Supplementary Information for

Translational control of E2f1 regulates the Drosophila cell cycle

Jan Inge Øvrebø¹, Mary-Rose Bradley-Gill², Norman Zielke³, Minhee Kim², Marco Marchetti¹, Jonathan Bohlen⁴, Megan Lewis¹, Monique van Straaten⁴, Nam-Sung Moon², Bruce A. Edgar¹∗.

¹ Huntsman Cancer Institute, Department of Oncological Sciences, University of Utah, Salt Lake City, UT 84112, USA. ² McGill University, Montreal, QC H3A 0G4, Canada. ³ University of Helsinki, Helsinki, Finland. ⁴ Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany.

*Author for correspondence (jan.ovrebo@hci.utah.edu; bruce.edgar@hci.utah.edu)

This PDF file includes:
  - Supplementary Materials and Methods
  - Figures S1 to S10
  - Tables S1 to S4
  - SI References
Supplementary materials and methods:

Quantification of endogenous E2f1 expression from immunohistochemistry samples.
Images for measuring dE2F1 in WDs were acquired on a Nikon Eclipse Ti widefield fluorescence microscope. dE2F1 was stained by guinea pig anti-dE2F1 primary antibody and anti-guinea pig secondary antibody conjugated with Alexa 568. Average A568 and Hoechst fluorescence levels were measured in posterior and anterior WD compartment using freehand selection tool in Fiji(V1.0). en-Gal4 UAS-GFP was used to define this boundary. The A568:Hoecst ratio was calculated for both posterior and anterior compartment to get an estimate of average E2F1 levels per DNA unit. The ratio between $E2F1_{\text{posterior}}/DNA_{\text{posterior}}$ and $E2F1_{\text{anterior}}/DNA_{\text{anterior}}$ was then calculated to observe relative change in E2F1 expression levels upon dMyc, Rheb, Ras$^{V12S35}$, or S6K$^{TE1}$ overexpression compared to GFP-negative control cells of the anterior wing pouch. For measuring dE2F1 in eye discs, on the clone marker channel, using the polygon tool, three polygons were drawn manually inside either wild type or mutant clones in randomly selected areas of each eye disc. Average intensity was calculated for the three polygons and averaged out for an average intensity for either wild type or mutant clones per eye disc. For GFP and dE2F1 calculations, polygons were restricted to the posterior domain marked by ELAV photoreceptors.

Quantification of pH3± cells.
Quantification of pH3+ cells in WD compartments was done by defining posterior and anterior regions with the freehand tool in Fiji. The number of pH3 puncta was defined by signal thresholding (same threshold for anterior and posterior WD), followed by “analyze particles” (size threshold was used to exclude noise). The number of pH3+ cells in adult midguts were manually quantified, blinded, using a Nikon Eclipse Ti widefield fluorescence microscope. Quantifications of E2F1 levels in larval imaginal discs were performed using ImageJ to measure GFP, dE2F1, and DAPI intensities in wing and eye discs. Statistical significance of pH3+ cells were assessed in Prism 8 by Student’s t test ($P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).
cDNA Synthesis and qRT-PCR on Drosophila S2R+ Cells

For quantification of gene transcription by real time qRT-PCR, whole RNA was isolated from S2-R+ cells using the QIAGEN RNAeasy kit. cDNA was synthesized from total RNA using the QIAGEN reverse transcription kit. qRT-PCR analyses were carried out using the QIAGEN SYBR Green PCR kit and the Roche Lightcycler 480 II. Analyses were performed as four technical replicates, and primer pair efficiency and specificity were verified beforehand. Absence of genomic DNA was verified by using reverse transcriptase free reactions as controls. Relative expression levels were determined by using housekeeping gene expressions as a reference in each experiment.

Real time measurement of translational sensors in S2R+ cells:
For transfection of S2-R+ cells with plasmid DNA the classic calcium phosphate transfection technique was carried out. 5 ml S2-R+ cells were seeded into a 25 cm² flask at and incubated over night at 25°C. The transfection mix was prepared by mixing 60 μl of 2 M CaCl2 with 20 μg plasmid DNA and water was added to a total volume of 500 μl. This mix was added dropwise to a 2x HEPES-Buffered-Saline solution (50 mM HEPES, 1.5 mM Na2HPO4, 280 mM NaCl, pH 7.1). The resulting solution was incubated for 30-40 minutes at room temperature to allow precipitate to form. It was mixed and added to the cells dropwise. Cells were incubated for 14 to 18 hours at 25 °C, subsequently, the calcium phosphate solution was removed and cells were washed with complete Schneider’s medium. Fresh medium was added. After 2-3 days the selection substance(s) (Geneticin, Puromycin, etc.) were added to the cells. For FACS sorting, cells were harvested as described above. After centrifugation, cells were resuspended in 3 ml Schneiders medium. To remove cell aggregates (clumps) cells were then pipetted into the filter of a FACS cell strainer tube. Next, cells were sorted, using appropriate gating settings. After sorting cells were seeded in 25 cm² tissue culture flasks in conditioned medium. For live GFP recording, cells were seeded in either 8-well or 24-well Ibidi plates at concentrations between 1*105 and 1*104 cells/ml in conditioned Schneiders Medium containing the appropriate selection substances. TMP, Rapamycin and Cycloheximide were added shortly before starting the acquisition. Incubation chambers were set to 25°C. On these systems, cells were usually imaged for 48 hours, with image acquisition every 20 minutes. Images were acquired in each of the required
fluorescence channels in Z stacks. Typically, in 7 z-slices at 1.5μm distance were acquired. Images acquired from Olympus IX81 Inverted Microscope were processed using Image J/Fiji. First, they were merged to hyperstacks with 2 to 3 channels (fluorescence channels), 7 z-slices and 144 timeframes. These hyperstacks were subsequently reduced in dimensionality by projecting the 7 z-slices with the maximum intensity projection function. Then the background was removed using the rolling ball background subtraction function with an estimated cell radius of 20 pixels. Live recording produced a large amount of data that was processed and analyzed with an ImageJ makro written by Dr. Aliakbar Jafar Pour (ZMBH Imaging Facility). This makro allowed for automation of the following steps. Images were processed by creating maximum intensity projections from the seven Z slices that were acquired in each fluorescence channel at each time point and well position. These were then subjected to rolling ball background subtraction and simple background subtraction. From these processed images time-lapse movies were created. Next, they were used to automatically detect objects in the RFP channel and track them over time. Afterwards the fluorescence intensity of these objects was automatically quantified in the green channel. For each of the acquired conditions 15-20 such tracks were quantified and then averaged.

**Construct generation**

**Construction of GFP-E2F1 constructs:**

The pUASt_RA-sfGFP-E2F1_attB construct were generated by cloning the RA-E2F1 5’UTR, CDS and 3’UTR into the UASTattB vector. 5’UTR truncations were generated by PCR, followed by circularization by self-ligation, excluding regions of interest using the following primers:

**pUASt ∆(1-414)-sfGFP-E2F1_attB**

fw: PHO-TGCAAGTAAAAGATTCAAGTCGCTG
rv: GCCCATCGCAATATCACAAATG
pUASt_\(\Delta(207-621)\)-sfGFP-E2F1_attB  
fw: PHO-CACCACCGACGTACTCACACTC  
rv: GAGTCGTCTACGTCAGACGAG  

pUASt_\(\Delta(414-828)\)-sfGFP-E2F1_attB  
fw: PHO-TTGGTTGTGGTTGCGTGTGATATATAG  
rv: GAGGCAGTCTTTTGCGGC  

pUASt_\(\Delta(621-1035)\)-sfGFP-E2F1_attB  
fw: PHO-GCAGTGCTTAAAAGTATGCTACAATATAGTGC  
rv: TGGGAACTTTAACTTTACTACGC  

pUASt_\(\Delta(828-1242)\)-sfGFP-E2F1_attB  
fw: PHO-ATGCGTAAAGGCGAGGAGCTG  
rv: ATTGCTCTGCTTTTTTTCCACC  

pUASt_\(\Delta\text{uORF}\)-sfGFP-E2F1_attB and pUASt_\(\Delta\text{uORF}\Delta(414-828)\) were generated by replacing the pUASt_RA-sfGFP-E2F1_attB 5'UTR with a synthesized \(\Delta\text{uORF}\) 5'UTR or pUASt_\(\Delta\text{uORF}\Delta(414-828)\) with partial sfGFP sequence (gBlocks\textsuperscript{®}) ordered from Integrated DNA Technologies flanked with NotI and KpnI restriction sites (Table S3), allowing complete replacement of the RA-E2F1 5'UTR with the \(\Delta\text{uORF}-E2F1\) 5'UTR or pUASt_\(\Delta\text{uORF}\Delta(414-828)\) by NotI and KpNI (New England Biolabs) restriction digestion followed by T4 ligase (New England Biolabs) reaction.

sfGFP E2F1 transgenes:  
To generate sfGFP translational sensors, the constructs generated above were altered by removing the E2F1 DNA binding domain and the PIP box. This region was removed by restriction digestion using Bpu10I (New England Biolabs). This left two incompatible overhangs that were bridged by a short dsDNA fragment formed by hybridizing two ssDNA oligos:
Oligo1(5’-3’): TGACAAAAAGAAACGTAAGGTT
Oligo2 (5’-3’): TGAAACCTACGTTTTTTTTGG

The ligation reaction was carried out using T4 ligase.
Successful vector construction was confirmed by Sanger sequencing by the DNA Sequencing Core Facility, University of Utah.

Sequencing of 5’UTR
sfGFP_rv GTACGTGCGTCATCCTT
UASt fwd GAGCGCCGAGTATAAATAGAG

Sequencing of E2F1 deletion:
sfGFP fwd GTTCAGTGCTTTGCTCGTTATC
RFP
RFP-sfGFP bicistronic reporters:
pUASt_RA-sfGFP-E2F1_attB were first cut with AatII (New England Biolabs) to replace the E2F1 CDS and the sfGFP with a sfGFP possessing a nuclear localization signal (sfGFP\textsubscript{NLS}) (Table S3). This new vector, pUASt_RA-sfGFP\textsubscript{NLS}-attB, were further modified by replacing the RA-E2F1 5’UTR with RA-RFP\textsuperscript{414} using the NotI and KpnI restriction sites, giving us the pUASt__RA-RFP\textsuperscript{414}-sfGFP\textsubscript{NLS}-E2F1_attB and pUASt_RA-RFP\textsuperscript{414}-sfGFP\textsubscript{NLS}-E2F1_attB vectors. The RA-RFP\textsuperscript{414} sequences were synthesized by Twist Bioscience (Table S3) and inserted into a pTwist Amp High Copy vector. All ligations were carried out using T4 ligase (NEB), and final constructs were sequenced by the DNA Sequencing Core Facility, University of Utah.

RD and RD\textsuperscript{ΔuORF} GFP translational reporters.
RD and RDmut 5’UTR sequences were ordered from BioCorps (Montreal, QC, Canada) using the FlyBase annotated sequence. For RDmut, sequence, each ATG of the uORFs was mutated to TTG. RB and RC sequences were cloned from cDNA made from RNA isolated from eye discs. Sequences were first cloned into pENTR vector using pENTR-D-TOPO Cloning Kit (Invitrogen). Next,
sequences were cloned into pTWG vector using Gateway LR Clonase Enzyme Mix (Invitrogen). Plasmids were microinjected into yw embryos and transformant lines were isolated. Mitotic clones were generated by using FRT-FLP mediated recombination (as reviewed in (Blair 2003). FLP-recombinase was expressed using the eyeless (ey) promoter combined with a marker for clone visualization using the following stock: ey-FLP; FRT82B GFP. To generate eye discs entirely of mutant tissue, mutants and a control FRT82B were crossed to ey-FLP; 82B [W+] l(3)cl-R3 which cause cell death in wild type clones.

**Protein Extraction and Western Blot**

10 fly heads were dissected per genotype and crushed in SDS loading buffer. Samples were run on an SDS-polyacrylamide gel and transferred to a PVDF membrane. Western blot was performed by first blocking with 5% milk solution in 0.1% Tween20-PBS for at least 1 hour. Primary antibodies used were anti-GFP (Santa Cruz, 1:1000) and anti-β-tubulin (DSHB, 1:1000). Anti-mouse-HRP (GE Healthcare UK Limited, 1:2000) secondary antibody was used.
Figure S1. Overexpression of Rheb, dMyc, Ras\textsuperscript{V12S35} and S6K\textsuperscript{TE1} in posterior larval wingdisc.

Myc, Rheb, Ras\textsuperscript{V12S35} and S6K\textsuperscript{TE1}, were overexpressed in the posterior WD (P) using the enGal4 UAS-GFP driver. Posterior wing-compartment were identified by GFP expression and DNA were stained with DAPI. E2F1 levels, stained with anti-E2F1 antibody, were enriched in cells overexpressing Rheb. Scalebar = 100 µm.
Figure S2. E2f1 Isoforms and mutants.

Overview of E2f1 isoforms and mutants. Six isoforms of E2f1 as reported by Fly base. The six isoforms differ by alternative splicing of the 5’UTR. Isoform variation also include alternative splicing of an exon internal to the coding sequence and transcriptional truncation of the 3’UTR. Because annotated transcripts do not represent all possible combinations of alternative exons and/or alternative promoters, full length CDS and 3’UTR is displayed for simplicity. The bottom two models display isoform specific E2f1 mutants. E2f1RSS possesses a P-element insertion that specifically blocks expression of the RA, RE and RD-E2F1 isoforms. The E2F1ΔRA mutant harbors a deletion of the RA/RE/RD exon.
Figure S3. Multiple sequence alignment of E2f1 5’UTRs of multiple Drosophila species.

E2f1 5’UTR sequences of 17 Drosophila species aligned using Genious 10.0.7. The E2f1 isoforms with highest sequence similarity to D. melanogaster E2f1-RA isoform were used in this alignment.
Figure S4. *E2f1* transgenes.

Overview of *E2f1* transgenes. A) *E2f1* translational sensors, expressed through an Act promoter, expressed in S2R+ cells. *E. coli* dihydrofolate reductase (ecDHFR) serve to destabilize GFP protein, unless supplemented with Trimethoprim. B) GFP-*E2F1* transgenes expressed through a UASt promoter in vivo. Transgenes possess variations in the 5’UTR, which includes truncations and single nucleotide substitutions that removes uORF start codons. C) UASt driven *E2f1* translational sensors for in vivo expression. Transgenes differ from transgenes displayed in B by the lack of *E2f1* coding sequence to prevent cell cycle dependent degradation. In addition, a bicistronic translational sensor with RFP insertion in the *E2f1-RA* 5’UTR were designed to assay cap-independent translation.
Figure S5. Gating scheme for flow cytometry.

Gating scheme for measurement of E2f1 translational sensors expressed in WDs and ISCs, assayed by GFP intensity. **A)** Debris was excluded from analysis of WD cells by gating cells of similar size and complexity by Forward (Fwd) and side scatter. Cell aggregates were excluded by gating single cells by Fwd scatter area (A) versus width (W). Nucleated cells were gated on Hoechst signal, before GFP intensity was recorded. **B)** For ISC GFP measurements, clustered cells were first excluded by a FSC-A:FSC-W gate. Nucleated cells were then selected using a Hoechst+ gate. Nucleated cells expressing RFP were identified as ISCs/EBs, due to the esgGal4 driven RFP expression. The ISC/EB population of larger size was measured as we assume they have not divided during the period of translational sensor expression.
Figure S6. uORFs attenuate E2f1 translation.

uORFs in the RD-E2F1 5’UTR reduce translational efficiency. A) A schematic of reporter constructs created by fusing the RD-E2F1 5’UTR to GFP. RD^ΔuORF-GFP contains mutated Start codons to disrupt the uORFs B) Western blot shows higher GFP levels expressed in RD^ΔuORF compared to RD, indicating increased translation. C) RD and RD^ΔuORF display similar levels of expression, measured by qRT-PCR.
Figure S7. E2f1 translation through IRES.

UASt-RA-RFP^{414}-GFP translational sensor (Figure S4C) expressed in Drosophila larvae and adult midguts using tubGal4 driver. Salivary glands and WDs from 3^{rd} instar larvae display RFP expression. Salivary glands also display GFP expression. Adult midguts display RFP expression, while rare cells also express GFP expression.
Confocal images of RA-E2F1 and Δ(414-828)-E2F1 transgenes expressed in Drosophila midgut ISCs, using esg-Gal4ts UAS-RFP driver. ISCs/EBs visualized by esgargot driven expression of RFP. Only weak GFP signal can be detected guts expressing Δ(414-828)-E2F1 (marked with arrows). Scalebars = 30µm.
Figure S9. *E2f1* transgene transcript levels.

qRT-PCR analysis of *E2f1* transgenes expressed in *Drosophila* midgut display similar levels of transcription. Temperature sensitive expression controlled by *esgGal4*<sup>ts</sup>-UAS-RFP 24 h expression induced by 29°C temperature shift. Student’s T-test (*p>0.05, **p>0.01, ***p>0.001, ****p>0.0001).
Figure S10. Protein binding site prediction of eIFs on E2f1 5’UTR.

Binding sites of eukaryotic initiation factors (eIFs) were predicted using RBP suite (1). One potential binding site for eIF3D, and three binding sites for eIF3G, eIF3H and eIF4G were found.
| Abbreviated name | Genotype | Used in Figure: | Source |
|------------------|----------|----------------|--------|
| w^{118}          | w^{118}  | 1, 3, 5, 6, S1, S9 | RRID:BDSC_3605 |
| RAS^{V12,S55}    | yw, hsFlp/22; UAS-RAS^{V12,S55} | 1, S1 | (2, 3) |
| enGal4, UAS-GFP  | w; enGal4, UAS-GFP; + | 1, S1 | (4) |
| dS6K^{Te1}       | yw, hsFlp/22; UAS-dS6K^{Te1}; + | 1, S1 | RRID:BDSC_6912 |
| RHEB             | w; UAS-RHEB; + | 1, S1 | (5) |
| dMyc             | w;+;UAS-dMyc^{G2} | 1, S1 | RRID:BDSC_9675 |
| $E2F^{1^{G8A}}$  | Frt82B d$E2F1^{G8A}$/TM6B, Sb, Tb | 2 | (6) |
| $E2F^{1^{G8A}}$ tsc1^{-} | Frt82B d$E2F1^{G8A}$/Tsc1^{G87X}/TM6B, Sb, Tb | 2 | This paper |
| $E2F^{1^{G8S}}$ tsc1^{-} | Frt82B d$E2F1^{G8S}$/Tsc1^{G87X}/TM6B, Sb, Tb | 2 | This paper |
| Tsc1^{-}         | Frt82B Tsc1^{G87X} | 2 | (7) |
| ey-Flip; FRT82B, GFP | w; ey-Flip; FRT82B, GFP | 2 | (8) |
| tubGal4         | y-w^{+}; tubGal4^{414}/TM3, sb, Ser | 3, 4, S7 | RRID:BDSC_5138 |
| RA-GFP           | w; w^{+} UAS-RA-GFP | 3, 5, 4 | This paper |
| $\Delta u ORF$-GFP | w; w^{+} pUASt-$\Delta u ORF$-GFP | 3, 5, 4 | This paper |
| $\Delta (1-414)$-GFP | w; w^{+} pUASt-$\Delta (1-414)$-GFP | 3, 5 | This paper |
| $\Delta (207-621)$-GFP | w; w^{+} pUASt-$\Delta (207-621)$-GFP | 3, 5 | This paper |
| $\Delta (414-828)$-GFP | w; w^{+} pUASt-$\Delta (414-828)$-GFP | 3, 5 | This paper |
| $\Delta (621-1035)$-GFP | w; w^{+} pUASt-$\Delta (621-1035)$-GFP | 3, 5 | This paper |
| $\Delta (828-1242)$-GFP | w; w^{+} pUASt-$\Delta (828-1242)$-GFP | 3, 5 | This paper |
| enGal4^{m6} UAS-GFP | w; enGal4 UAS-RFP; tubGal80^{p}, UAS-GFP | 3, 4 | This paper |
| RA-2EF1          | w; w^{+} UAS-RA-GFP-2EF1 | 3, 4, 5, 6, S8, S9 | This paper |
| $\Delta u ORF$-2EF1 | w; w^{+} UAS-RA-GFP-2EF1 | 3, 4, 5, 6, S9 | This paper |
| $\Delta (1-414)$-2EF1 | w; w^{+} UAS-RA-GFP-2EF1 | 3, 5, 6, 59 | This paper |
| $\Delta (207-621)$-2EF1 | w; w^{+} UAS-RA-GFP-2EF1 | 3, 5, 6, 59 | This paper |
| $\Delta (414-828)$-2EF1 | w; w^{+} UAS-RA-GFP-2EF1 | 3, 5, 6, 59 | This paper |
| $\Delta (621-1035)$-2EF1 | w; w^{+} UAS-RA-GFP-2EF1 | 3, 5, 6, 59 | This paper |
| $\Delta (828-1242)$-2EF1 | w; w^{+} UAS-RA-GFP-2EF1 | 3, 5, 6, 59 | This paper |
| esgGal4^{m6} UAS-GFP | w; esgGal4 UAS-GFP tubGal80^{p} | 5 | (9) |
| esgGal4^{m6} UAS-RFP | w; esgGal4 UAS-RFP; tubGal80^{p} | 5, 6, S8, S9 | (10) |
| $MEK^{RNAi}$    | w; UAS-$MEK^{RNAi}$; + | 6 | (11) |
| $MEK^{RNAi}$ RA-2EF1 | w; UAS-$MEK^{RNAi}$; w^{+} UAS-RA-GFP-2EF1 | 6 | This paper |
| $MEK^{RNAi}$ $\Delta uORF$-2EF1 | w; UAS-$MEK^{RNAi}$; w^{+} UAS-RA-GFP-2EF1 | 6 | This paper |
| $MEK^{RNAi}$ RA-GFP | w; UAS-$MEK^{RNAi}$; w^{+} UAS-RA-GFP | 6 | This paper |
| $MEK^{RNAi}$ $\Delta uORF$-GFP | w; UAS-$MEK^{RNAi}$; w^{+} UAS-RA-GFP-2EF1 | 6 | This paper |
| RD-GFP           | UAS-RD-GFP | S6 | This paper |
| RD$\Delta uORF$-GFP | UAS-RD$\Delta uORF$-GFP | S6 | This paper |
| GMR-Gal4         | w; GMR-Gal4; + | S6 | BDSC |
| RA-RFP^{G14}GFP^{NLS} | w; w^{+} UAS-RA-RFP^{G14}GFP^{NLS} | S7 | This paper |
| $\Delta uORF$(414-828)-2EF1 | w; w^{+} UAS-RA-GFP[(414-828)]-2EF1 | 6 | This paper |

Table S1: Fly stocks used in this paper, sorted by the order of which they appear in the paper.
| Primer | Sequence (5’-3’) | Target | Used in fig.: |
|--------|-----------------|--------|---------------|
| P01    | GCTCAACGTGGATCTCTTCAA | E2F1_fw | 1             |
| P02    | CGCTTTCACTAAAACTCGC | E2F1_rv | 1             |
| P03    | ACCAGATGAGCGTGTTAAAG | Rheb_FW | 1             |
| P04    | CTGTACTGCAACCGGAATATG | Rheb_RW | 1             |
| P05    | CGCAACATATGCTTTTCTTG | dDP Fwd | 1             |
| P06    | CCGCAATTTCCGACAGAGAC | dDP Rv  | 1             |
| P07    | GGATGCTCTCTAGGGATTTG | Gal4 Fwd | 3, 4, S9 |
| P08    | CAACATCTAGCTGCTGGAGAG | Gal4 Rv  | 3, 4, S9 |
| P09    | GTTCAGTGCTTTGCTCGTATC | sfGFP Fwd | 3, 4, S9 |
| P10    | GTACGGGCGGTCATCTTTT | sfGFP Rv  | 3, 4, S9 |
| P11    | CATCCGTTGACCAACCAG | Total de2f1 forward: | 2             |
| P12    | TTATATCCAGCTGGACTGC | Total de2f1 reverse: | 2             |
| P13    | AACACAAAGCTACCCCAAGG | RB/RF-de2f1 forward: | 2             |
| P14    | TGCCTTATCAACTAATTAGC | RB/RF-de2f1 reverse: | 2             |
| P15    | TGTTCAATCAAGGCTTCAATTG | RC-de2f1 forward: | 2             |
| P16    | GAATTCCAGCTCTCGAGTCG | RC-de2f1 reverse: | 2             |
| P17    | GCGAGCAGAAAAACACGTTAC | RE-de2f1 forward: | 2             |
| P18    | GCACGGTGGACCTTCTCAAC | RE-de2f1 reverse: | 2             |
| P19    | TTGCAAATATGAAACGTGTGC | RA/RD-de2f1 forward: | 2             |
| P20    | TCTTTCTCTCTCTCTGAGCTTG | RA/RD-de2f1 reverse: | 2             |
| P21    | AGCACAACCTCTATATCATGG | GFP forward: | 1, S6          |
| P22    | GTGTCTCTGCTGTTAGTGGTC | GFP reverse: | 1, S6          |

Table S2: qRT-PCR primers.
ΔuORF sequence:

```
GCGGCCGC CACACACATTGTCGAGCAGACAAACACTGACGTTACAGGGGAAAATTCGACGGA
AAAATTCGCCGAAGTTAAATTAAATATGTTAATATGAACTAAATAATAG
GAGCCGTTATATTAAAGTGTCATACGGTGTCTACGTCAATTCTCCGGCTGCTAGTGAAAGACGACTCTCCTCTCCGGCT
CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
```

ΔuORF (414-828) sequence:

```
GCGGCCGC CACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
GAGCCGTTATATTAAAGTGTCATACGGTGTCTACGTCAATTCTCCGGCTGCTAGTGAAAGACGACTCTCCTCTCCGGCT
CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
```

ΔuORF (414-828) sequence:

```
GCGGCCGC CACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
GAGCCGTTATATTAAAGTGTCATACGGTGTCTACGTCAATTCTCCGGCTGCTAGTGAAAGACGACTCTCCTCTCCGGCT
CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
```

RA-RFP m3GFP sequence:

```
GCGGCCGC CACACACATTGTCGAGCAGACAAACACTGACGTTACAGGGGAAAATTCGACGGA
AAAATTCGCCGAAGTTAAATTAAATATGTTAATATGAACTAAATAATAG
GAGCCGTTATATTAAAGTGTCATACGGTGTCTACGTCAATTCTCCGGCTGCTAGTGAAAGACGACTCTCCTCTCCGGCT
CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
AAAAGACCCAGCCACACACACATGTCGAGCAGACAAACACTGACTCAAATGTTAATATGAACTAAATAATAG
```

RA-RFP m3GFP sequence:
GTGATATTCAAAAAGAGCAAGAAGAAGCAGGCGGCGGTGGAAAAAAAAGCAGAGCAATTTGTTGTGGTTGCGTGTG
ATATATAGAAAGCAAGTAAAACCAACAAAAATTCCATTTTTAGATGTGAAGAATCCTTAGGTGCTAATTTAGTTGA
TAAGCGCAAGCTGCAAAGAGAGGAAGATAGAGAGCGATAAACAGAAACAGGCAGTACAATAAGTCAATCGACTCGA
GAGCTGGAAATTCCGACTGGGTGGCAACTTTTCGAAGGCAGTGCTTAAAAGTAGTCTACAATATAGTGCCAGCGAT
GATTGCCGACTGAGGCGAACCTAACAGAATCGGCATATTTATAGAATATATACAGAAGATATATACACGGAGCAAAGAG
AGAGAGAGAGGAGAGAGAGAGCGGAGAAGAGAGAGGGAGAGAGTAAGAGTCAGGCGGAGAATCGCCGAGGAATCACGA
TAGAACCCACGACGCAAAATGCGTAAAGGCGAGGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGT
GATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGT
TCATCTGTACTACTGGTAAACTGCCGGTACC

Table S3: Synthetic constructs used to generate E2F1 transgenes. **Yellow:** NotI, **Turquoise:** KpnI, **Pink:** AatII, **Green:** GFP start codon.

| Plasmid                  | Vector          | Antibiotics resistance |
|--------------------------|----------------|------------------------|
| pUAST_RA-GFP_attB        | pUAST_attB     | Ampicillin             |
| pUAST_AuORF-GFP_attB     | pUAST_attB     | Ampicillin             |
| pUAST_A(1-414)-GFP_attB  | pUAST_attB     | Ampicillin             |
| pUAST_A(207-621)-GFP_attB| pUAST_attB     | Ampicillin             |
| pUAST_A(414-828)-GFP_attB| pUAST_attB     | Ampicillin             |
| pUAST_A(621-1035)-GFP_attB| pUAST_attB     | Ampicillin             |
| pUAST_A(828-1242)-GFP_attB| pUAST_attB     | Ampicillin             |
| pUAST_RA-E2F1_attB       | pUAST_attB     | Ampicillin             |
| pUAST_AuORF-E2F1_attB    | pUAST_attB     | Ampicillin             |
| pUAST_A(1-414)-sfGFP-E2F1_attB| pUAST_attB     | Ampicillin             |
| pUAST_A(207-621)-sfGFP-E2F1_attB| pUAST_attB     | Ampicillin             |
| pUAST_A(414-828)-sfGFP-E2F1_attB| pUAST_attB     | Ampicillin             |
| pUAST_A(621-1035)-sfGFP-E2F1_attB| pUAST_attB     | Ampicillin             |
| pUAST_A(828-1242)-sfGFP-E2F1_attB| pUAST_attB     | Ampicillin             |
| pUAST_RA-RFPsfGFP_attB   | pUAST_attB     | Ampicillin             |
| pUAST_AuORFsfGFP(414-828)-sfGFP-E2F1_attB| pUAST_attB     | Ampicillin             |

Table S4: new plasmids produced under this work.
SI references:

1. Pan X, Fang Y, Li X, Yang Y, & Shen HB (2020) RBPsuite: RNA-protein binding sites prediction suite based on deep learning. *BMC Genomics* 21(1):884.
2. Xiang J, et al. (2017) EGFR-dependent TOR-independent endocycles support Drosophila gut epithelial regeneration. *Nat Commun* 8:15125.
3. Karim FD & Rubin GM (1998) Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in Drosophila imaginal tissues. *Development* 125(1):1-9.
4. Zhang S, et al. (2017) Hippo Signaling Suppresses Cell Ploidy and Tumorigenesis through Skp2. *Cancer Cell* 31(5):669-684 e667.
5. Saucedo LJ, et al. (2003) Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol* 5(6):566-571.
6. Bradley-Gill MR, et al. (2016) Alternate transcripts of the Drosophila "activator" E2F are necessary for maintenance of cell cycle exit during development. *Dev Biol* 411(2):195-206.
7. Tapon N, Ito N, Dickson BJ, Treisman JE, & Hariharan IK (2001) The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* 105(3):345-355.
8. Krivy K, Bradley-Gill MR, & Moon NS (2013) Capicua regulates proliferation and survival of RB-deficient cells in Drosophila. *Biol Open* 2(2):183-190.
9. Jiang H, Grenley MO, Bravo MJ, Blumhagen RZ, & Edgar BA (2011) EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in Drosophila. *Cell Stem Cell* 8(1):84-95.
10. Korzelius J, et al. (2014) Escargot maintains stemness and suppresses differentiation in Drosophila intestinal stem cells. *EMBO J* 33(24):2967-2982.
11. Jin YH, et al. (2015) EGFR/Ras Signaling Controls Drosophila Intestinal Stem Cell Proliferation via Capicua-Regulated Genes. *Plos Genetics* 11(12).