Discussion

Here we describe a novel mechanism for inhibiting activator E2F function. We demonstrate that the destruction of *Drosophila* E2f1 during S phase requires PCNA and a Cul4^{Cdt2} E3 ubiquitin ligase. We identified a region in E2f1 that when mutated stabilizes E2f1 during S phase, resulting in cell cycle acceleration, apoptosis, and aberrant development. These data suggest that replication-coupled degradation provides important, pRb-independent negative regulation of E2f1 activity during normal development.

The mechanism of E2f1 destruction during the cell cycle

The mechanism of E2f1 destruction during S phase is similar to that recently described for the pre-RC component, Cdt1, which interacts with chromatin-bound PCNA via a PIP box (Arias and Walter, 2006). This PCNA-Cdt1 interaction recruits Cul4$^{Ddb1-Cdt2}$, leading to the ubiquitylation and subsequent destruction of Cdt1, particularly after DNA damage (Higa et al., 2006; Hu and Xiong, 2006; Jin et al., 2006; Senga et al., 2006). While we did not determine whether E2f1 binds PCNA directly or is ubiquitylated on chromatin, we did observe that Dp, which is necessary for E2f1 to bind DNA as a E2f1/Dp dimer (Dynlacht et al., 1994a), is required for E2f1 destruction during S phase. Replication fork movement could bring PCNA to E2f1/Dp that is bound to specific sites throughout the genome. However, stalling replication forks with chemical inhibitors of DNA synthesis did not affect the kinetics of E2f1 destruction (V.T. and B.A.E; S.T.S. and R.J.D., unpublished). We therefore favor a model where the nucleoplasmic pool of E2f1/Dp, in equilibrium with the DNA bound pool, is the relevant Cul4^{Cdt2} substrate and is recruited to PCNA bound at replication forks once S phase begins. *Drosophila* Cdt1 also contains a PIP box and is destroyed during S phase in a replication-dependent manner (May et al., 2005). Therefore, the Cul4/PIP box mechanism is conserved and has been co-opted by different proteins during *Drosophila* evolution to couple destruction with ongoing DNA synthesis.

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Genetic depletion of *Drosophila* $Cul1^{Slmb}$ E3 ligase activity was previously reported to stabilize E2f1 during S phase (Heriche et al., 2003). Cul1 and Cul4 act redundantly to trigger Cdt1 destruction in human S phase cells (Nishitani et al., 2006). By analogy, multiple Cullin complexes may target E2f1. Our experiments did not reveal a major role for a Cul1-based E3 ligase in S phase destruction of E2f1, but neither did they exclude the possibility that Cul1 regulates E2f1 levels at other times in the cell cycle. Perhaps Cul1 restrains E2f1 accumulation during G1, such that reduction of Cul1 function results in elevated levels of E2f1 prior to S phase, and this excess E2f1 cannot be depleted as rapidly as in wild type cells once S phase begins.

pRb-independent regulation of activator E2Fs during S phase

There is not an obvious PIP box in mammalian activator E2Fs, and human E2F1 is targeted by a Cul1 E3 ubiquitin ligase (Marti et al., 1999; Ohta and Xiong, 2001). In addition, human E2F1 stability is modulated by interaction with pRb (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996), whereas our data indicate that the regulation of E2f1 protein accumulation during the cell cycle is independent of Rbf1. Thus, the mechanism for ubiquitin-mediated activator E2F destruction evolved differently in *Drosophila* than in mammals.

What appears to be evolutionarily conserved is a requirement to inhibit activator E2Fs during S phase independently of pRb family proteins. This can be achieved by different mechanisms. Furthermore, the failure of this inhibition results in apoptosis. In mammals, the phosphorylation of E2F1-bound-DP via Cyclin A/Cdk2, which interacts with the NH_2-terminus of E2F1, blocks DNA binding of E2F1/DP (Dynlacht et al., 1994b; Krek et al., 1994). *Drosophila* achieves the same effect by rapidly destroying E2f1 during S phase. Much like our $E2f1^{PIP-3A}$ results, the expression of an E2F1 allele that can't bind Cyclin A results in an increase in the S phase population and apoptosis (Krek et al., 1995). *Dp* mutant wing imaginal discs do not display elevated apoptosis (Moon et al., 2005), suggesting that any free E2f1 that accumulates during S phase in this situation is not detrimental. Thus, cells may possess an S phase-specific sensing mechanism to detect chromatin bound E2f1/Dp and trigger apoptosis.

What functions of activator E2Fs might necessitate their inhibition, or more specifically their removal from chromatin, during S phase? One possibility is that this provides a means to down-regulate E2F transcriptional targets in S/G2. Consistent with this, the simultaneous mutation of the mouse E2F7 and E2F8 repressors, which lack a pRb interaction domain, results in a failure to down-regulate the E2F1 and CDC6 genes in S/G2 in embryonic fibroblasts and causes widespread apoptosis in embryos (Li et al., 2008). E2F also controls the expression of genes at the G2/M transition in flies and mammals (Ishida et al., 2001; Neufeld et al., 1998). Perhaps the precocious activation of G2/M targets because of persistent E2F activity during S phase prevents the normal coordination of events needed to progress from interphase to mitosis, contributing to the accumulation of S/G2 cells that we observe. Additionally, the interplay between activator and repressor E2Fs may be disrupted when chromatin bound E2f1 persists during S phase. E2f1 prevents E2f2-mediated repression in *Drosophila* (Frolov et al., 2003), likely by blocking access of E2f2 to specific DNA binding sites. Consequently, excess chromatin bound E2f1 during S phase may antagonize the function of dREAM/MMB, a recently described E2f2-containing complex that regulates the expression of many genes that control both the cell cycle and development (Dimova et al., 2003; Georlette et al., 2007; Korenjak et al., 2004; Lewis et al., 2004; Stevaux et al., 2005). An analysis of whether E2f1 transcriptional activity is required for the cell cycle defects caused by stabilized E2f1 and a description of what transcriptional changes occur will be necessary to explore these questions.

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pRb-independent regulation of activator E2Fs during development

Is replication-coupled destruction of E2f1 necessary for normal fly development? Because our experiments involve ectopic over-expression of E2f1^{PIP} mutants and not replacement of endogenous E2f1, we cannot definitively answer this question. However, E2f1^{PIP-3A} expression in the larval salivary gland blocks endocycle progression (V.T. and B.A.E., unpublished), suggesting that at least in some tissues this regulatory mechanism is necessary. We also cannot unambiguously determine whether phenotypes caused by E2f1^{PIP-3A} result from changes in the timing (i.e. present in S phase) or total amount of E2f1 accumulation. In either case, coupling destruction of E2f1 to replication provides a possible explanation for previous data indicating that Cyclin E/Cdk2 activity is inversely correlated with E2f1 accumulation (Reis and Edgar, 2004). This negative regulatory relationship is at the heart of a mechanism that maintains overall cell cycle timing. Cyclin E/Cdk2 may indirectly reduce E2f1 protein by triggering DNA replication. In this way, E2f1 destruction during each S phase would keep E2f1/Dp activity “in check” during the cell cycle by counteracting the positive feedback loop that occurs during the G1-to-S transition, in which E2f1 induces *Cyclin E* transcription and Cyclin E/Cdk2 phosphorylates and inhibits Rbf1, resulting in more E2f1 activity. Without replication-coupled destruction of E2f1 to break or dampen this loop, stable E2f1 may gradually accumulate over multiple cycles, thereby inappropriately accelerating the cell cycle in a proliferating cell population. Such cell cycle acceleration is incompatible with *Drosophila* development, and may constitute a form of “oncogenic stress” in mammals that contributes to the onset of cancer.

This model may also explain our prior observation that *Drosophila* E2f1 actually accumulates during S phase in the blastoderm embryo (Shibutani et al., 2007). How E2f1 avoids destruction during these very earliest S phases of development is not known. At this stage of development there is no zygotic transcription and no G1 phase. Consequently, positive feedback amplification between Rbf1, Cyclin E/Cdk2 and E2f1-induced transcription is not needed for cell cycle progression. Thus, replication-coupled E2f1 destruction is not necessary for S phase per se, but may rather provide an intrinsic rheostat to dampen the positive feedback loop that is necessary to trigger the G1-to-S transition in canonical G1-S-G2-M cell cycles.

Experimental Procedures

Molecular Biology

All gene products were expressed in cell culture or as transgenes using Gateway compatible vectors(see Supplemental Data for details).

Cell culture and transfection

S2 cells were grown at 28 C in Schneider’s *Drosophila* medium (Gibco) supplemented with 10% Fetal Bovine Serum, 5 units/ml Penicillin, 5 μg/ml Streptomycin (Sigma). 5×10^5 freshly plated cells/ml were transfected 24 hours later with 0.4 μg of plasmid using Effectene® (Qiagen) and analyzed 2–3 days later. Stable cell lines were generated by co-transfection with pCoHygro (Invitrogen) and growing cells for >18 days in medium containing 500 μg/ml hygromycin B (Invitrogen).

Flow cytometry

Transfected S2 cells were fixed with ice-cold 1% paraformaldehyde in PBS (30 min), permeabilized with PBS + 0.1% Tween20 (15 min at room temperature), and treated with 1.5 μg DNase-free RNase (Roche; 30 min at 37 C). DNA was stained with 15 μM propidium iodide in PBS overnight at 4 C. DNA content was determined with a CyAn flow cytometer using Summit 4.3 software (Dako). Percentage of G1, S, and G2 cells was calculated using the ModFit

LT™ software (Verity Software House). FACS analysis of wing imaginal discs was performed as described (Neufeld et al., 1998).

RNAi

Double stranded RNAs were transcribed with the RiboMAX™ Large Scale RNA Production System-T7 (Promega) from PCR products (see Supplemental Data for primers). 1×10^6 freshly diluted cells grown in Sf-900II serum free medium (Gibco) supplemented with Penicillin-Streptomycin were grown for one day at 28 C and then treated with dsRNAs for 2 days before analysis.

RT-PCR

1 μg or 0.75 ug of total RNA extracted from S2 cells or 30 3rd instar wing discs, respectively, using TRIzol® was used for reverse transcription with M-MLV reverse transcriptase (Invitrogen), and 1/40 (S2 cells) or 1/40, 1/200, 1/1000, or 1/5000 (discs) of the resulting cDNA was used for PCR.

Western blots

The following primary antibodies were used: rabbit anti-Cul1 (1:250, Invitrogen-Zymed), rabbit anti-Cul4 (1:10000)(Hu et al., 2008), mouse anti-Ddb1 (1:100)(Invitrogen-Zymed), mouse anti-Dp (YUN1–3, 1:4)(Du et al., 1996b), and mouse anti-α Tubulin (1:2000, Sigma). Secondary antibodies were ECL™ sheep anti-mouse HRP (1:2000) and ECL™ donkey anti-rabbit HRP (1:2000) from GE Healthcare.

Drosophila developmental genetics

UAS-*E2f1*, UAS-$E2f1^{PIP\text{-}3A}$, UAS-$E2f1^{PIP\text{-}7A}$, and UAS-$E2f1^{PIP\text{-}7del}$ transgenic flies were created by injecting w^{1118} embryos (Rainbow Transgenic Flies, Inc., Newbury Park, CA). UAS-GFP, *prd*-Gal4/*TM3*, and *arm*-Gal4 were obtained from the Bloomington Stock Center. *en*-Gal4 was a gift from Dr. Steve Crews. In situ hybridization, BrdU labeling (1 mg/ml BrdU for 5 min) and immunostaining in embryos were performed as described (Shibutani et al., 2007). Rabbit anti-GFP (1:10000, Upstate) was detected with a biotin-conjugated anti-rabbit secondary antibody (1:1000, Chemicon) and the TSA™ Fluorescein System (Perkin Elmer). Rat anti-phosphotyrosine (1:100, R&D Systems) was visualized with donkey anti-rat-Cy5 (1:500, Jackson). Wing discs were incubated with 100 μg/ml BrdU for 30 min, and fixed with 4% formaldehyde. Mouse anti-GFP (1:500, Upstate) was visualized with goat anti-mouse-Oregon Green (1:500, Invitrogen-Molecular Probes). BrdU was detected by acid denaturation of chromosomes using mouse anti-BrdU (1:100, Becton Dickinson) and goat anti-mouse-Cy3 (1:500, Jackson) for embryos, and rat anti-BrdU (1:200, Abcam) and goat anti-rat-Cy3 (1:500, Jackson) for wing discs. Apoptotic cells in wing discs fixed with 6% paraformaldehyde were detected with rabbit anti-cleaved Caspase-3 (Asp175) (1:200, Cell Signaling Technology) and goat anti-rabbit-rhodamine (1:1000, Invitrogen-Molecular Probes). Tissue samples were analyzed with a Zeiss LSM 510 scanning confocal microscope or a Nikon Eclipse E800 microscope. Cell doubling time (CDT) was determined as described previously (Prober and Edgar, 2000) by generating clones of GFP-E2f1-expressing wing disc cells. CDT is calculated as (log 2/log N)hr, where N = median number of cells/clone and hr = elapsed time from clone generation to disc fixation.

Supplementary Material

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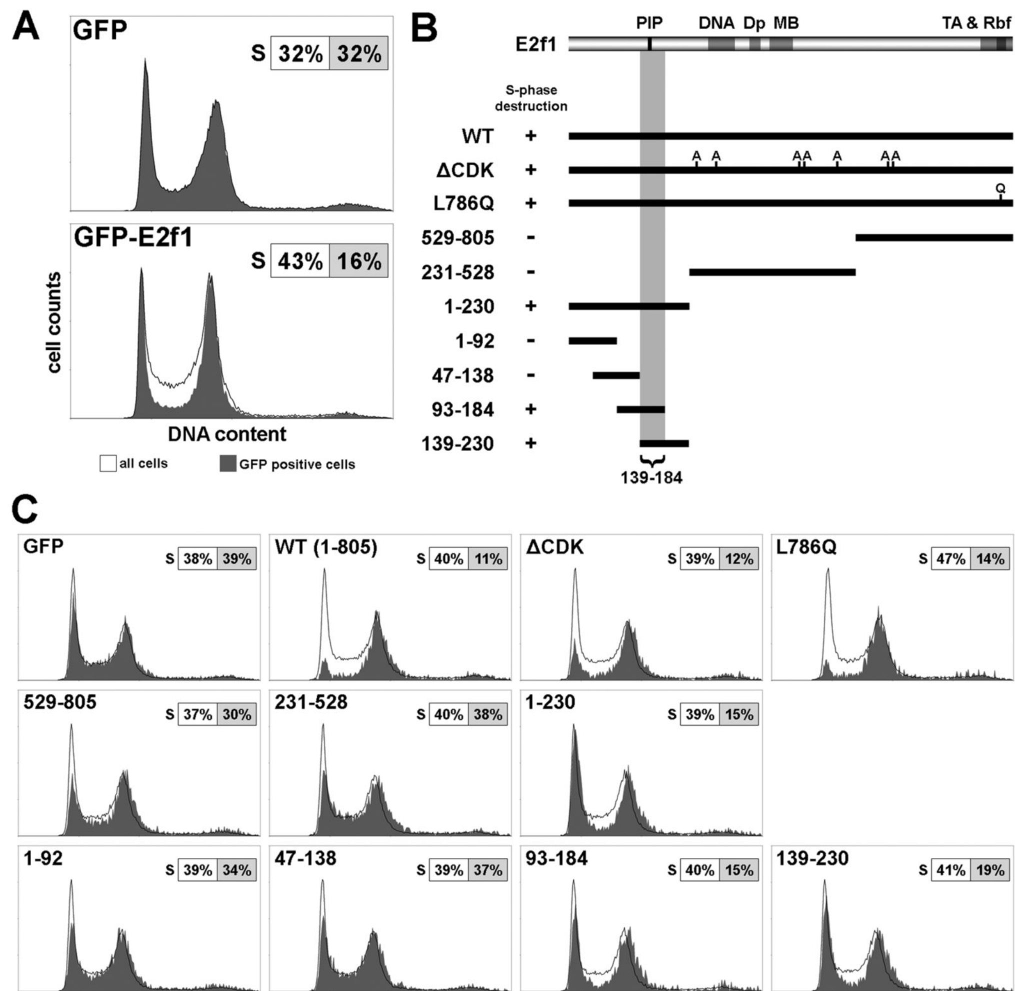

Figure 1. An NH_2-terminal motif in E2f1 mediates S phase-specific destruction

(A) Cell cycle profiles of S2 cells stably transfected with *Actin5C*-GFP or *Actin5C*-GFP-E2f1. DNA content profile of GFP positive cells (gray area: defined where 99.9% of non-transfected cells are excluded) is overlaid on the profile of all cells in the population (unfilled solid line). The Y axis is shown as a relative scale between "all cells" and "GFP-positive cells". Insets show percentages of S phase cells for the entire population and for GFP-positive cells. (B) Schematic of E2f1 mutants analyzed by FACS. + and − indicates S phase-specific destruction of GFP-E2f1 (WT) and mutant variants. Destruction motif within residues 139–184 is shadowed. PIP=PCNA-interaction protein box. DNA=DNA-binding domain. Dp=Dp-dimerization domain. MB=marked box. TA&Rbf=transactivation and Rbf1 binding domain. (C) Cell cycle profiles of S2 cells transiently transfected with plasmids expressing *Actin5C*-GFP-E2f1 variants.

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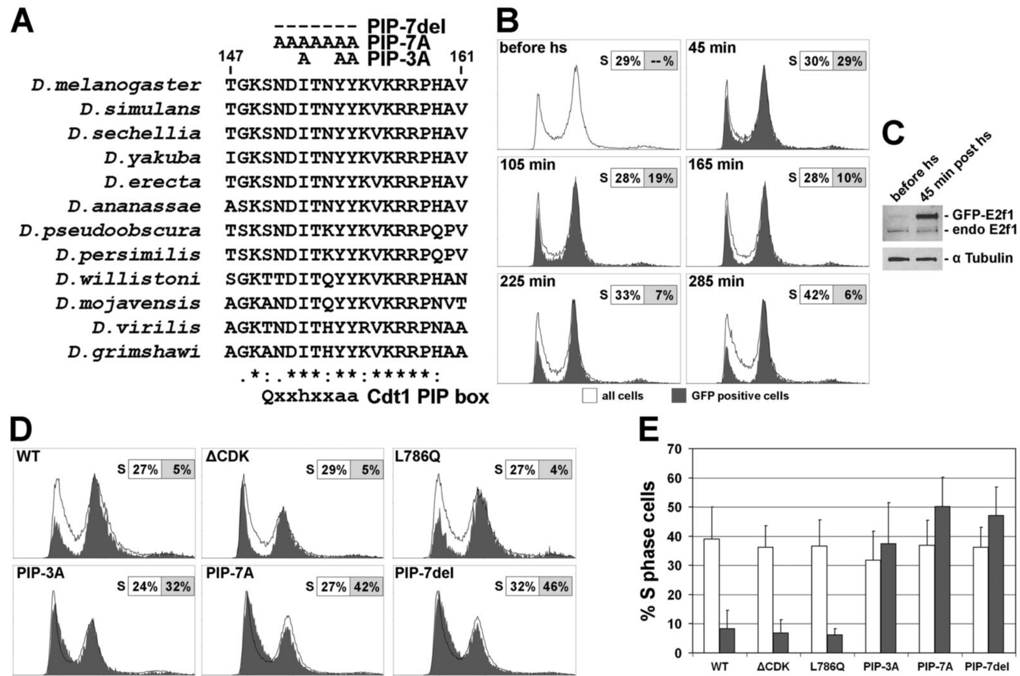

Figure 2. A PIP box is required for S phase-specific destruction of *Drosophila* E2f1

(A) ClustalW2-derived multiple sequence alignment of the PIP box-containing region of E2f1 orthologs from 12 *Drosophila* species. The consensus Cdt1 PIP box is shown below, where h=hydrophobic, a=aromatic, and x=any amino acid. The E2f1 PIP mutant alleles are shown above, where “A” and “-” indicate alanine substitution and deletion, respectively. (B) Cell cycle profiles of stably transfected S2 cells that were fixed at the indicated times after a 30 min at 37 C heat shock. (C) α-E2f1 western blot of extracts from cells treated as in B. (D) Cell cycle profiles of the *Hsp70*-GFP-E2f1 or the indicated *Hsp70*-GFP-E2f1 mutant cell lines at 225 min after a 30 min at 37° C heat shock. (E) Average and standard deviation for three independent heat shock experiments indicating the percentage of S phase cells for the entire population (open bars) and for GFP positive cells (filled bars).

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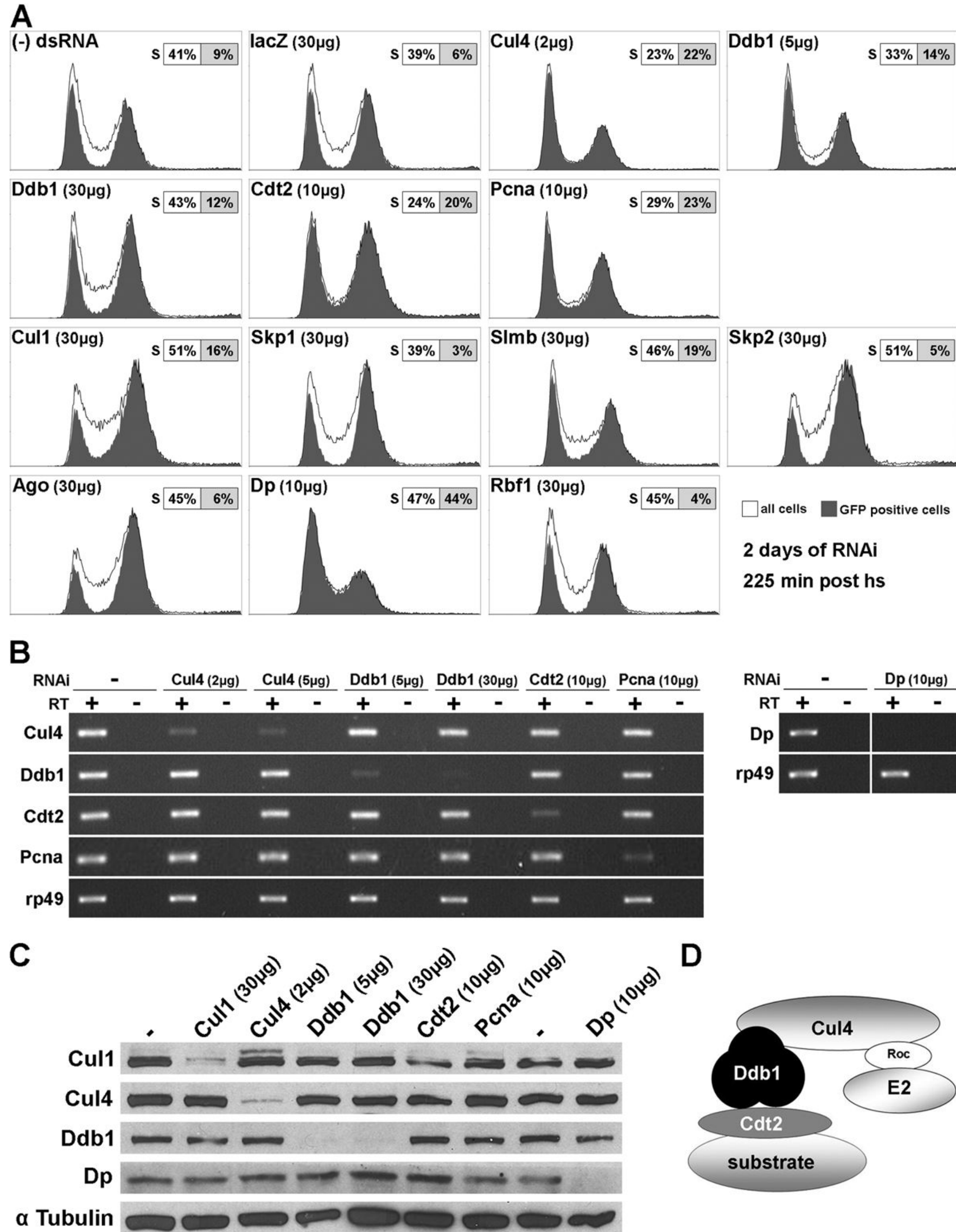

Figure 3. RNAi analysis of E2f1 destruction

(A) *Hsp70*-GFP-E2f1 cells were treated with the indicated dsRNAs for 2 days and analyzed by FACS 225 min after a 30 min at 37° C heat shock. (B) mRNA levels of RNAi-targeted genes was analyzed by RT-PCR. RT indicates the presence (+) or absence (−) of reverse transcriptase. *rp49* is a ubiquitously expressed control. RNAi for *Cul4*, *Ddb1*, *Cdt2*, or *Pcna* does not affect the other genes of the Cul4 complex. (C) Western blot of the RNAi-treated cells. α-Tubulin is a loading control. (D) Diagram of the $\text{Cul4}^{\text{Cdt2}}$ E3 ligase predicted to target E2f1.

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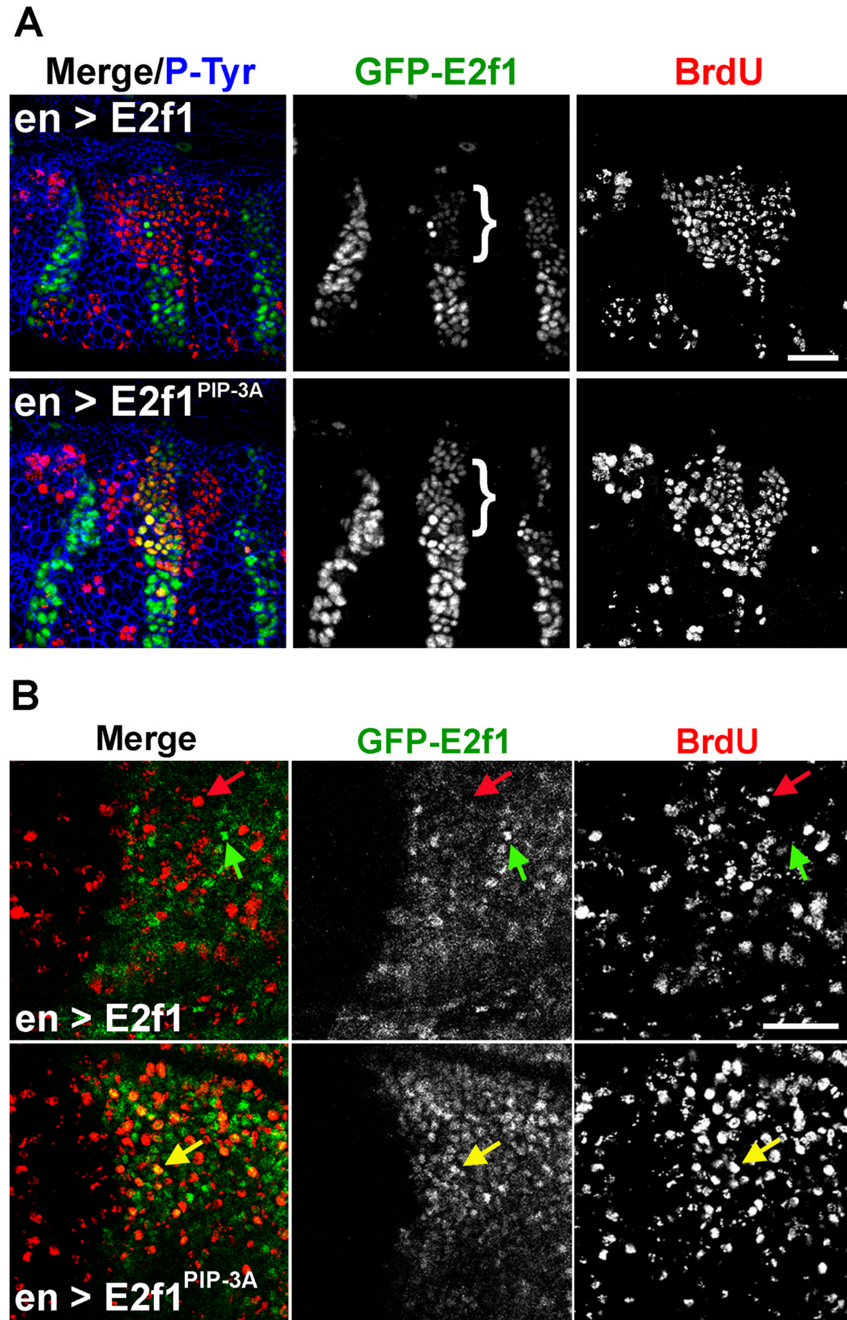

Figure 4. Mutations in the PIP box stabilize E2f1 during S phase in vivo

(A) Stage 11 *en*-Gal4>UAS-GFP-E2f1 or *en*-Gal4>UAS-GFP-E2f1$^{PIP\text{-}3A}$ embryos were stained for GFP (green), BrdU incorporation (red), and phosphotyrosine (P-Tyr, blue) to visualize cell boundaries. Brackets indicate cells undergoing S phase that overlap with the dorsal portion of the *en*-Gal4 expressing cells. (B) 3rd instar *en*-Gal4>UAS-GFP-E2f1 or *en*-Gal4>UAS-GFP-E2f1$^{PIP\text{-}3A}$ wing discs were stained for GFP (green) and BrdU incorporation (red). Green and red arrows indicate cells that are positive only for GFP-E2f1 or BrdU, respectively. Yellow arrows indicate GFP-E2f1$^{PIP\text{-}3A}$ and BrdU double positive cells. Scale bars are 20 μm.

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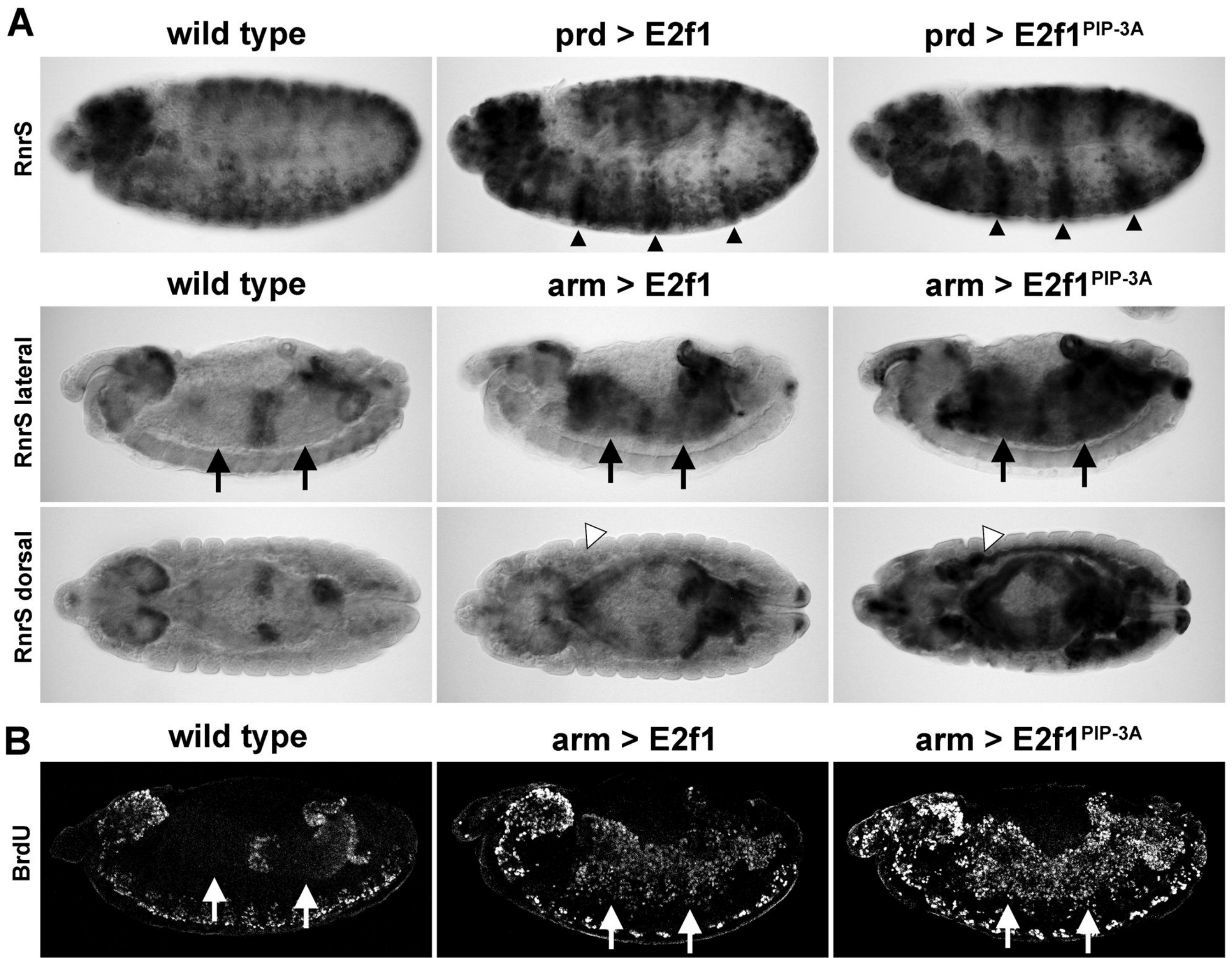

Figure 5. GFP-E2f1 and GFP-E2f1^{PIP} can induce transcription

(A) *w^{1118}* control (wild type), prd>GFP-E2f1, prd>GFP-E2f1^{PIP-3A}, arm>GFP-E2f1, or arm>GFP-E2f1^{PIP-3A} embryos were hybridized with an *RnrS* probe. The top two and bottom rows are from the lateral and dorsal perspective, respectively. Arrowheads indicate induction of *RnrS* expression where the *prd* promoter is active (top). (B) BrdU incorporation in *w^{1118}* control (wild type), arm>GFP-E2f1, or arm>GFP-E2f1^{PIP-3A} embryos. Arrows indicate ectopic *RnrS* expression (A) and DNA replication (B) in endocycling midgut cells. Unfilled arrowheads indicate endocycling cells with ectopic *RnrS* expression in arm>E2f1^{PIP-3A} but not arm>E2f1 embryos.

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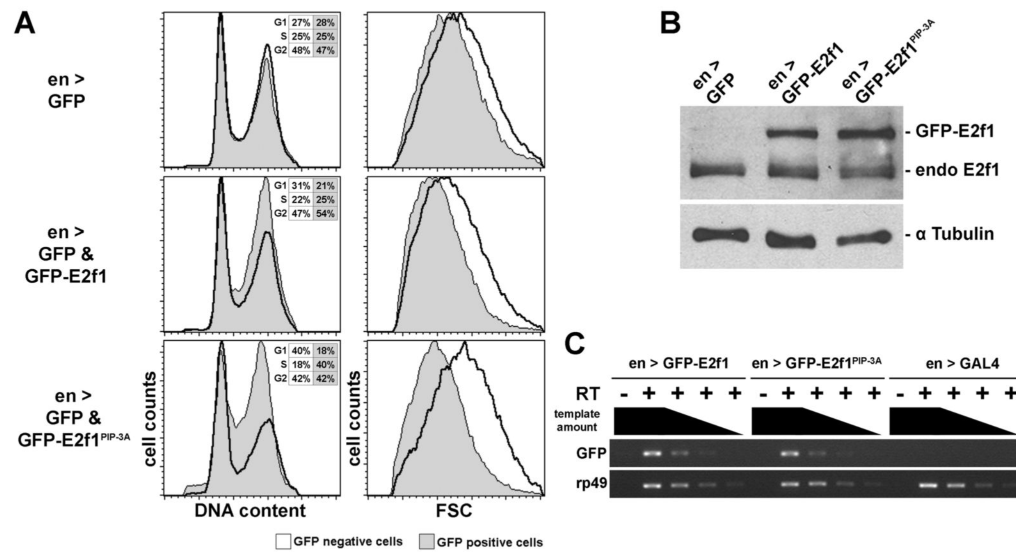

Figure 6. Stabilization of E2f1 during S phase induces cell cycle acceleration

(A) 3rd instar larval wing discs of the indicated genotypes were dissociated with trypsin and analyzed by FACS for DNA content (left panels) or forward scatter to measure cell size (right panels). (B,C) α-E2f1 western (B) and RT-PCR with GFP primers (C) of wing discs shown in (A). RT as in Fig. 3. The right 3 lanes in each set are 5-fold serial dilutions of the cDNA input in the first lane.

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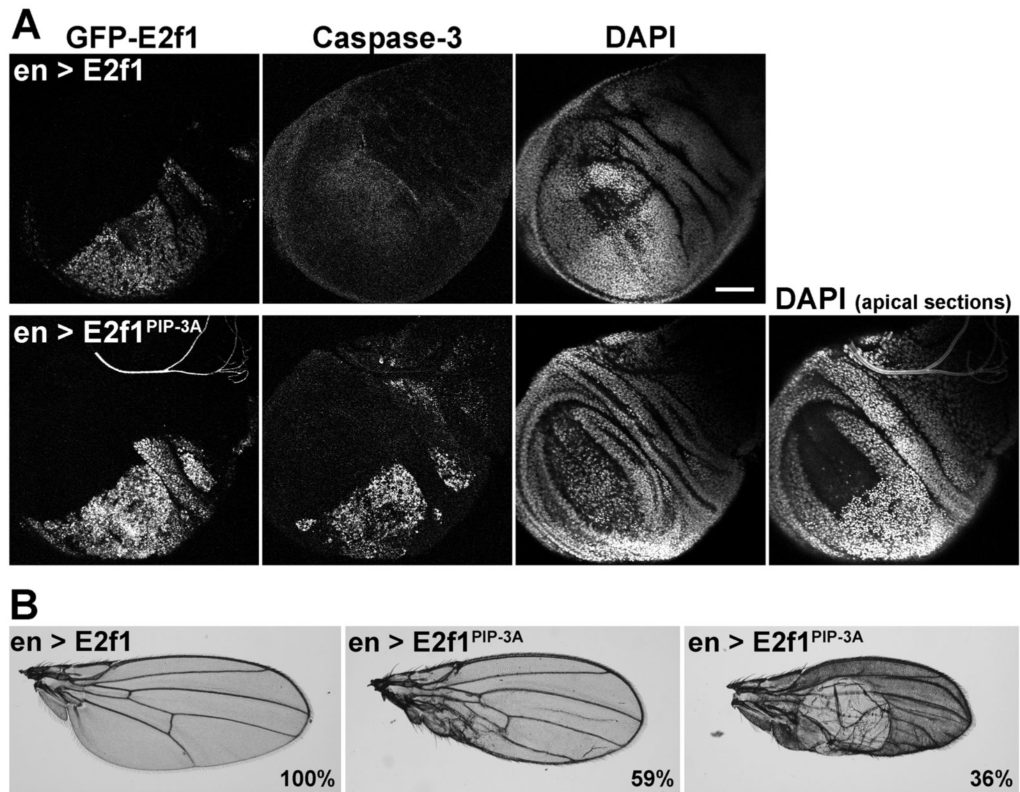

Figure 7. Stabilization of E2f1 during S phase induces apoptosis

(A) 3rd instar *en*-Gal4>UAS-GFP-E2f1 or *en*-Gal4>UAS-GFP-E2f1^{PIP-3A} larvae wing discs were stained for cleaved Caspase-3 (red) to detect apoptosis and DNA (DAPI). Anterior: top left. Posterior: bottom right. Caspase-3-positive cells are extruded from the surface of the disc, as evident by the DAPI staining image of apical confocal sections. Scale bar is 50 µm. (B) Wings of adult flies eclosed from *en*-Gal4>UAS-GFP-E2f1 or *en*-Gal4>UAS-GFP-E2f1^{PIP-3A} cultures. The percentages indicate the fraction of total wings scored. Note that 5% of the *en*-Gal4>GFP-E2f1^{PIP-3A} wings appeared normal.