Developmental Cell *15*

Supplemental Data

Intrinsic Negative Cell Cycle Regulation Provided by PIP Box- and Cul4^{Cdt2}-Mediated Destruction of E2f1 during S Phase

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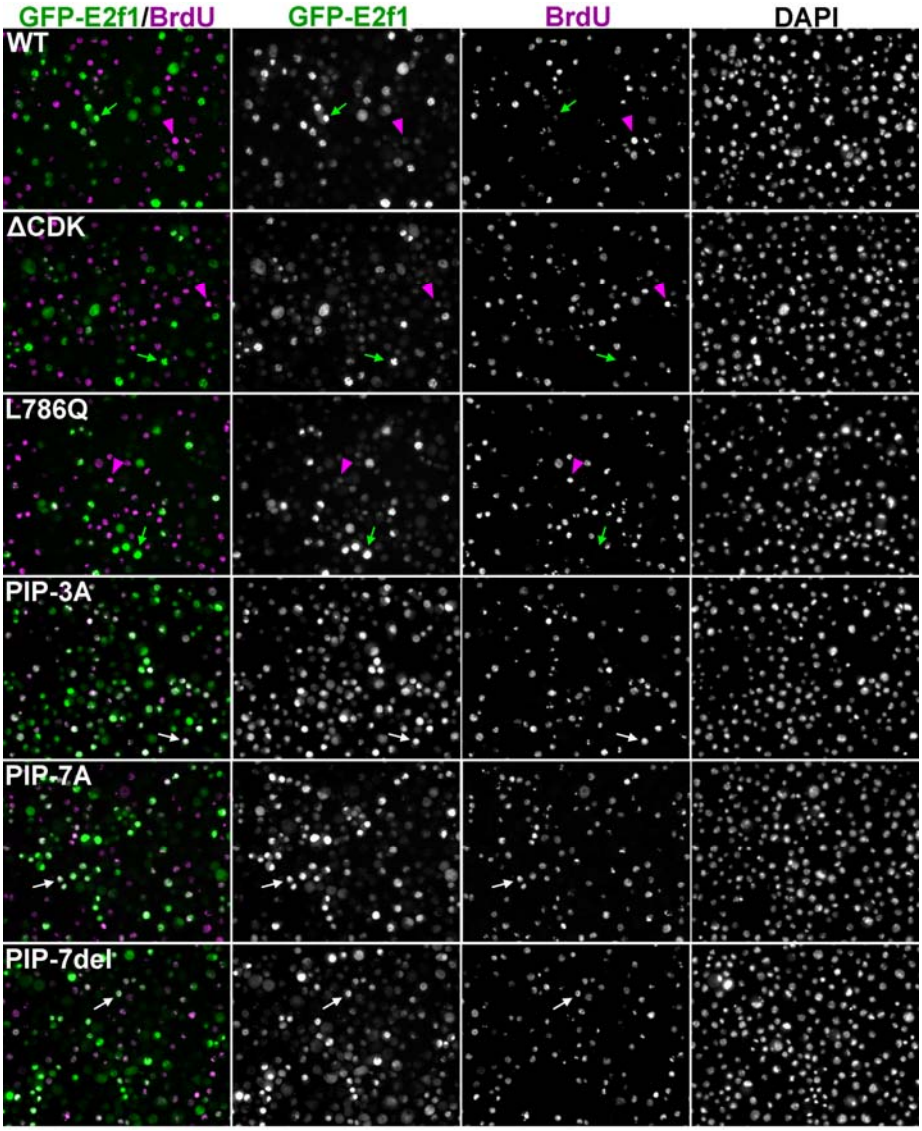


Figure S1. The GFP-E2f1^{PIP} Mutants Accumulate in the Nucleus during S Phase in S2 Cells

S2 cells stably transfected to express GFP-E2f1 variants under the *Hsp70* promoter were heat shocked for 30 min at 37˚C, pulse-labeled on a Concanavalin A-treated cover glass with 10 μg/ml BrdU in growth medium for the last 45 min prior to fixation, which occurred with 4% paraformaldehyde for 20 min at room temperature 225 min after the end of the heat shock. Cells were then permeabilized with PBT for 15 min at room temperature, and the chromosomes

were denatured by incubation with 50 units/ml RNase-free RQ1 DNase (Promega) in RQ1 DNase buffer (Promega) for 30 min at 37˚C. After blocking with 5% normal goat serum in PBT for 30 min at room temperature, cells were incubated with mouse anti-BrdU (1:100, Becton Dickinson) overnight at 4˚C followed by goat anti-mouse-Cy3 (1:500, Jackson), and 5 µg/ml DAPI. BrdU positive nuclei are in magenta. E2f1 detection by GFP auto fluorescence is shown in green. Wild type, ΔCDK mutant, and L786Q mutant E2f1 accumulate in the nucleus in BrdU negative cells (green arrows) and are not present during S phase (magenta arrowheads). In contrast, PIP mutant E2f1s are stabilized and accumulated in the nucleus during S phase (white arrows).

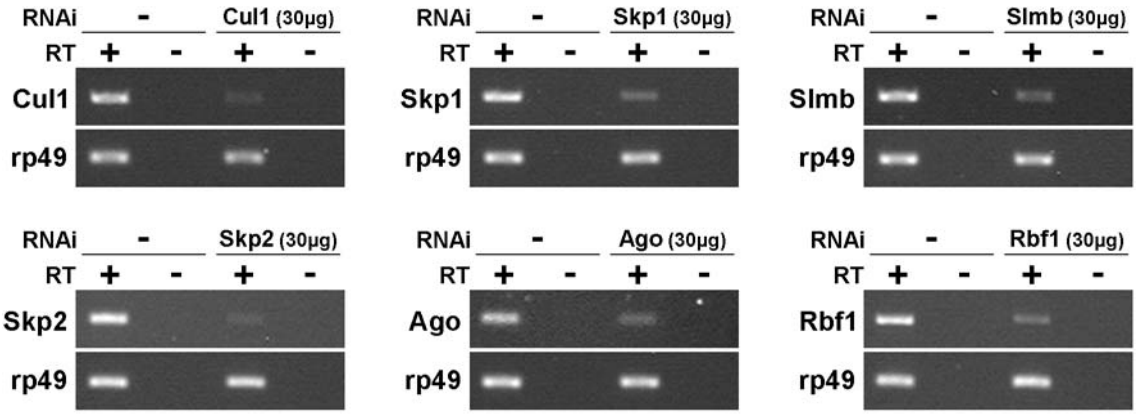


Figure S2. mRNA Levels of RNAi-Targeted Genes Analyzed by RT-PCR

Hsp70-GFP-E2f1 cells were treated with the indicated dsRNAs for 2 days, and 1 µg of total RNA was extracted using TRIzol® (Invitrogen) and used for reverse transcription with M-MLV reverse transcriptase (Invitrogen). 1/40 of this reaction was used for 24, 27, or 30 cycles of PCR with GoTaq® Flexi (Promega). RT indicates the presence (+) or absence (-) of reverse transcriptase. The ubiquitously expressed *rp49* is used as a control.

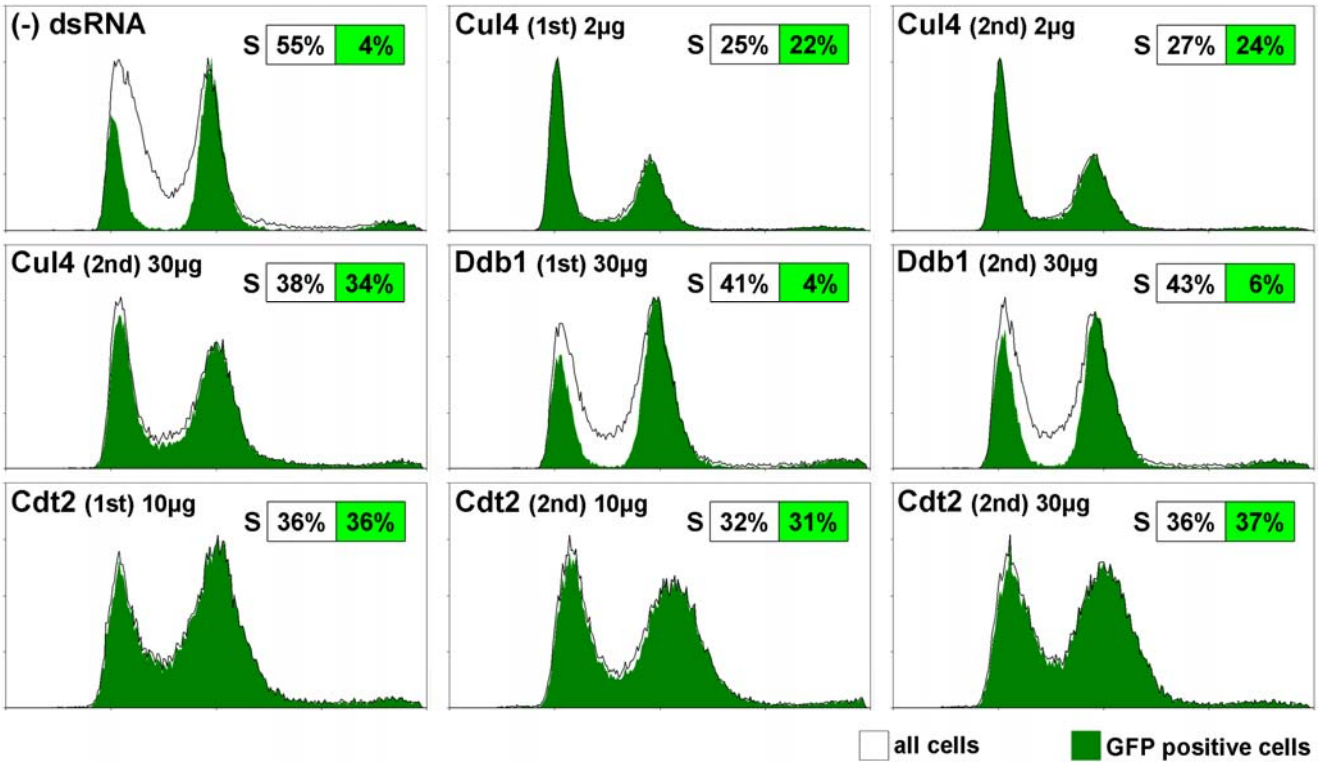


Figure S3A. RNAi Knockdown of Cul4, Ddb1, and Cdt2 with Two Independent dsRNAs

Double stranded RNAs (dsRNAs) were transcribed with the RiboMAX™ Large Scale RNA Production System-T7

(Promega) from PCR products amplified using gene-specific primers containing the T7 promoter and genomic DNA or cDNA clones (listed in Supplemental Experimental Procedures). 1×10^6 freshly diluted S2 cells grown in Sf-900II serum free medium (Gibco) supplemented with Penicillin-Streptomycin and stably transfected with pHGW-*E2f1* (*hsp70*-GFP-E2f1) were grown for a day at 28˚C and treated with the indicated amounts and types of dsRNAs. After 2 days of incubation at 28˚C, a subset of the cells was used for RT-PCR and western blot (Supplemental Figure 3B), and the rest were heat-shocked for 30 min at 37˚C, fixed at 225 min after the end of the heat-shock, and subjected to flow cytometry. The GFP positive gate was determined where 99.97% of non-heat-shocked transfected cells are excluded. "1st" and "2nd" refer to dsRNAs derived from a different portion of the Cul4, Ddb1, and Cdt2 genes (see Supplemental Experimental Procedures). The "2nd" dsRNAs were also used in Figure 3.

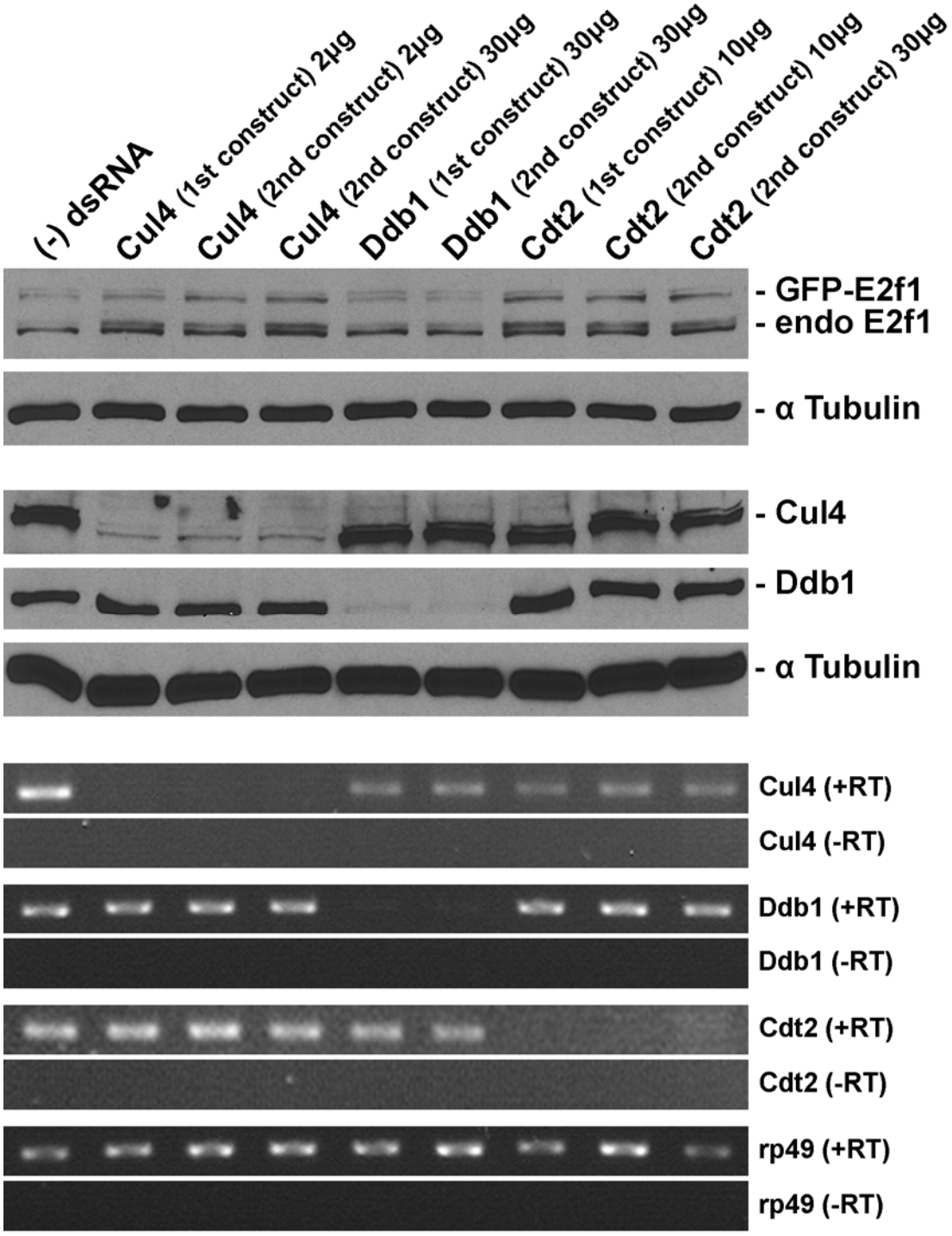


Figure S3B. E2f1 Protein Is Stabilized by Cul4 or Cdt2 Knockdown

For western blot (upper 5 panels), *hsp70*-GFP-E2f1 cells were treated for 2 days with the indicated types and amounts of dsRNA (as in Fig. S3A) and lysed before heat shock with ice-cold lysis buffer (50mM Tris-HCl pH8.3, 150mM

NaCl, 0.5% NP40) containing 1 μg/ml leupeptin, 0.5 μg/ml pepstatin and 1 mM PMSF. Primary antibodies were rabbit anti-E2f1 (1:100)(see Experimental Procedures), rabbit anti-Cul4 (1:10000)(Hu et al., 2008) mouse anti-Ddb1 (1:100)(Invitrogen-Zymed), and mouse anti-α Tubulin (1:10000 (upper) or 1:2000 (lower), Sigma). Secondary antibodies were ECL™ sheep anti-mouse HRP (1:2000) and ECL™ donkey anti-rabbit HRP (1:2000) from GE Healthcare. For RT-PCR (lower 8 panels) 1 μg of total RNA extracted from S2 cells using TRIzol® (Invitrogen) was used for reverse transcription with SuperScript™ II reverse transcriptase (Invitrogen), and 1/40 of this reaction was used for 27 (Ddb1 and rp49) or 33 (Cul4 and Cdt2) cycles of PCR with Taq DNA polymerase (Fermentas).

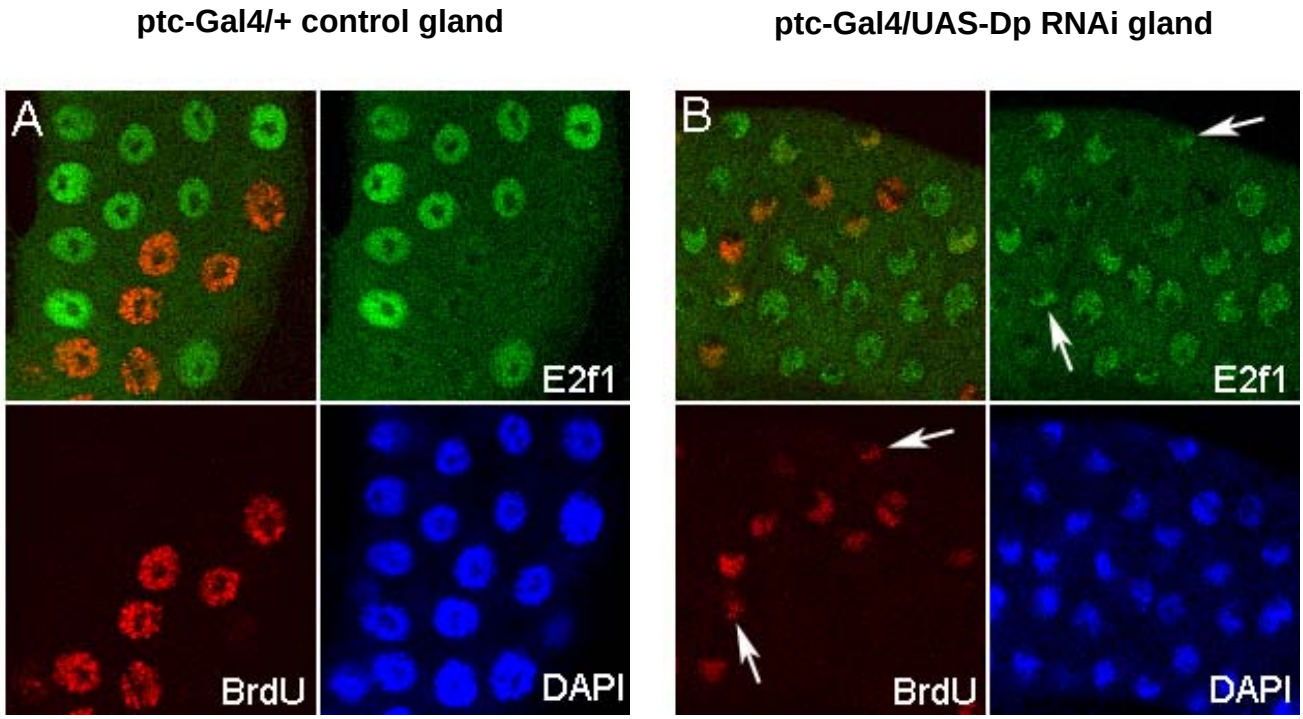


Figure S4A,B. E2f1 Accumulates during S Phase in Salivary Glands Depleted of Dp

A UAS-haripin RNAi construct targeting Dp (stock 4654R-3 from the National Institute of Genetics, Kyoto, Japan) was expressed in salivary glands using ptc-Gal4. Salivary glands from larvae 72 hours after egg deposition (AED) grown at 25ºC were labeled with BrdU, dissected and stained for E2f1 protein and BrdU incorporation.

(A) ptc-Gal4/+ control glands.

(B) Dp RNAi glands. Arrows indicate representative nuclei double positive for BrdU and E2f1.

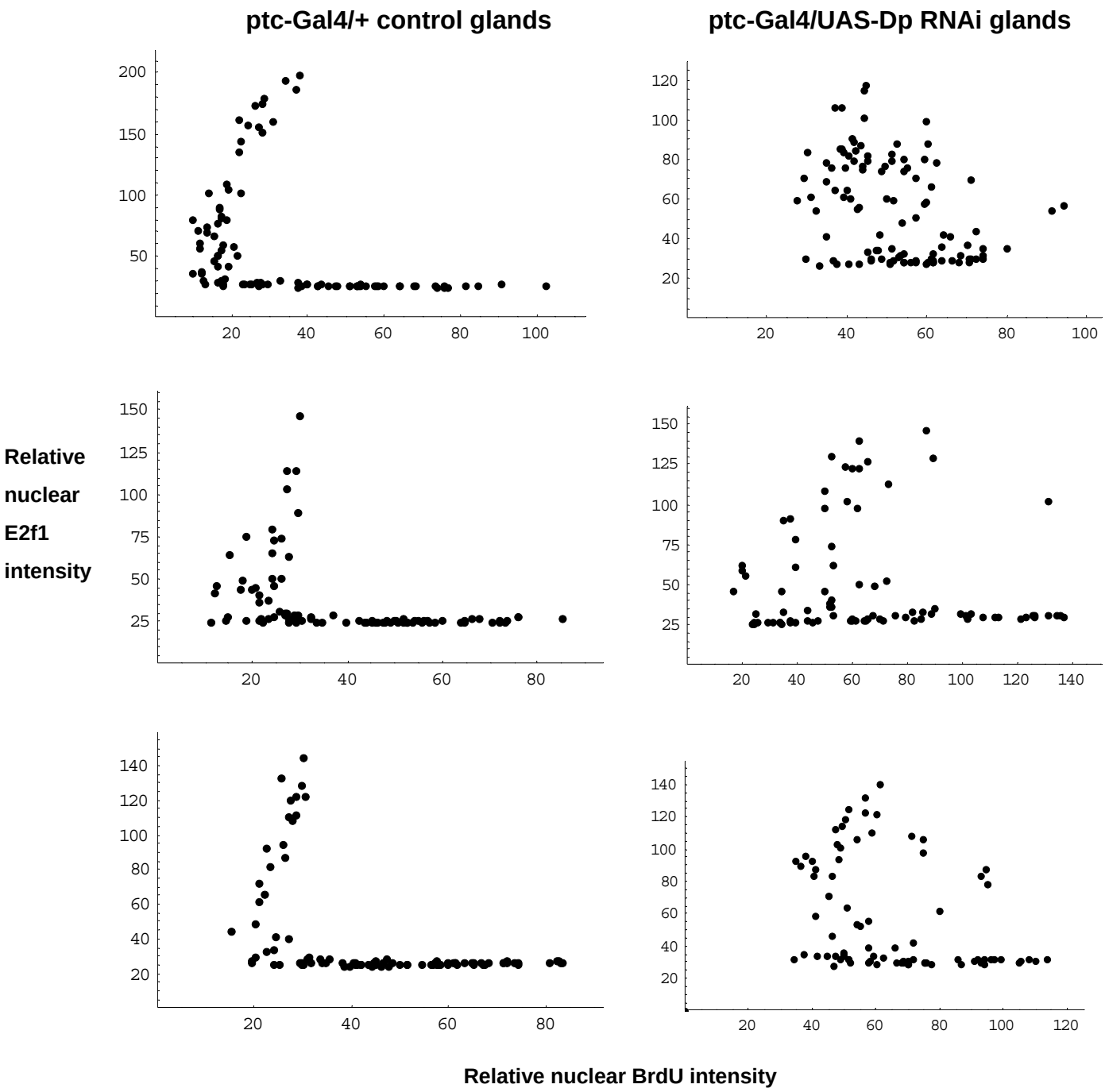


Figure 4C. E2f1 Accumulates during S Phase in Salivary Glands Depleted of Dp

ImageJ was used to quantify the amount of BrdU and E2f1 in individual nuclei from confocal image stacks from three different glands of each genotype. Each scatter plot shows the resulting values from a different gland. The X axes indicate the relative nuclear BrdU intensities, and the Y axes the relative nuclear E2f1 intensities. The left panels show data derived from control ptc-Gal4/+ glands, and the right panels show data from ptc-Gal4-UAS Dp RNAi glands. Each dot indicates an individual nucleus. Note that in wild type E2f1 is high only in nuclei that do not label with BrdU, and that many BrdU-E2f1 double positive nuclei are found upon depletion of Dp.

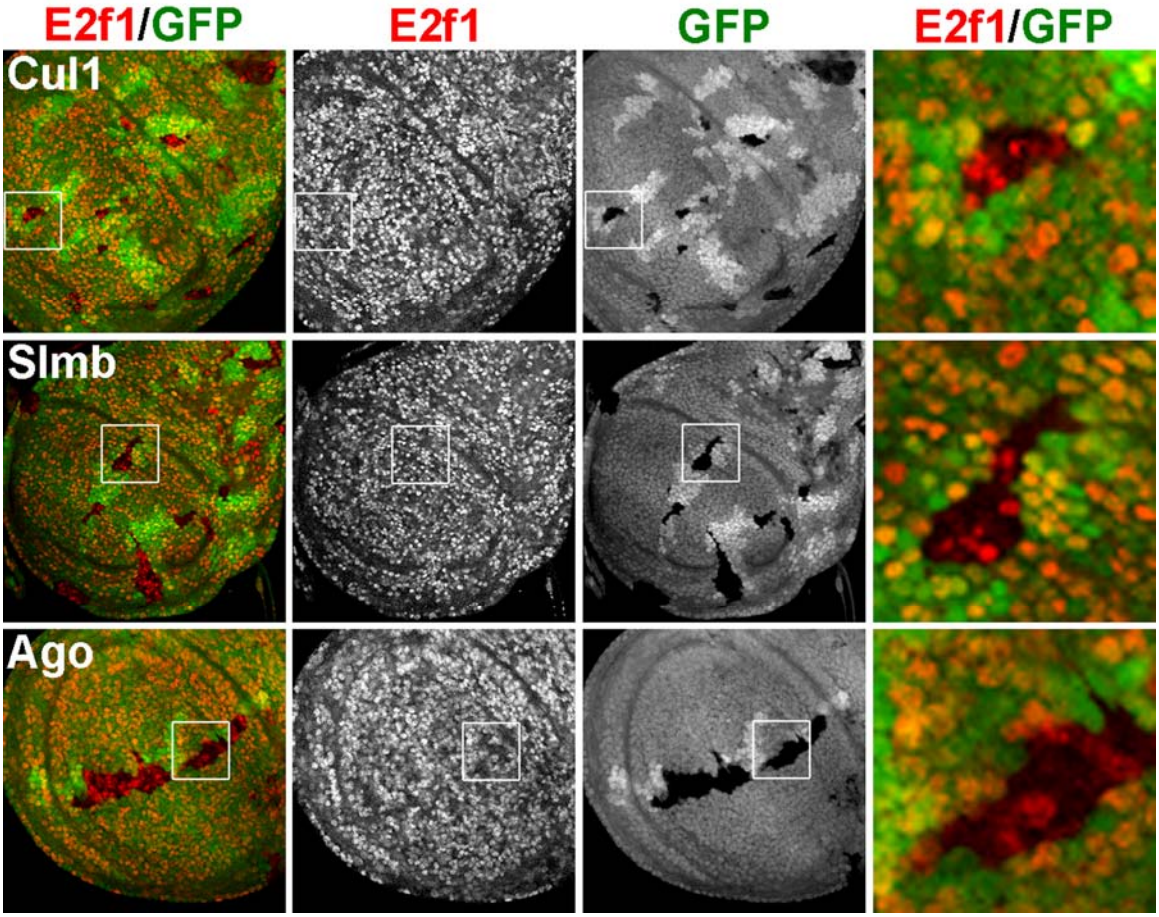
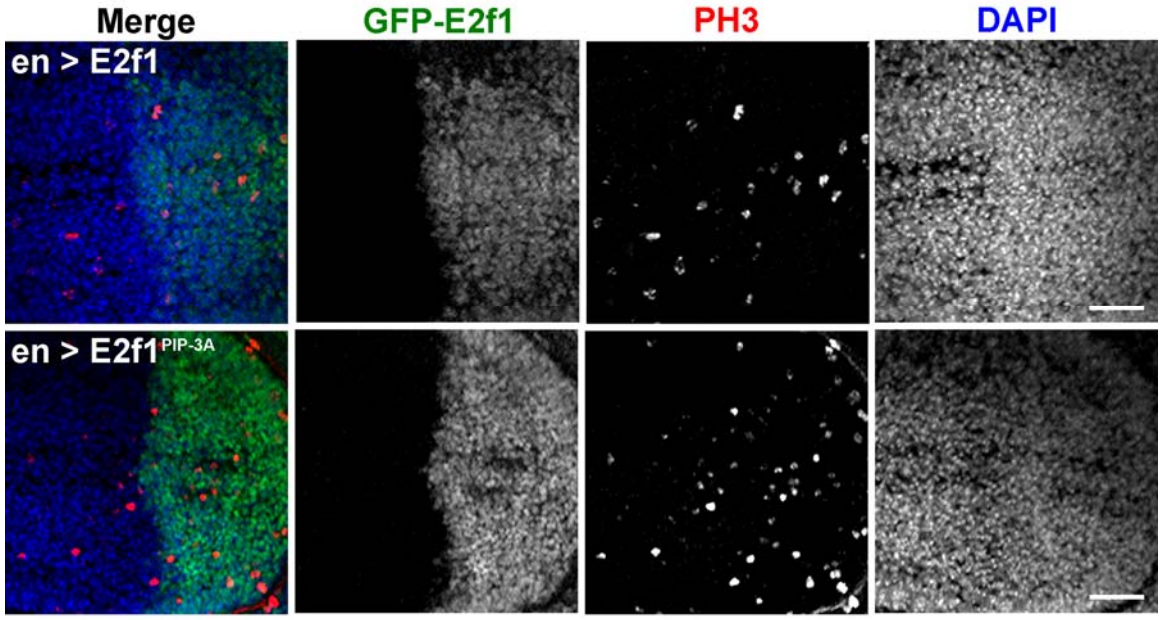


Figure S5. Patterns of E2f1 Accumulation in Wing Discs Are Unaffected by Loss of Cul1, Slmb, or Ago

The indicated mutant mitotic cell clones were generated in early third instar larval wing imaginal discs by the heat shock FLP/FRT method (Xu and Rubin, 1993) as described (Neufeld et al., 1998). Larvae were heat-shocked in a water bath for 30 min at 48 hrs AED to induce clones, dissected at 96 hrs AED, and stained with guinea pig anti-E2f1 antibodies (red; gift from Terry Orr-Weaver). Wild type sibling clones are marked with two copies of GFP (green). GFP-negative homozygous mutant clones for *Cul1*, *Slmb* or *Ago* are shown. Note that some cells within the mutant clones are positive for E2f1 and some are not, indicating the cyclic accumulation of E2f1 during the cell cycle. Fly strains used for mitotic recombination were *hs-FLP^{122}; TM3/TM6B, hs-FLP^{122}; Sp/SM6-TM6B, w; FRT 42D GFP, w; FRT 82B GFP,* and *yw; FRT 82B slmb^{P1493}/TM6B. w; FRT 42D Cul1/CyO* and *w; FRT 80B ago^{3}/TM6B* were gifts from Cheng-Ting Chien and Ken Moberg, respectively.



Genotype & location in wing disc	No. of PH3-positive nuclei		
en > E2f1 anterior	3.7 ± 2.5	P < 0.05	P > 0.05
en > E2f1 posterior	7.0 ± 4.6		
en > E2f1^{PIP-3A} anterior	6.3 ± 1.2	P < 0.0001	P < 0.05
en > E2f1^{PIP-3A} posterior	17.7 ± 4.0		

Figure S6. PH3 Positive Mitotic Cells Are Elevated in the Posterior Compartment after Expression of GFP-E2f1^{PIP-3A}

Wing discs of the indicated genotypes were dissected, fixed in 6% paraformaldehyde for 20 minutes at room temperature, permeabilized in PBT containing 5% normal goat serum for an hour at room temperature, and incubated with rabbit anti-phospho-Ser10-histone H3 (1:500, Upstate) overnight at 4ºC. Discs were then incubated with goat anti-rabbit-rhodamine (1:500, Invitrogen-Molecular Probes) for 30 minutes at room temperature, and with 1 µg/ml DAPI (blue) for 5 min at room temperature. Confocal images were compressed into 3 µm thick stacks, and PH3 positive cells were counted within an area of 47.7 µm x 47.7 µm for both the anterior and posterior sides of each wing disc (anterior at left, dorsal at top). PH3 positive nuclei are shown in red. GFP detection of GFP-E2f1 is shown in green. Scale bars are 20 µm. (Lower panel) The averages and standard deviations are shown. P values were calculated by using Poisson generalized estimating equations. n=3 for each genotype/location in wing disc.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis

pIE4-E2f1 (gift from Dr. Nick Dyson) was used as a template for QuikChange® XL Site-directed Mutagenesis (Stratagene) to engineer E2f1$^{\Delta Cdk}$ containing T234A, S273A, S430A, S434A, T486A, S583A and T586A mutations. The open reading frame (ORF) of the wild type or ΔCdk mutant *E2f1* was cloned into pUAST, then re-amplified (primer sequences available in Supplemental Experimental Procedures) from pUAST-*E2f1* plasmids and cloned into pENTR™/D-TOPO® (Invitrogen). *E2f1* PIP box and L786Q mutants were created from pENTR-*E2f1* clones using a QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). The E2f1 deletion mutants were amplified from pENTR-*E2f1* and re-cloned into pENTR. The *Cul4* and *Cdt2* (*l(2)dtl*) ORFs were amplified from Open Biosystems cDNA clones LP02965 and LD21681, respectively, and cloned into pENTR. The *Dp* ORF was amplified from a cDNA (Duronio et al., 1998) and cloned into pDONR™221 (Invitrogen). ORFs in pENTR or pDONR were confirmed by sequencing and then recombined with pAGW (*Actin5C* promoter, N-term GFP), pHGW (*Hsp70* promoter, N-term GFP), pAMW (*Actin5C* promoter, N-term myc), pAHW (*Actin5C* promoter, N-term HA), or pPGW (UASp, N-term GFP) (provided by Dr. Terence Murphy, Carnegie Institute).

PCR Primers and Templates for Cloning

Primers for pENTR-D-TOPO cloning

E2f1 (1-) pENTR forward:
CACCATGTCCAAGTTCTTTGTGAATGTTGCC

E2f1 (47-) pENTR forward:
CACCATGGTGGCCCGCAGACTCAACTA

E2f1 (93-) pENTR forward:
CACCGGCGGCGTGGCAGCCCACC

E2f1 (139-) pENTR forward:
CACCCAAAATCAGCAGCAACGCAAGG

E2f1 (231-) pENTR forward:
CACCTCGCTGTCGACGCCCCAGCAAC

E2f1 (529-) pENTR forward:
CACCCAGCAACAACAACAGTTGCTACAGC

E2f1 (-92) pENTR reverse:
TCAGTTGCTGTTGCTGTCGCTGCTGC

E2f1 (-138) pENTR reverse:
TCAGTGGTGGTGCTGCTGCTGCAG

E2f1 (-184) pENTR reverse:
TCACGTCTGGTGGTGGGCGCTCTG

E2f1 (-230) pENTR reverse:
TCAAAAGGGGTGATGCGATGCCGG

E2f1 (-528) pENTR reverse:
TCACTGCTGCTGCTGGTTCAGATTATG

E2f1 (-805) pENTR reverse:
TTAGGGTCCATAGGCATCCGAACCGAA

Cul4 pENTR forward:
CACCATGAGTGCGGCCAAGAAGTACAAG
Cul4 pENTR reverse:
TTATGCCACATAGTTGTATTGGTTTTG

Cdt2 (l(2)dtl) pENTR forward:
CACCATGAACATTTACAACAAGTTGCGGGC
Cdt2 (l(2)dtl) pENTR reverse:
TCAATCGCTGCCCACCGCCGTC

Primers for pDONR221 cloning
Dp pDONR forward:
GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGCATTCGACGGGCGGTAC
Dp pDONR reverse:
GGGGACCACTTTGTACAAGAAAGCTGGGTTCAATCAATGTCGTCGTCCAGCTC

Primers for mutagenesis
E2f1 L786Q mutagenesis forward:
CTATCCGTATGCGCAGAACGCGAACGAGG
E2f1 L786Q mutagenesis reverse:
CCTCGTTCGCGTTCTGCGCATACGGATAG

E2f1 PIP-3A mutagenesis forward:
GACCGGCAAATCCAACGATGCCACAAATGCCGCCAAGGTCAAACGTCGGCCAC
E2f1 PIP-3A mutagenesis reverse:
GTGGCCGACGTTTGACCTTGGCGGCATTTGTGGCATCGTTGGATTTGCCGGTC

E2f1 PIP-7A mutagenesis forward:
GGCGACCGGCAAATCCGCCGCAGCAGCGGCCGCGGCCAAGGTCAAACGTCGG
E2f1 PIP-7A mutagenesis reverse:
CCGACGTTTGACCTTGGCCGCGGCCGCTGCTGCGGCGGATTTGCCGGTCGCC

E2f1 PIP-7del mutagenesis forward:
GGCGACCGGCAAATCCAAGGTCAAACGTCGGC
E2f1 PIP-7del mutagenesis reverse:
GCCGACGTTTGACCTTGGATTTGCCGGTCGCC

Primers and templates for dsRNA synthesis

lacZ dsRNA forward:
TAATACGACTCACTATAGGG ACGCCGAACGATCGCCAGTTCTG
lacZ dsRNA reverse:
TAATACGACTCACTATAGGG CGAGCCAGTTTACCCGCTCTGC
lacZ dsRNA template: genomic DNA extracted from *w; Sco/CyO wg-lacZ*

Cul4 dsRNA (1st amplicon) forward:
TAATACGACTCACTATAGGG TTGGCCAAACGATTACTTGTGGG
Cul4 dsRNA (1st amplicon) reverse:
TAATACGACTCACTATAGGG GAGAAGATTATGGCTCAGCG
Cul4 dsRNA (1st amplicon) template: cDNA GM14815

Cul4 dsRNA (2nd amplicon) forward:
TAATACGACTCACTATAGGG TGAACATGTGTAGCCACAAGATG
Cul4 dsRNA (2nd amplicon) reverse:
TAATACGACTCACTATAGGG ACAACAGTGTAAGGTCACTCAGTC
Cul4 dsRNA (2nd amplicon) template: cDNA GM14815

Ddb1 dsRNA (1st amplicon) forward:
TAATACGACTCACTATAGG CCCCGCTCCATTCTGATGACC
Ddb1 dsRNA (1st amplicon) reverse:
TAATACGACTCACTATAGGG CTGCAGCAGCGTGATGGAGCGC
Ddb1 dsRNA (1st amplicon) template: genomic DNA from w^{1118} flies (Fig. 3) or cDNA LD08715 (Supplemental Fig. 3)

Ddb1 dsRNA (2nd amplicon) forward:
TAATACGACTCACTATAGGG GAGCTAAACGTGTACGATGTTGAG
Ddb1 dsRNA (2nd amplicon) reverse:
TAATACGACTCACTATAGGG TGCAGGCATGCTCCTGAATGCC
Ddb1 dsRNA (2nd amplicon) template: cDNA LD08715

Cdt2 (l(2)dtl) dsRNA (1st amplicon) forward:
TAATACGACTCACTATAGGG GCGGGCTCCGGCATACGCGGC
Cdt2 (l(2)dtl) dsRNA (1st amplicon) reverse:
TAATACGACTCACTATAGG CGTGGCTGGAGCCCCAGGCCACG
Cdt2 (l(2)dtl) dsRNA (1st amplicon) template: genomic DNA from w^{1118} flies

Cdt2 (l(2)dtl) dsRNA (2nd amplicon) forward:
TAATACGACTCACTATAGGG GCAGGCCGATGTGCTGGCATC
Cdt2 (l(2)dtl) dsRNA (2nd amplicon) reverse:
TAATACGACTCACTATAGGG AGTTGGTTACCAACTCGTCC
Cdt2 (l(2)dtl) dsRNA (2nd amplicon) template: genomic DNA from *w*^{1118} flies

Pcna dsRNA forward:
TAATACGACTCACTATAGGG CAGGCCATGGACAACTCCCATG
Pcna dsRNA reverse:
TAATACGACTCACTATAGGG TGTCTCGTTGTCCTCGATCTTGGG
Pcna dsRNA template: genomic DNA from *w*^{1118} flies

Cul1 dsRNA forward:
TAATACGACTCACTATAGGG CTGCTCAACGCAGACCG
Cul1 dsRNA reverse:
TAATACGACTCACTATAGGG TGTCCTGCAGTTGCTGG
Cul1 dsRNA template: genomic DNA from *w*^{1118} flies

Skp1 (SkpA) dsRNA forward:
TAATACGACTCACTATAGGG TGCCCAGCATCAAGTTGCAATCTTC
Skp1 (SkpA) dsRNA reverse:
TAATACGACTCACTATAGGG CTAGCTGTTTCAACTTAATGTTGGTC
Skp1 (SkpA) dsRNA template: genomic DNA from *w*^{1118} flies

Slmb dsRNA forward:
TAATACGACTCACTATAGGG GGCCGCCACATGCTGCG
Slmb dsRNA reverse:
TAATACGACTCACTATAGGG CGGTCTTGTTCTCATTGGG
Slmb ds RNA template: cDNA LD08669

Skp2 (CG9772) dsRNA forward:
TAATACGACTCACTATAGGG TAGACCAGGTGCCCTCG
Skp2 (CG9772) dsRNA reverse:
TAATACGACTCACTATAGGG GGTTGCTGGAATAAGATAGC
Skp2 (CG9772) dsRNA template: cDNA GM13370

Ago dsRNA forward:
TAATACGACTCACTATAGGG GATGCCCAGCTGCTAGC
Ago dsRNA reverse:
TAATACGACTCACTATAGGG TGCTGGCGAGGGATTCG
Ago dsRNA template: genomic DNA from *w*^{1118} flies

Dp dsRNA forward:

TAATACGACTCACTATAGGG GGCCCAGAACAAGTCCGAAATGG

Dp dsRNA reverse:

TAATACGACTCACTATAGGG GGCAAGGTTTGGAGGCACCCAC

Dp dsRNA template: Dp cDNA (Duronio et al., 1998)

Rbf1 dsRNA forward:

TAATACGACTCACTATAGGG AAGCTGGCGAAGAGGTAATAGCC

Rbf1 dsRNA reverse:

TAATACGACTCACTATAGGG GCACACATAATATTTTGATCGAGGTG

Rbf1 dsRNA template: genomic DNA from w^{1118} flies

Primers for RT-PCR

Cul4 RT-PCR forward:

CGTCACTATCTGGACTCGAGCAC

Cul4 RT-PCR reverse:

CCAGATCGCAGTTTCATATCTACG

Ddb1 RT-PCR forward:

GGTCTCTAAAGGTGGGAGTCG

Ddb1 RT-PCR reverse:

GTAGATGGTCTCAAGATCAGGCAG

Cdt2 (l(2)dtl) RT-PCR forward:

AATATTTTCGATGCCTCGAAGGTTG

Cdt2 (l(2)dtl) RT-PCR reverse:

CACCTCCCACAGTCTGGCAGTG

Pcna RT-PCR forward:

CTTCGATTGCAGCGACTCCGGC

Pcna RT-PCR reverse:

AAATGTCAGCGTCACCGGCTCC

Cul1 RT-PCR forward:

GCATCTGCAACTACCTGAATCGG

Cul1 RT-PCR reverse:

CAGATTCTGAAACTCGGTGTGAAAG

Skp1 (SkpA) RT-PCR forward:

TGCCCAGCATCAAGTTGCAATCTTC

Skp1 (SkpA) RT-PCR reverse:

CAAATCCAATTCGTTCCCGAATCC

Slmb RT-PCR forward:

CAACCACTATGCTGTACGACCCG
Slmb RT-PCR reverse:
TCCGCCAGTTGTTCTCTATGCTG

Skp2 (CG9772) RT-PCR forward:
TCACTGTCGATGTCCCGTCAATC
Skp2 (CG9772) RT-PCR reverse:
TTGTCCGCAAACCCAAATCTAGCC

Ago RT-PCR forward:
GCTCGCGAGACGCAACCTTGAG
Ago RT-PCR reverse:
GACCGCAGAATGATGCTTATTTGG

Dp RT-PCR forward:
GTACGACAACAACTGTGATCAAAAG
Dp RT-PCR reverse:
TTCGCCAGTCTTGCCGGTGCC

Rbf1 RT-PCR forward:
GTGGTCAAGGGTAATTGTGTGTCC
Rbf1 RT-PCR reverse:
CGAAGTTTTCGTTAGCCAATAGGCC

GFP RT-PCR forward:
GGAGTACAACTACAACAGCC
GFP RT-PCR reverse:
CTTGTACAGCTCGTCCATGCCG

SUPPLEMENTAL REFERENCES

Duronio, R.J., Bonnette, P.C., and O'Farrell, P.H. (1998). Mutations of the Drosophila dDP, dE2F, and cyclin E genes reveal distinct roles for the E2F-DP transcription factor and cyclin E during the G1-S transition. Mol Cell Biol *18*, 141-151.

Hu, J., Zacharek, S., He, Y.J., Lee, H., Shumway, S., Duronio, R.J., and Xiong, Y. (2008). WD40 protein FBW5 promotes ubiquitination of tumor suppressor TSC2 by DDB1-CUL4-ROC1 ligase. Genes Dev *22*, 866-871.

Neufeld, T.P., de la Cruz, A.F., Johnston, L.A., and Edgar, B.A. (1998). Coordination of growth and cell division in the Drosophila wing. Cell *93*, 1183-1193.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117*, 1223-1237.