



**SIGNALING PATHWAY REGULATION OF TRANSCRIPTION FACTOR E2F1
ALTERS CELL CYCLE PROGRESSION IN *DROSOPHILA* MIDGUT**

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Introduction

Colorectal cancer is ranked as the third leading cause of cancer related death [1]. Cancer arises from the loss of control of cell growth and proliferation. To better understand the development of cancer, a more robust understanding of the regulation of this process is needed. The transcription factor, E2F1, regulates cell cycle progression by driving cells into the DNA synthesis phase. However, the regulation of E2F1 is not fully understood. E2F1 regulates transcription of Cyclin E (CycE), which binds to cyclin dependent kinase 2 (CDK2) to trigger transition into S phase. S phase then promotes Cullin-RING-4 in conjunction with substrate recognition factor CDT2 (CRL4CDT2) ubiquitin ligase that destroys E2F1 [2]. This explains how E2F1 is removed, but not how it is added to the cycle. E2F1 is post transcriptionally regulated, as its mRNA levels remain steady while its protein levels fluctuate [3]. This regulation is carried out by the target of rapamycin (TOR) [4] and epithelial growth factor receptor (EGFR) [5] pathways. It is common for regulatory mechanisms to be contained in the 5' untranslated region (5' UTR) [6]. Therefore, two forms of E2F1 were examined in this project; the endogenous E2F1-RA isoform, and a mutation with all upstream open reading frames (uORFs) removed from the 5' UTR, called E2F1- Δ uORF.

Differentiating two forms of E2F1 enables more robust study of possible regulatory pathways than one isoform alone. In the model organism, *Drosophila melanogaster*, the TOR pathway is a nutrition sensing pathway that regulates growth based on available energy [7]. The TOR pathway is regulated upstream by Ras homolog enriched in the brain (Rheb) [8] and contains many regulatory components. 4E-BP (Thor) is an inhibitor of 5' cap dependent translation [9], while eIF4A promotes cap dependent translation [10]. General translation is promoted by eukaryotic elongation factor 2 (eEF2) [11] and dS6K [12]. EGFR regulates the PI3-K pathway, offering additional growth control based on available nutrition [13]. There may also be other factors outside of these pathways regulating the translation of E2F1. The *D. melanogaster* homolog of Myc, a human oncogene [14], regulates apoptosis of damaged cells [15]. Lastly, fl(2)d and Mett14 are 5' cap independent translation regulators that methylate mRNA during post transcriptional modifications [16].

While it is known that E2F1 is regulated by translation, the precise mechanism behind this is yet to be discovered. To obtain a list of possible mechanisms, we quantified the effects of these signaling pathways on E2F1 levels. To do so, we conducted a panel of RNAi knockdown of genes from these pathways in conjunction with the two forms of E2F1. Flies with these genetic alterations were exposed to healthy and stressed conditions. The resulting changes in proliferation of intestinal stem cells were then quantified in the *D. melanogaster* midgut. Immunohistochemistry with Anti-phospho-Histone H3 (Ser10) antibody was used to visualize intestinal stem cells that were currently dividing or had recently divided. Proliferation was

quantified by counting the dividing cells per gut by fluorescent microscopy. The gene knockdowns that significantly alter proliferation may participate in the regulation of E2F1. Knowing how these pathways affect proliferation through E2F1 directs further research regarding precise cancer treatments.

Materials

A. Buffers and Cultures

Washing buffers (PBST) were made with either 0.015% Triton X-100 or 0.15% Triton X-100 in phosphate buffer solution (PBS). Blocking buffer was composed of 10% goat serum and 3% bovine serum albumin in 0.15% Triton X-100 in PBS. Pathogenic stress was induced by *Pseudomonas entomophila*. *P. entomophila* cultures were grown in Luria Broth for 24 hours, centrifuged at 2500 RPM at 4°C for 20 minutes, then resuspended in 5% sucrose to an optical density of 50.

B. Immunohistochemistry

The primary antibody used was Anti-phospho-Histone H3 (Ser10) from Sigma-Aldrich, diluted 1:1000 in blocking buffer. The secondary antibody was AlexaFluor 568, diluted 1:1000 in blocking buffer with nuclear stain, 4',6-diamidino-2-phenylindole (DAPI), at 1:1000. For the expression reporter, LacZ-Thor, an anti-beta-Galactosidase mAb antibody was used, diluted 1:1000 in blocking buffer as the primary antibody.

C. Fly Lines

RNAi and E2F1-construct containing lines were obtained from Bloomington Drosophila Stock Center and the Vienna Drosophila Resource Center.

Targeted gene expression was driven by a two-component system of GAL4-upstream activating sequence (UAS) and GAL80. RNAi expression is driven by *escargot*, an intestinal stem cell (ISC) specific driver [17] with the UAS-GAL4 system [18]. The UAS-GAL4 system is inhibited by GAL80, so a temperature sensitive version of GAL80, GAL80TS, that is inactive at higher temperatures was used to inhibit GAL4 and thus RNAi expression at normal temperatures [19]. Knockdown was activated by shifting flies to 29°C for 24 hours.

RNAi lines were homozygous on either the second or third chromosome. The E2F1 constructs were homozygous on the second or third chromosome, whichever did not have RNAi. The endogenous construct, E2F1-RA, contained all 11 uORFs, while E2F1- Δ uORF had them deleted.

D. Fly Husbandry

Flies were kept at 25°C and given standard food. RNAi line flies were crossed with E2F1 line flies by selecting 15 virgin females from the E2F1 lines and crossing with 5 males from the RNAi lines. Male offspring homozygous for RNAi and the E2F1 constructs on separate chromosomes were crossed with virgin females from the driver line. First generation offspring from this cross were collected three to six days after emerging from pupae as adults.

Methods

A. Tissue Collection

Flies were placed in the incubation vials and incubated at 29°C for 24 hours. Incubation vials were made with wide drosophila vials and fly plugs. For healthy conditions, the fly plug was soaked with 5 mL of 5% sucrose. For pathogenically stressed conditions, corrugated filter paper is added to the vial and soaked with 0.75 mL 50 OD *P. entomophila* 6 hours after flies were shifted to 29°C to infect them for 18 hours. After incubation, fly guts were dissected in PBS.

B. Immunohistochemistry

Tissue was fixed in 4% paraformaldehyde (PFA) for 30 minutes, then washed six times with 0.015% PBST for 15-minute intervals. Guts were blocked for two hours in blocking buffer. Primary antibody was added for two hours, excess was washed away with 6 washes in 0.015% PBST. Secondary antibody and DAPI were added for 1.5 hours. Samples were washed three times with 0.015% PBST and once with 0.15% PBST. Guts were mounted using VectaShield Anti-Fade mounting medium.

C. Quantification

The primary antibody is a mitosis marker, used as an indicator of proliferation. The quantity of cells in mitosis in each gut were counted by hand using fluorescent microscopy. Cells were identified by DAPI stain, and the presence of E2F1 was confirmed by the presence of GFP in the gut. Cells were counted using an RFP filter and 20X and 40X dry objectives.

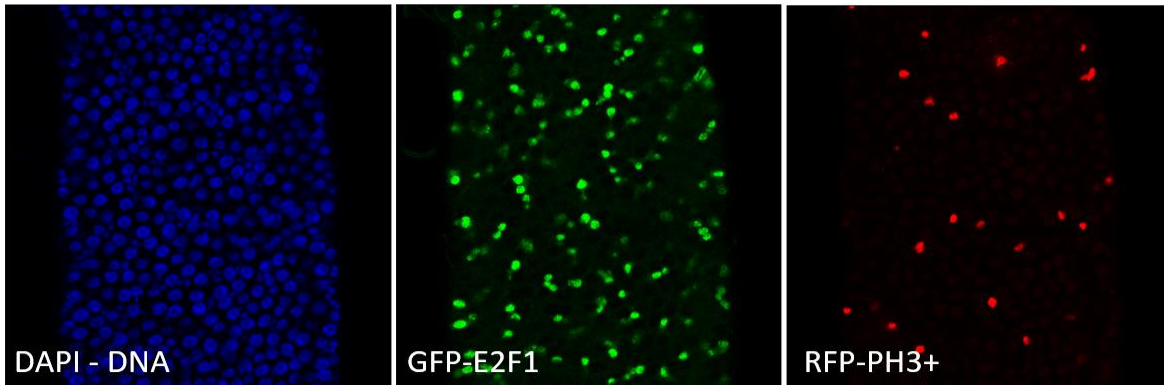


Fig. 1. Confocal Imaging of Quantification Demonstrates the Appearance of Mitosis-Marked Cells. These images were taken using a confocal microscope and a 40X oil objective. DAPI shows the presence of intestinal stem cells. GFP shows the presence of the E2F1 constructs. RFP shows the presence of mitosis-marked intestinal stem cells.

D. Statistical Analysis

Data are presented as individual values with mean and standard deviation error bars. The quantities of proliferating cells were analyzed for statistical significance against corresponding controls using a two-tailed, unpaired, Student's T-test with an alpha value of 0.05 with GraphPad Prism 8.0.1. P values less than 0.05 are indicated with a single asterisk, those under 0.01 are indicated with two asterisks, under 0.001 with three, and 0.0001 with four.

Results

Five RNAi lines returned significant results. Knockdown of Thor, PTEN, eEF2, Mett14, and fl(2)d altered the proliferation of intestinal stem cells. Fly line genotypes are written as first chromosome; second chromosome; third chromosome, and the fourth chromosome is omitted. For example, W; Thor-RNAi; E2F1-RA indicates White on the first chromosome, Thor-RNAi on the second chromosome, and the RA isoform of E2F1 on the third.

The response of the RNAi lines is compared to lines with White on the first chromosome, wild type second chromosome, and one of each isoform of E2F1 on the third chromosome. First, Fig. 2 displays the effect of Thor. During healthy conditions, there was no significant change in proliferation. However, knockdown of Thor promoted proliferation upon stress. The effect was stronger with the E2F1- Δ uORF isoform, though the degree of significance was the same for both isoforms with P values under 0.001 Fig. 2 displays the difference between the isoforms. The

average quantity of marked cells per gut was slightly over 200 for the endogenous isoform, and over 500 for the Δ uORF form.

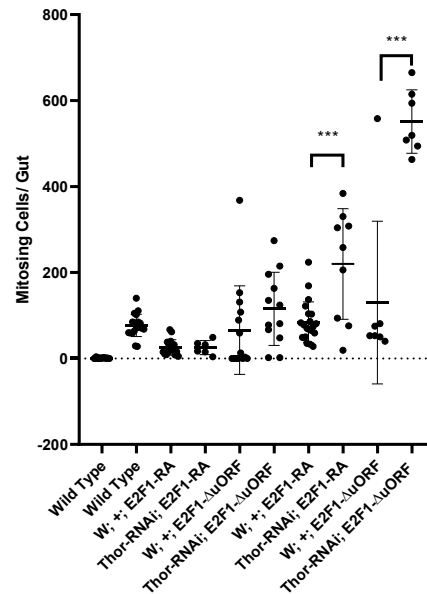


Fig. 2. Knockdown of Translation Inhibiting Thor Promotes ISC Proliferation Upon Stress. This plot displays single values of the quantity of mitosis-marked cells in each gut. Means are shown as horizontal lines, and standard deviation is shown with error bars. If multiple guts within a sample have the same number of mitosis-marked cells, those dots will be in a horizontal line. Sample size varies between 5 and 20, depending on the number of offspring produced in crosses. Black dots represent guts from flies fed sucrose, while orange dots represent guts from flies under pathogenic stress from *p. entomophila*. Data from wild type flies are shown for reference of the effect of stress. The magnitude of significance is shown with asterisks.

Upon further examination with confocal microscopy and the expression reporter gene, LacZ-Thor, we found that Thor was not present in the midgut under healthy conditions. This is shown in Fig. 3. The DAPI staining shows the midgut cells, and the LacZ staining shows the absence of Thor. Thor was, however, present under stressed conditions. Fig 4 shows the midgut cells with DAPI stain, and the presence of RFP positive cells in the LacZ stain indicates that Thor appeared in the midgut under stressed conditions.

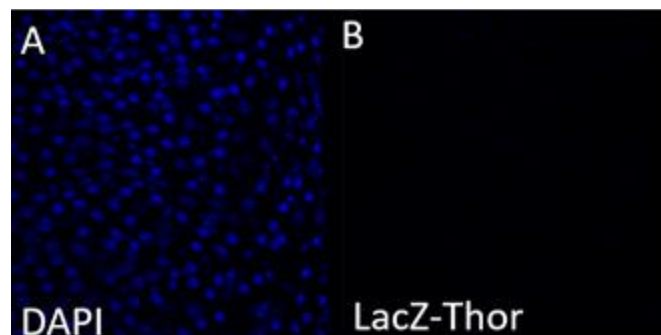


Fig. 3. Confocal Microscopy with Expression Reporter LacZ-Thor shows Thor not present in healthy response. Images were obtained on a confocal microscope with a 40X oil objective. The tissue sample is a section of fly midgut stained by standard protocol. Fig. 3A displays the

intestinal cells in blue from the DAPI stain. Fig. 3B displays LacZ tagged with red fluorescent protein. The lack of fluorescence in Fig. 3B demonstrates that Thor is completely absent in the midgut under healthy conditions.

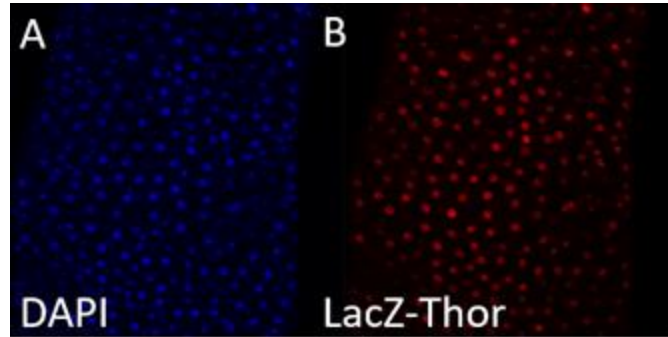


Fig. 4. Thor is Present in Midgut Under Stress Response. Images were obtained from a confocal microscope using a 40X oil objective as in Fig. 3. The tissue sample was a section of midgut stained by the standard protocol. Fig. 4A shows the locations of intestinal cells, while Fig. 4B displays the RFP tagged Lac Z. The presence of RFP in this sample indicates the presence of Thor in the midgut under stress conditions.

The knockdown of PTEN produced similar effects. Fig. 5 displays increased proliferation in response to stress and PTEN knockdown. The effect is much more significant with E2F1- Δ uORF ($P < 0.0001$), as indicated by the four asterisks over the data. The control Δ uORF line had an average of approximately 100 marked cells per gut. The PTEN knockdown line had an average nearly triple that. The control and knockdown lines featuring the endogenous isoform of E2F1 only differed in means by about 50 marked cells. Additionally, the knockdown of PTEN did not affect the level of mitotic activity in flies fed sucrose. One difference to be noted from the Thor assay is the controls used. The control lines contain the balancers, MKRS and TM6B on the third chromosome. These were the available stocks.

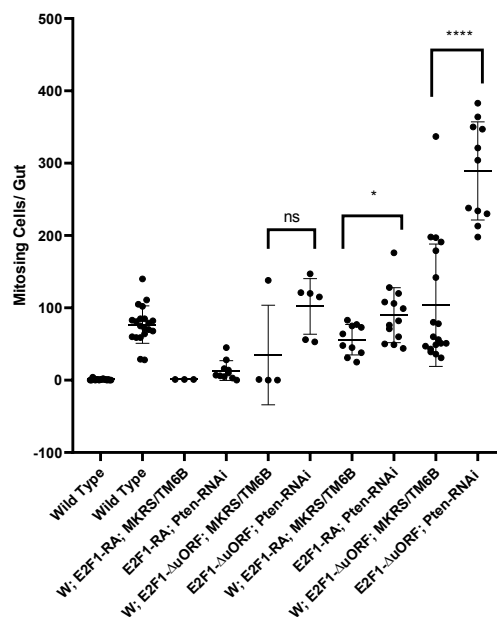


Fig. 5. Knockdown of Tumor Suppressor PTEN Promotes ISC Proliferation Upon Stress. This figure is an individual value plot, with each dot representing the number of mitosis-marked cells in one gut. Means are shown as horizontal lines, while standard deviations are represented by error bars. The magnitudes of significance are shown with asterisks.

Knockdown of transcription factor eEF2 significantly reduced proliferation upon stress, as shown in Fig. 6. The effect was more significant with the E2F1-RA isoform ($P < 0.0001$) than the E2F1- Δ uORF isoform ($P < 0.05$). The mean quantity of marked cells was reduced by about 200 when knockdown of eEF2 was combined with E2F1-RA. For the E2F1- Δ uORF lines, the mean value of marked cells dropped by about 150. Levels of proliferation under healthy conditions did not respond to eEF2 knockdown.

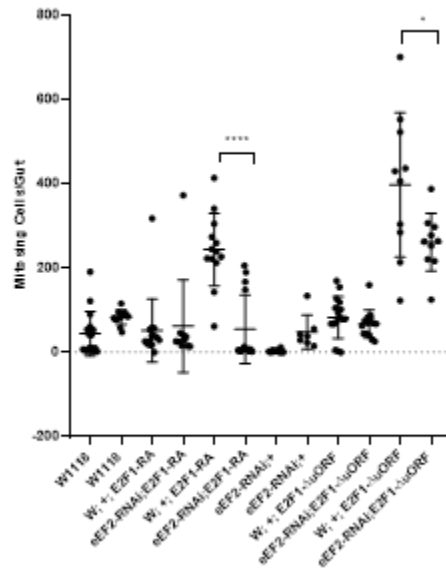


Fig. 6. Knockdown of eEF2 Decreases Proliferation Upon Stress. This is an individual value plot that displays the level of proliferation for each gut in the RNAi knockdown and controls. Means are shown as horizontal lines, and the error bars show standard deviation. The additional control of RNAi-eEF2 without either E2F1 construct was added for reference. Degrees of significance are shown with asterisks.

The next two target genes are related to the modification of mRNA. First is Mettl14, a promoter of N6 adenosine methylation [20]. The knockdown of Mettl14 promotes proliferation with the E2F1-RA isoform, though not with the E2F1- Δ uORF isoform. Fig. 7 displays the high degree of significance with E2F1-RA, as indicated by four asterisks above the data points, and the lack of significance with E2F1- Δ uORF, indicated by 'ns' above the data. It is important to notice that, although the Mettl14; E2F1- Δ uORF genotype appears to be significantly different from its control, the T Test returned a P value above 0.05. The E2F1- Δ uORF control line returned a large amount of variance, with the quantity of marked cells ranging from 50 to 500. The E2F1- Δ uORF knockdown line also had a large amount of variability.

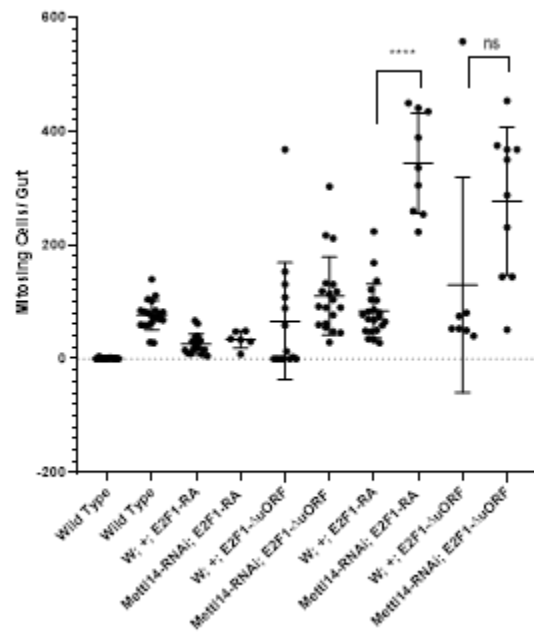


Fig. 7. Knockdown of Mettl14 Promotes ISC Proliferation with Endogenous E2F1 Isoform. Plot shows an individual data point for each gut. Means are shown with horizontal lines and standard deviations are shown with error bars.

The other mRNA modifying gene examined was *fl(2)d*. Fig. 8 shows that knockdown of *fl(2)d* suppressed proliferation upon stress. The effect was more significant with E2F1- Δ uORF ($P < 0.0001$). Knockdown of *fl(2)d* decreased the mean value of dividing cells per gut from nearly 600 to approximately 250. The contrast between the control and knockdown lines was not as large with E2F1-RA. Proliferation was unaffected under healthy conditions.

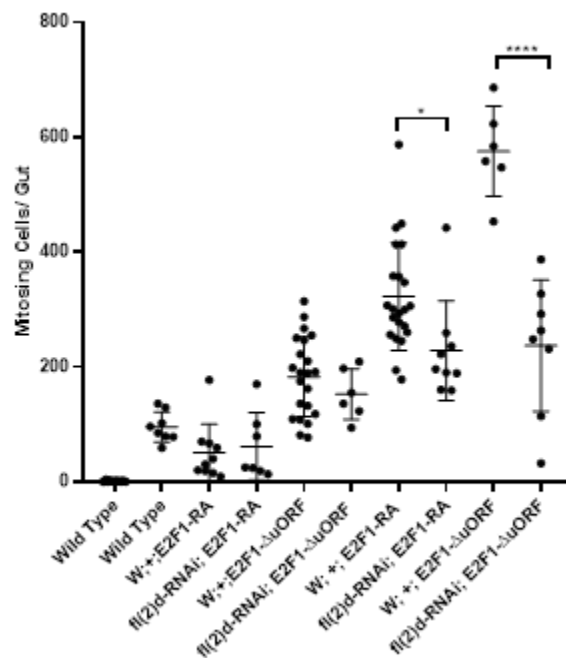


Fig. 8. ISC Proliferation is Suppressed by Knockdown of fl(2)d. Individual values of mitosis-marked cells per gut are shown, means and standard deviations shown as in previous plots.

Discussion

A. Figures and Tables

The loss of control of cell growth and proliferation results in cancer, and E2F1 is a key regulator of these processes. Our aim was to identify likely mechanisms of the post transcriptional regulation of E2F1. Through a panel of RNAi knockdown of target genes, a list of relevant genes was identified. RNAi knockdown was combined with the overexpression of two forms of E2F1, one with the endogenous 5' untranslated region (UTR) and one with the upstream open reading frames (uORFs) in the 5' UTR removed, and the resulting levels of proliferation in *D. melanogaster* midguts were quantified. The knockdown of 4E-BP (Thor), PTEN, eEF2, Mettl14, and fl(2)d all resulted in significant ($P < 0.05$) changes in proliferation. The mechanisms of these growth regulating genes give insight into the regulation of E2F1.

4E-BP (Thor)

Knockdown of Thor resulted in significant ($P < 0.001$ from Fig. 2) increase in proliferation under stressed conditions for both forms of E2F1. Thor is a component of the TOR pathway, and decreases levels of cap-dependent translation during stressed conditions. Translation is hindered by 4E-BP blocking 40S ribosomal subunit from binding to the 5' cap in response to insufficient nutrients [21]. In doing so, 4E-BP inhibits the eIF4F complex [22], which is implicated in malignancy and tumorigenesis [23]. Reducing metabolic rate in response to decreased nutrients is one way an organism can adapt to sub-optimal environments. Because Thor decreases cap dependent translation during stressed conditions, we assumed that knocking down Thor would remove this expected impairment. Our results confirmed this assumption, as proliferation sharply increased without Thor function. Proliferation in the gut is clearly sensitive to Thor expression.

Despite its effect on proliferation under stressed conditions, knockdown of Thor had no significant effect during healthy conditions. Thor was absent in the midgut during healthy conditions, shown in Fig. 3B, explaining why knockdown had no effect. Pathogenic stress induces Thor expression in the gut, shown in Fig. 4B.

PTEN

Knockdown of PTEN significantly increased proliferation under stressed conditions ($P < 0.05$ for E2F1-RA and $P < 0.0001$ for E2F1- Δ uORF, Fig. 5), but not under healthy. PTEN is a known tumor suppressor [24], [25] and is one of the most common mutated or deleted genes in human cancers, second only to p53 [24]-[38]. PTEN is a component of the PI3K-Akt pathway [37] and inhibits PI3K signaling [39]. PI3K can promote proliferation during stressed conditions [40]. PTEN suppresses E2F1-mediated transcription, and the loss of this function is oncogenic [41]. Because the knockdown of PTEN promotes proliferation only during the stress response, and more significantly with E2F1- Δ uORF, these data indicate that PI3K signaling can affect E2F1-mediated transcription only upon stress by a process sensitive to the presence of uORFs.

EEF2

The knockdown of eEF2 inhibited proliferation in response to stress ($P < 0.0001$ for E2F1-RA and $P < 0.05$ for E2F1- Δ uORF, Fig. 6). This elongation factor is a GTPase that works by catalyzing the translocation of peptidyl-tRNA from the A site to the P site on the ribosome [42]. Removing this elongation factor reduces the ability for epithelial cells to divide. It is rational that removing a general translation promoter would decrease a tissue's ability to generate new cells. However, it was unexpected that knockdown of eEF2 did not cause a significant change in proliferation under healthy conditions. This suggests that the level of translation required under

healthy conditions is able to be supplemented by other pathways or mechanisms. Furthermore, the inhibition of proliferation during the stress response was much more significant in the E2F1-RA form ($P < 0.0001$). This finding may indicate that eEF2 acts through some mechanism that is lost by removing the uORFs in the 5' UTR.

Mettl14

The knockdown of *Mettl14* increased the stress response, but only with the E2F1-RA isoform ($P < 0.0001$). *Mettl14* is a component of the methyltransferase complex, which modifies mRNA by methylating adenosine residues at the nitrogen-6 position, creating m6A sites. The transferase complex also contains *Mettl3* and may aggregate with William's Tumor Associated Protein (WTAP) in mammals [20]. Fig. 7 appears to have a significant difference between the E2F1- Δ uORF form and its control, however, the T test returned a P value over 0.05. Such a result was most likely due to the high variance in the sample, which may be due to impurities in the fly line. Because the E2F1-RA isoform returned a significant increase in proliferation upon *Mettl14* knockdown ($P < 0.0001$), it appears that *Mettl14* suppresses proliferation during stress and may rely on mechanisms contained in the uORFs. It is worth mentioning, however, that one data point in the Δ uORF control deviated far from the rest in that sample. There may be some significant change from *Mettl14* knockdown with this form of E2F1, though likely not as significant as with E2F1-RA.

Fl(2)d

Lastly, the knockdown of *fl(2)d* shown in Fig. 8 resulted in significant decrease in proliferation under stressed conditions ($P < 0.05$ for E2F1-RA and $P < 0.0001$ for E2F1- Δ uORF). Although the effect was more significant with the Δ uORF form, both W; *fl(2)d*-RNAi; E2F1-RA and W; *fl(2)d*-RNAi; E2F1- Δ uORF result in similar levels of proliferation under stress. The control, W; +; E2F1- Δ uORF had unusually high proliferation in this sample. This control did not exceed an average of 400 marked cells per gut in any of the previous test. Therefore, it is more likely that this control was exposed to some unintended stress through contaminants in the food, and that the decrease in proliferation is not as drastic.

That being said, the decrease in proliferation was still significant. *Fl(2)d* is the *D. melanogaster* homolog of WTAP [43], and is involved in the methyltransferase complex mentioned in respect to *Mettl14*. *Fl(2)d* is involved in alternative splicing of several proteins like *Sxl* and *Ultrabithorax (Ubx)* [16], [44]. *Ubx* is a transcription factor that directs haltere differentiation in *D. melanogaster* [45]. If one transcription factor can be regulated through alternative splicing by *fl(2)d*, it is reasonable to assume that *fl(2)d* may have additional downstream targets like E2F1.

Throughout each experiment, obtaining adequate sample sizes posed a challenge. While *D. melanogaster* is a strong genetic model due to higher sample sizes than larger models, there is still an element of difficulty in obtaining enough flies of the correct genotype. Because genetic drivers are required to express the knockdown and overexpressed genes, crosses needed to be maintained. With each generation, the parent flies produce fewer offspring. Additionally, to reduce variability in gut viability from aging, flies were collected within a two-day period. This decreased the quantity of adult offspring from the crosses that were available during collection. Of the adult flies that were able to be collected in the shorter time window, not all of the offspring were of the correct phenotype. The flies used in these experiments needed to be over expressing E2F1, indicated by the presence of GFP. If guts from the samples were GFP negative, they were thrown out.

Also, there are inherent inconsistencies with quantification of marked cells. When examining the samples, cell size can vary dramatically. There may also be clusters of marked cells that are

difficult to separate into individual cells. For guts containing over 500 marked cells, many of the cells overlap and are again difficult to differentiate.

The knockdown of Thor, PTEN, and Mettl14 increased proliferation in response to pathogenic stress. These genes are all involved in restraining growth from amplifying out of control. The ratio between Thor and eIF4E has been proposed as a biomarker for the efficacy of mTOR related cancer therapies [46]. By knowing that E2F1 translation is sensitive to levels of Thor, the mechanism of Thor function can be further investigated for more specific biomarkers. Because PTEN appears to inhibit E2F1-mediated translation under stress, alternative methods of inhibiting this translation may be developed to compensate for PTEN mutations or deletions. Mettl14 may be blocking mechanisms of E2F1 translation, and could potentially be used to restrict excess growth.

Of the opposite effect, the knockdown of eEF2 and fl(2)d decreased proliferation during stress. The proliferation induced by overexpression of E2F1 appears more sensitive to eEF2 knockdown when uORFs are present, therefore, it appears that eEF2 is important to overcoming uORFs during translation. Fl(2)d, like Mettl14, promotes the methylation of adenines in RNA. However, knockdown of fl(2)d had the opposite effect of Mettl14, indicating that m6A sites can both promote and repress proliferation. Better understanding of how fl(2)d and eEF2 promote translation can lead to more treatments associated with them.

While this project identified a small list of genes of interest with regards to E2F1 regulation, further studies are required to understand their connections. To further quantify how E2F1 translation is initiated, three new transgenes were established adjacent to this project. Each transgene contains a form of the E2F1 5' UTR, either the endogenous form, the Δ uORF form, or a form with base pairs 414-828 removed. The E2F1 5' UTR is preceded by an RFP coding region, and followed by a GFP coding region. The RFP will serve as a sensor for cap dependent translation and GFP will mark cap independent translation.

These transgenes will be combined with RNAi knockdown of Thor, PTEN, eEF2, Mettl14, and fl(2)d. The RNAi lines will also be combined with another set of transgenes containing the three forms of the E2F1 5' UTR and GFP without RFP. The quantity of fluorescence in the midgut will be quantified by flow cytometry. This future experimentation will determine the quantity of cap dependent translation of E2F1 when combined with the knockdown of the target genes identified in this project.

D. melanogaster is a powerful model for studying the genetics of cancer. There are many tools that may be used with flies, such as the MARCM system to mutate genes of interest [47], the UAS-GAL4 system for targeted gene expression [18], and the RNAi used in this project. Flies are a powerful model for understanding how human diseases function as there are functional homologs in *D. melanogaster* for nearly 77% of human disease-related genes [48].

By identifying genes that affect the translation of E2F1, cancer therapies can be tailored to target these genes and regain control of cell growth. In-depth knowledge of the regulatory pathways that control tissue growth, especially in response to carcinogens, is critical to effective treatment development.

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