Student Responses to RENEWAL Questions

The following apply only for renewal applications. Please consider these responses in making your assessment of the application.

1. Please describe how successful you were in achieving the intended outcomes of and adhering to the plan/timeline of your original proposal.

Mostly successful. The RNAi screen was completed and identified several genes of interest. 4E-BP, fl(2)d, and eEF2 knockdown altered levels of proliferation in the D. melanogaster midgut. These genes will be further examined in this semester's work.

The largest challenge with the previous work was coordinating the crossing of fly lines. Some lines were more proliferative than others, so offspring were ready for testing at different times.

2. Please describe how successful you think your relationship with your mentor was during your first semester of UROP.

It was very successful. Dr. [redacted] and Dr. [redacted] are both willing to guide my experiments, explain concepts to me, and challenge me to produce the best work possible. They are both responsive and approachable.

3. Please explain what you think the impact of an additional semester in UROP would be to your educational and career goals.

An additional semester of UROP will provide me with more lab experience. This will help me further understand genetics and expand my skillset. Lab work has greatly improved my ability to understand what I am learning in my coursework, and to actually apply these techniques to research. My time in this lab has greatly improved my motivation and dedication to science, because I see the direct impact of our work. More time in this lab will help me publish work, which will help me in employment after graduation. I am also hoping to present on capital hill this spring, and another semester of UROP funding will help me create more content to present.

UROP Proposal

Title of Proposal

Examining the Mechanism of Translational Regulation of Transcription Factor E2F1

Problem/Topic of Research or Creative Work

Colorectal cancer is the third leading cause of cancer related deaths in the united states [1]. Cancer arises from uncontrolled cell growth and proliferation. To better understand and treat the development of cancer, a more robust understanding of the mechanisms controlling these processes is required. Many oncogenes, such as target of rapamycin (TOR) [2], Myc [3], and epithelial growth factor receptor (EGFR) [4], are identified regulators of cell growth. The transcription factor, E2F1 is post transcriptionally regulated by TOR and EGFR [4,5]. E2F1 regulates cell cycle progression by driving cells into the DNA synthesis phase [6]. While it is understood that E2F1 is translationally regulated, the precise mechanisms controlling this process are not fully identified.

Translation of proteins is carried out by ribosomes, and may or may not depend on the 5' cap of messenger RNA (mRNA), depending on the mRNA and cellular conditions. It is of great interest

to know if E2F1 translation is dependent on the 5' cap or other regulatory mechanisms that may be contained in the 5' untranslated region (UTR). For example, upstream open reading frames (uORFs) allow the ribosome to initiate translation outside of the main protein coding open reading frame (ORF). Internal ribosomal entry sites (IRES) provide areas for the ribosome to bind to mRNA and bypass parts of the UTR, affecting the translation of the downstream protein coding region [7].

To determine whether E2F1 translation is initiated through a cap-dependent mechanism or via an IRES, three transgenes were established, each containing a different form of the E2F1 5'UTR. The transgenic transcripts all possess an ORF encoding a red fluorescent protein (RFP) at the 5' end, serving as a sensor for cap-dependent translation, followed by E2F1 5'UTR. Downstream of the E2F1 5'UTR, we inserted an ORF encoding green fluorescent protein (GFP). We assume ribosomes detach from transcript as soon as RFP translation is finished, and thus GFP expression is dependent on ribosomes binding downstream of the RFP ORF, within the E2F1 5'UTR. GFP signal thus suggests translation through a cap independent manner, presumably through an IRES. The forms of E2F1 examined are the endogenous isoform, a form with all uORFs removed, and a truncated form with bases 414-828 removed. This area has been identified critical to translation in preliminary studies.

These transgenes are a powerful tool to examine the presence of an IRES, as cap dependent translation will appear red, while cap independent translation will appear green. These transgenes will be used to examine cap- independent translation of E2F1 in several types of tissues under several conditions to identify the environments in which the translation mechanism of E2F1 is altered.

Relevant Background/Literature Review

E2F1 regulates transcription of Cyclin E, which binds and activates cyclin dependent kinase 2 (CDK2), triggering the cell to transition into S phase. This promotes activation of the Cullin-RING ubiquitin ligase complex 4 in conjunction with substrate recognition factor CDT2 (CRL4CDT2), which targets E2F1 for proteasomal degradation. The oscillation of E2F1 is critical to cell cycle regulation. E2F1 is post transcriptionally regulated, as mRNA levels remain constant while protein levels fluctuate. [5]. This regulation is carried out by target of rapamycin (TOR) [2] and epithelial growth factor receptor (EGFR) [4].

Our work last semester identified several genes of interest. Within the TOR pathway is the cap- dependent translation inhibitor, 4E-binding protein 1 (4E-BP1). [8]. 4E-BP blocks eukaryotic translation initiation factor 4G (eIF4G) from binding to eukaryotic translation initiation factor 4E (eIF4E), blocking the initiation of translation [9]. Knockdown of 4E-BP resulted in increased proliferation under stressed conditions. This indicates the E2F1 dependent proliferation in the midgut during stressed conditions is sensitive to 4E-BP expression. Also of interest is the general translation factor, eukaryotic elongation factor 2 (eEF2), a GTPase that promotes the translocation of mRNA-tRNA complexes within the ribosome [10]. Results from last semester show that the knockdown of eEF2 decreases proliferation under stress.

Knockdown of female-lethal(2)d (fl(2)d) also decreased proliferation under stress. Fl(2)d is a required component of female-specific splicing of Sex-lethal (Sxl). [11]. Fl(2)d is the drosophila homolog of Wilms'- Tumor-1-Associated Protein (WTAP) [12]. WTAP forms a complex with methyltransferase like 3 and 14 (METTL3 and METTL14, respectively) that modifies mRNA by N6-adenosine methylation (m6A) [13].

This project also examines components of the mitogen-activated protein kinase (MAPK) pathway

and TOR pathways on cap independent translation. MAPK promotes Ras/Raf signaling, promoting proliferation [14]. We will also examine the Ras homolog enriched in the brain (RHEB), an upstream regulator of TOR [15].

One genetic tool utilized in this project is the Gal4/UAS system. Gal4 is a protein derived from yeast that can be added to the fly genome. An upstream activation sequence (UAS) is paired with a specific gene in one fly line, while its target, Gal4, is kept in another. When UAS and Gal4 are combined by crossing the fly lines, Gal4 activates the target gene by binding to UAS [16]. Another tool used is knockdown by RNAi interference (RNAi). RNAi serves as part of an organism's viral immune response to target foreign RNA for degradation. Double stranded RNA (dsRNA) will be recognized as a foreign genome and can be designed so that digestion by the Dicer enzyme produces small interfering RNAs (siRNAs) that serve as templates to recognize invasive genes. Argonuate protein, part of the RNA-induced silencing complex (RISC) binds the template siRNA. One strand of the siRNA is removed, while the other guides RISC to the targeted mRNA, where Argonuate cleaves the mRNA, silencing the gene. [17].

Pseudomonas entomophilia (p.e.), a gram-negative entomopathogenic bacteria, is one source of stress. As with most other insects, D. melanogaster does not have an adaptive immune system and depends on innate immune system functions such as antimicrobial peptides (AMPs) produced in the fat body and phagocytosis by macrophages. The fly innate immune system is not enough to combat the effects of p.e, however in wild type flies, proliferation increases in response to infection [18].

Lastly, one technology used to quantify these effects is flow cytometry. Flow cytometry analyzes a cell suspension for fluorescence, size, and granularity. The cell suspension is hydrodynamically focused through a cytometer, passing a laser. Fluorescence from the cells is detected, and light scattered off the cells from the laser is also detected and sorted into two categories, forward scatter and side scatter. Forward scatter relates to the size of the cells, while side scatter relates to the granularity. Different cell types will have different FS/SS profiles, allowing the quantification of fluorescence intensity and cell type present in a sample [19].

Specific Activities to be Undertaken and Timeframe for Each Activity

Throughout the semester I will attend weekly lab meetings, continue to review literature, and consult with mentors about my progress.

January 6th: Cross transgenes with w;tubGal4;tubGal80ts.

January 20th - 24th: Collect 3rd instar larvae and activate expression of translational sensors by shifting to 29C. RFP should be present in all larvae. Dissect tissue of choice and purify RNA. Make a cDNA library and run PCR with GFP and RFP primers to ensure both are expressed in each transcript. May also sequence.

January 27th - February 7th: Collect samples of several tissues of interest from RFP+ larvae and from adult flies. Examine these tissues by confocal microscopy to identify a list of tissues expressing GFP (cap independent translation).

February 10th - 14th: Collect adult flies, infect some with p.e. Compare with non-infected.

February 17th - 28th: Quantify differences in GFP expression between endogenous and truncated transcript in tissues displaying GFP. Quantify differences by confocal microscopy and flow cytometry.

March 2nd - 13th: Quantify differences in GFP expression between endogenous and uORF-free transgenes in tissues displaying GFP. Quantify differences by confocal microscopy and flow cytometry.

March 16th - 20th: Cross endogenous transgene with eEF2-RNAi, 4E-BP- RNAi, UASt-4E BP, and fl(2)d - RNAi.
March 20th - April 3rd: Dissect tissues expressing GFP and quantify GFP expression.
April 6th - 10th: Cross endogenous transgene with UASt-RafGOF, UASt-Ras, UASt - Rheb. April 10th - 24th: Dissect tissues that do not normally express GFP and quantify GFP expression.

Relationship of the Proposed Work to the Expertise of the Faculty Mentor

Dr. [redacted] has been working with D. melanogaster for about 33 years. Dr. [redacted]'s focuses have been on cancer, cell cycle, and developmental biology, genetics, and cell growth and proliferation. His lab focuses both on the mechanisms of cell growth and proliferation as well as epithelial self-renewal in drosophila intestine. The proposed project focuses on the signals used when cells transition from G1 to S phase, part of the cell growth and proliferation focuses.
Dr. [redacted] is a Post-Doctoral Fellow in the [redacted] lab. His research focuses on regulation of the cell cycle. Dr. [redacted] completed his PhD on a cell cycle project using a marine urochordate as a model organism. Dr. [redacted] now investigates G1/S transition in D. melanogaster, and has also published a review article on how polyploidy contributes to wound healing in mammalians and insects.

Relationship of the Proposed Work to Student's Future Goals

This spring, I will be completing my final semester of undergraduate studies in Biomedical Engineering. With this degree, I aim to study tissue and genetic engineering. I had not planned on a future in genetics until I worked in this lab. Realizing the vast amount of tools and focus areas within genetic engineering has helped me realize how well I fit in this field. I also want to work with tissue engineering because of its great potential for advancement. Combining genetic engineering with tissue engineering may lead to powerful technologies and medical advancements. The ability to grow and reproduce healthy mammalian cells is critical to the field of regenerative medicine. Understanding the signaling pathways that drive cell proliferation may help develop more effective methods by which cells can be grown in culture, whole tissues can be grown in vitro, and eventually how artificially manufactured tissues can function in the body.
Working in the [redacted] lab has provided me with priceless experience in benchwork, genetics, and molecular biology. While my coursework covers a wide variety of theory behind biological research methods, working here has given me the hands-on experience and guidance needed to fully understand them. In my time spent here, I have learned to design primers for and perform polymerase chain reaction (PCR), perform Western Blots, quantitative or reverse transcriptase PCR, immunostaining, gel electrophoresis, and statistical analysis of data. I have gained experience using a variety of microscopes, such as dissecting scopes and fluorescent scopes. I have also gained experience in scientific writing, applying for funding, presentation design, and the peer review process that will greatly help me in my future career.
I plan on continuing my education into graduate school, starting with a combined BS/MS degree, then potentially a PhD. By attending weekly lab meetings, I have seen excellent presentations and from those I am learning how to present scientific information. I have also been able to participate in questioning the presenter throughout their presentation to both get a taste for the intensity of a thesis dissertation, and to experience the level of attention to detail required in peer reviewed research. The proposed work would give me the opportunity to see a project through from development to presentation. This would be an incredibly valuable

experience to prepare me for graduate school and a possible career in research.

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