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Dynamic regulation of mRNA decay during *Drosophila* neural development

A Thesis submitted in partial satisfaction of the requirements for the degree of Masters of Science

in

Quantitative and Systems Biology

by

Maxine C. Umeh

Committee in charge:

Assistant Professor Michael D. Cleary, Chair
Assistant Professor David Ardell
Assistant Professor Fred Wolf

2013
The Thesis of Maxine C. Umeh is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, Merced

2013
DEDICATION

First and foremost I dedicate this to God. He is my strength in times of weakness, my joy in times of sorrow, and my peace in times of turmoil. Without Him I would not be who I am or where I am.

Secondly, I dedicate this to my husband, my mother, my family, and friends in recognition of their enduring love and constant support. They are my beautiful inspiration, and unrelenting encouragement.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>viii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xii</td>
</tr>
</tbody>
</table>

## CHAPTER 1: INTRODUCTION

OVERVIEW OF mRNA DECAY ................................................................. 1

- mRNA DECAY PATHWAYS AND MACHINERY ................................. 2
- PROCESSING BODIES AND STRESS GRANULES ............................ 5

REGULATION OF mRNA DECAY .......................................................... 5

- CIS-ACTING ELEMENTS: SECONDARY STRUCTURE ............... 5
- CIS-ACTING ELEMENTS: SEQUENCE .................................... 7
- TRANS-ACTING ELEMENTS: RNA-BINDING PROTEINS ........... 9
- TRANS-ACTING ELEMENTS: MICRO-RNAs ......................... 15
- ARE-mRNAs: AN INTERPLAY BETWEEN AUBPS AND miRNAs .... 16

IMPORTANCE OF mRNA DECAY .......................................................... 20

- mRNA DECAY IN NERVOUS SYSTEM DEVELOPMENT .............. 20
- RBPs IN NERVOUS SYSTEM DEVELOPMENT ......................... 21
- miRNAs IN NERVOUS SYSTEM DEVELOPMENT ..................... 21
- EXTENDED 3’UTRs IN NERVOUS SYSTEM DEVELOPMENT ....... 23

BIOLOGICAL SIGNIFICANCE OF mRNA DECAY ......................... 25

- AUBPs IN HEALTH AND DISEASE ........................................ 25
- miRNAs IN HEALTH AND DISEASE ...................................... 26
- REGULATION OF ARE-mRNAs IN HEALTH AND DISEASE ....... 27
PREVIOUS STUDIES OF mRNA DECAY KINETICS ................................. 30

LIMITATIONS: USE OF TRANSCRIPTIONAL INHIBITOR .................. 32

LIMITATIONS: LACK OF CELL-TYPE SPECIFICITY ............................ 32

TU-TAGGING: A METHOD FOR MEASURING CELL-TYPE SPECIFIC mRNA DECAY .......................................................... 34

RESEARCH AIMS ........................................................................... 35

CHAPTER 2: METHODS

FLY STOCKS .................................................................................. 36

4sUd / 4TU TREATMENT AND RNA EXTRACTION ......................... 36

PURIFICATION OF TU-TAGGED mRNA ........................................... 37

MICROARRAY ANALYSIS ................................................................ 37

cRNA SYNTHESIS, CY DYE LABELING, AND HYBRIDIZATION .......... 37

SPIKE-IN CONTROLS, NORMALIZATION, AND QUALITY CONTROL .......................................................... 38

CALCULATION OF mRNA DECAY KINETICS AND TRANSCRIPT HALF-LIFE .......................................................... 39

CHAPTER 3: RESULTS

4-THIOURIDINE PULSE-CHASE ALLOWS GLOBAL mRNA DECAY MEASUREMENTS IN INTACT DROSOPHILA EMBRYOS .................. 40

mRNAs EXHIBIT NEURAL-SPECIFIC DECAY KINETICS .................. 42

NEURAL mRNA STABILITY CORRELATES WITH LOCALIZATION AND FUNCTION ........................................................................ 44

IDENTIFICATION OF AU-RICH ELEMENTS ENRICHED IN DISTINCT NEURAL mRNA DECAY CLASSES .................................................. 47
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A:</td>
<td>DEADENYLATION DEPENDENT DECAY</td>
</tr>
<tr>
<td>1B:</td>
<td>DEADENYLATION INDEPENDENT DECAY</td>
</tr>
<tr>
<td>1C:</td>
<td>ENDONUCLEASE MEDIATED DECAY</td>
</tr>
<tr>
<td>2:</td>
<td>CIS-ELEMENTS THAT AFFECT mRNA STABILITY</td>
</tr>
<tr>
<td>3A:</td>
<td>AU-RICH BINDING PROTEINS</td>
</tr>
<tr>
<td>3B:</td>
<td>RECRUITMENT OF AUBPs TO ARE-mRNAs</td>
</tr>
<tr>
<td>4A:</td>
<td>CYTOKINE mRNAs WITH miRNAs AND ARE BINDING SITES</td>
</tr>
<tr>
<td>4B:</td>
<td>NETWORK OF TRANS-ACTING FACTORS THAT REGULATE CYTOKINE mRNA EXPRESSION</td>
</tr>
<tr>
<td>5:</td>
<td>miRNAs and AUBPs in HEALTH AND DISEASE</td>
</tr>
<tr>
<td>6A:</td>
<td>TU-TAGGING CHEMISTRY</td>
</tr>
<tr>
<td>6B:</td>
<td>SPATIAL AND TEMPORAL SPECIFICITY</td>
</tr>
<tr>
<td>7:</td>
<td>NORMALIZATION OF MICROARRAYS</td>
</tr>
<tr>
<td>8A:</td>
<td>PULSE-CHASE DESIGN</td>
</tr>
<tr>
<td>8B:</td>
<td>ACT D TREATMENT</td>
</tr>
<tr>
<td>8C:</td>
<td>GLOBAL DISTRIBUTION OF mRNA HL</td>
</tr>
<tr>
<td>8D:</td>
<td>FOLD CHANGE OF INDIVIDUAL mRNA HL</td>
</tr>
<tr>
<td>8E:</td>
<td>mRNAs IN DISTINCT FUNCTIONAL CLASSES</td>
</tr>
<tr>
<td>9:</td>
<td>NEURAL-SPECIFIC DECAY KINETICS</td>
</tr>
</tbody>
</table>
9A: GLOBAL DISTRIBUTION OF mRNA HL IN WHOLE EMBRYO VERSUS NEURAL-SPECIFIC ........................................ 43
9B: FOLD CHANGE OF INDIVIDUAL mRNA HL IN WHOLE EMBRYO VERSUS NEURAL-SPECIFIC .............................. 43
9C: GENE ONTOLOGY OF WHOLE EMBRYO ................................................................. 44
9D: GENE ONTOLOGY OF NEURAL-SPECIFIC .......................................................... 44

FIGURE 10: STABILITY CORRELATES WITH LOCALIZATION AND FUNCTION
10A: STABILITY CORRELATES WITH LOCALIZATION AND FUNCTION ...... 45
10B: ODDS RATIO ANALYSIS OF LOCALIZATION AND FUNCTION ........... 46

FIGURE 11: AU-RICH ELEMENTS IN DISTINCT DECAY CLASSES
11A: ODDS RATIO ANALYSIS OF AU-RICH ELEMENTS ................................. 49
11B: GENE VENN DIAGRAM OF ARE GROUP 5 ................................................. 49
11C: GENE VENN DIAGRAM OF EXTENDED 3’UTRs ........................................ 50

FIGURE 12: ACT D TREATMENT STABILIZES mRNAs ........................................... 53

FIGURE 13: LOCALIZATION AND FUNCTION KNOWN NEURAL mRNAs .......... 56

FIGURE 14: LOW STABILITY mRNAs DETERMINE CELL FATE ......................... 57

SUPPLEMENTAL FIGURE 1: AREs IN HIGH AND LOW STABILITY mRNAs
1A: GENE VENN DIAGRAM OF AREs ................................................................. 65
1B: GENE VENN DIAGRAM OF ARE-LOCAL .................................................... 65
1C: GENE VENN DIAGRAM OF ARE-CELL FATE ............................................. 66
1D: GENE VENN DIAGRAM OF ARE GROUP 1 ................................................. 66
1E: GENE VENN DIAGRAM OF ARE GROUP 2 ................................................. 67
1F: GENE VENN DIAGRAM OF ARE GROUP 3 ................................................. 67
1G: GENE VENN DIAGRAM OF ARE GROUP 4 ................................................. 68
1H: GENE VENN DIAGRAM OF ARE-miR-315 ................................................ 68
LIST OF TABLES

TABLE 1: AU-RICH ELEMENTS ............................................................... 9
TABLE 2: REGULATION BY miRNAs AND AUBPs ........................................ 18
TABLE 3: NERVOUS SYSTEM t_{1/2} > WHOLE EMBRYO t_{1/2} .......................... 43
TABLE 4: NERVOUS SYSTEM t_{1/2} < WHOLE EMBRYO t_{1/2} .......................... 43
TABLE 5: SYNAPTIC PROTEINS (LOCALLY TRANSLATED) ............................. 46
TABLE 6: CELL FATE DETERMINANTS ....................................................... 46
TABLE 7: HIGH STABILITY 3'UTR EXTENDED mRNAs ................................. 50
TABLE 8: LOW STABILITY 3'UTR EXTENDED mRNAs .................................. 51
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ABSTRACT OF THESIS

Models of gene expression during development traditionally focus on the regulation of mRNA transcription. However, an essential level of control occurs via mRNA decay. mRNA decay is likely to be important during nervous system development, where the structure of neurons requires localized translation of mRNAs far from their site of synthesis and the generation of cellular diversity requires rapid turnover of mRNAs that regulate proliferation and differentiation. The study of mRNA decay during embryonic development has previously been hindered by the lack of methods allowing in vivo, cell type-specific measurements of transcript stability. We have developed a technique, called TU-decay that overcomes this technical challenge and allows neural-specific, genome-wide measurements of mRNA decay in intact Drosophila embryos. This technique provides the foundation for a systems-level approach that we are using to construct a neural development mRNA decay network. Our comparisons of whole embryo and neural-specific mRNA half-lives have identified mRNAs that are selectively stabilized or destabilized in the nervous system. TU-decay analysis has also revealed transcript decay kinetics that correlate with the function of the encoded protein. For example, mRNAs that are known to be translated within axon growth cones or dendrites have long half-lives while mRNAs encoding signaling proteins and transcription factors that regulate cell fate decisions have short half-lives. AU-rich element (ARE) containing transcripts were analyzed to investigate the role of known cis-regulatory elements in determining neural mRNA stability. Examples of both low and high stability ARE-containing mRNAs were identified in this analysis. Also, this analysis provided evidence that other mRNA sequence features, including micro-RNA binding sites and alternative polyadenylation, may have combinatorial effects in determining the stability of ARE-containing mRNAs. This work lays the foundation for future analyses aimed at generating a comprehensive and predictive network map of neural mRNA decay dynamics, thus filling a significant gap in current models of gene expression during neural development.
CHAPTER 1: INTRODUCTION

OVERVIEW OF mRNA DECAY

Microarray analyses reveal that approximately 40-50% of changes in gene expression can be ascribed to altered rates of decay; and thus altered levels of mRNA stability (1,54,68). mRNA decay rates often change in response to external stimuli or cellular signals (5). Since mRNAs exhibit a wide range of differing stabilities this allows for rapid increases or decreases in mRNA abundance to properly meet the cell's need for specific proteins over short periods of time (1,5,68). When these rapid changes coincide with elevated mRNA synthesis and/or translation massive changes in gene expression are observed (1,68).

Steady-state levels of mRNA are determined by the balance between mRNA synthesis and mRNA decay. A delicate interplay of the rates of mRNA synthesis, splicing, transport, translation, and degradation determine the abundance of mRNA transcripts, and thus the amount of protein available to the cell (2,4,6,15,21). The rate at which genes are transcribed is called the transcription rate ($k_t$). The rate at which genes decay is called the decay rate ($k_d$). The change in mRNA abundance over time is measured as the rate of transcription minus the rate of decay. Indicating that the lifetime of an mRNA transcript is dependent on both synthesis and decay.

$$\frac{[\text{mRNA}]}{dt} = k_t - k_d [\text{mRNA}]$$

In most cases, $k_d$ is often replaced by a half-life (HL or $t_{1/2}$) measurement for a transcript and can be used to calculate mRNA stability. Half-life is the time required to reduce the mRNA concentration of a specific transcript to half of its original value.

$$\text{HL} = \frac{\ln(2)}{k_d}$$

Transcript half-life is inversely proportional to the decay rate constant. Thus, a transcript with a short half-life has a high rate of decay and exhibits faster changes in mRNA concentration; whereas a transcript with a long half-life has a low rate of decay and exhibits slower changes in mRNA concentration (4).

mRNA half-life has been found to vary greatly across eukaryotes. For example, in *Saccharomyces cerevisiae* mRNA half-lives range between 3 – 60 minutes, while half-lives in vertebrates, such as, *Xenopus*, and mice range between 20 minutes – 24 hours. Such variation in mRNA half-life, and thus stability, is caused by variations in mRNA decay rates. mRNA decay rates are determined by a wide range of stimuli and cellular signals, such as hormones, iron levels, cell cycle progression, cellular differentiation, and viral infection (6). mRNA decay rate is also affected by cellular stress caused by ultraviolet light exposure, heat shock, and glucose and amino acid starvation (6, 50). However, across all species there is an evolutionarily conserved link between mRNA decay and gene function supporting the idea that mRNA degradation plays an important role in cellular functions (50). Maintaining this idea, mRNA decay has been shown to regulate many cellular responses such as immune response, inflammation, cellular
stress, and cancer pathogenesis; and alterations in mRNA stability can lead to cancer, heart disease, and immune disorders (2,15).

**mRNA DECAY PATHWAYS AND MACHINERY**

Eukaryotic mRNAs are created with two fundamental stability determinants: 5’-methylguanosine cap and the 3’ poly(A)-tail, which interact with the cytoplasmic cap binding complex eIF4E and the poly(A)-binding protein (PABP), respectively; to protect the transcript from exonucleolytic attack and to enhance translation initiation. In order to initiate degradation either one of these two structures must be compromised by exonucleases, or the transcript body must be cleaved internally by endonucleolytic attack (1,65). Since an individual mRNA transcript can simultaneously be a substrate for multiple decay pathways, mRNA stability is a result of the summation of decay rates through each individual pathway (3,5). Therefore, differences in mRNA half-life specifically result from differences in rates of deadenylation, decapping, 5’ – 3’ or 3’ – 5’ exonuclease, and endonuclease activity (3,5).

In eukaryotes, 5’ – 3’ decay is initiated by the removal of the 5’ cap by DCP1/DCP2 decapping complex. The process of decapping requires the replacement of eIF4E with DCP1/DCP2. Since eIF4E promotes translation initiation and efficiency, decapping activity competes with translation, thus playing a role in regulation of gene expression through decay. Once the 5’ cap is removed the mRNA body is degraded in the 5’ – 3’ direction by XRN1 exoribonuclease. XRN1 is highly conserved in all eukaryotes. Mutations in *Drosophila XRN1* show defects in specific tissues during development (4). This suggests that XRN1 regulates the degradation of a specific subset of mRNAs.

In contrast, in eukaryotes, 3’ – 5’ decay is initiated by deadenylation, or loss of the poly(A) tail. Several eukaryotic deadenylases have been characterized including PAN2-PAN3, CCR4-NOT, and PARN (poly(A)-specific ribonuclease) (1,4). PAN2-PAN3 carries out the initial shortening of the poly(A)-tail (1). CCR4-NOT consists of nine protein subunits and is the main deadenylase in eukaryotes (1,5). PARN is unique in that it has cap-dependent deadenylase activity such that its efficiency is enhanced by the presence of the 5’ cap, and has been implicated in the mass deadenylation of maternal mRNAs in *Xenopus oocytes* (1). Once the poly-(A)-tail is eliminated, the now unprotected 3’ end of mRNA is attacked by a large complex of 3’ – 5’ exonucleases known as the exosome. Following the decay of the mRNA body the 5’ cap is metabolized by the scavenger-decapping enzyme DcpS (1,3,4). Although, exonucleolytic and endonucleolytic cleavage activities exists in all organisms, decapping and deadenylation are specific to eukaryotic mRNAs and may be a conserved decay mechanism (3,4). (Figure 1A)
Deadenylation-independent decay does occur; examples of this mechanism have been found in *S. cerevisiae* Rps28b and Edc3 mRNAs. In this case, structural characteristics of the mRNA transcript may prevent deadenylases from accessing the poly(A)-tail. An enhancer of decapping, *Edc1* or *Edc3*, recruits DCP1/DCP2 to eliminate the 5' cap, followed by 5'–3' exonucleolytic decay by XRN1 (1). (Figure 1B)

**Figure 1A. Deadenylation-Dependent Decay.** 5' – 3' decay (left) and 3' – 5' decay (right). Figure adapted from Gameau et al. *Nature Reviews Molecular Cell Biology* (2007)

**Figure 1B. Deadenylation Independent Decay.** Figure adapted from Gameau et al. *Nature Reviews Molecular Cell Biology* (2007)
In eukaryotes, the bulk of mRNAs undergo decay that is initiated by poly(A)-tail shortening (1,4). This indicates the importance of the poly(A)-tail in protecting the mRNA body from exonucleolytic attack. Physical interaction between the 5’ cap and 3’ poly(A)-tail allows the presence, or absence, of the poly(A)-tail to directly influence mRNA degradation. For example, the poly(A)-tail may inhibit decapping activity by forming or stabilizing the circular structure of mRNA through its interaction with eIF4E. In contrast, the absence of the poly(A)-tail has been shown to promote decapping. In *S. cerevisiae*, deadenylation to an oligo(A)-tail allows mRNA to become a substrate for decapping enzymes, thus promoting 5’–3’ exonucleolytic decay. Also, since mRNAs interact with the cytoskeleton through their poly(A)-tails, deadenylation may alter the subcellular localization of an mRNA; exposing it to localized decapping enzymes (3). In *S. cerevisiae*, the length of the poly(A)-tail has also been shown to influence decay activity, such that mRNAs that exhibit similar decay kinetics bear similar tail lengths (4). This indicates the critical role of the poly(A)-tail in gene expression control by regulation of mRNA decay.

In addition to 5’–3’ and 3’–5’ exonucleolytic decay, mRNA transcripts can also be decayed by endonucleolytic cleavage (1,3,4). Several endonucleases have been characterized: PMR1, IRE1, and RNase MRP (1). Endonucleolytic decay produces two fragments, which are susceptible to exonucleases, making it the most efficient means of degrading mRNA. The upstream mRNA fragment is degraded by the exosome, while the downstream mRNA fragment is degraded by XRN1. Due to their potency, endonucleases often exhibit high selectivity for mRNA targets and/or are highly regulated. (Figure 1C)

![Figure 1C. Endonuclease Mediated Decay](image)

In *S. cerevisiae*, knocking out components of either 5’–3’ or 3’–5’ decay pathways had minimal effects on the transcriptome implying functional redundancy of decay factors (1). However, decay factors like XRN1 and endonucleolytic factors, confirm the possibility that decay machinery is recruited to specific mRNAs targets and/or shows restricted spatial and temporal expression patterns. These findings imply the presence of a protective mechanism used by the transcriptome to ensure efficient large-scale mRNA degradation, while still maintaining spatial- and temporal-specific decay. Both forms of decay coincide to ensure proper cellular development and maintenance. Thus, all enzymes and modulating factors are putative targets for regulation of gene expression through mRNA stability.
PROCESSING BODIES AND STRESS GRANULES

Components of mRNA decay pathways are enriched in granular cytoplasmic foci known as Processing bodies (P bodies). Studies of decay kinetics currently propose P bodies as sites of cellular decay. Factors involved in deadenylation, 5'–3' exonucleolytic decay, exosome machinery, nonsense-mediated decay, and microRNA-mediated decay have been shown to colocalize to P bodies (1). Although the complete function of P bodies has not yet been characterized, what is known is that P bodies play an integral role in increasing the efficiency of mRNA decay. P bodies are thought to locally concentrate decay machinery in the cytoplasm, which simultaneously depletes decay machinery from other “non-decay” regions of the cytoplasm and enhances mRNA decay kinetics.

P bodies assemble when the 5'–3' exonucleolytic decay pathway is overloaded with mRNA substrates. P bodies also substantially decrease in size and number, or disappear when mRNA decay substrates are reduced (1,5). These findings coincide with the current model of P bodies not only localizing decay machinery, but also depleting decay-bound mRNAs from competing for translational machinery. Sequestration of mRNAs in P bodies may be a way to segregate mRNAs targeted for degradation but cannot be immediately degraded; and/or ensure that these mRNAs do not reassocia with ribosomes and generate aberrant proteins (1,4,5).

REGULATION OF mRNA DECAY

The regulation of mRNA decay is a major point of control in gene expression. Regulation of mRNA stability is a controlled interplay between the features of the mRNA transcript, such as sequence and secondary structure, often referred to as cis-elements; and diffusible trans factors, such as RNA binding proteins (RBPs) and microRNAs (miRNAs) that interact with mRNA to influence its fate (4,21,65,67). Both cis-elements and trans factors can regulate mRNA stability in a general and/or mRNA-specific manner. These diverse multi-layered networks of mRNA decay regulation help establish and maintain proper gene expression levels across the organism.

CIS-ACTING ELEMENTS: SECONDARY STRUCTURE

A subset of mRNA transcripts form defined stem-loop structures in UTRs, which are recognized by RBPs, and can either lead to rapid degradation or stabilization of the mRNA transcript (2). For example, the presence of stem-loop structures in either the 3' or 5' UTRs can protect mRNAs from exonucleolytic digestion; and deletion of intercistronic stem-loops has been shown to significantly reduce the half-life of long-lived mRNAs (21).

One well characterized example of RBPs mediating decay by binding to mRNA secondary structures is stem-loops formed in the 3’UTRs of mRNA transcripts, often those containing AU-rich element (AREs). Alterations in local mRNA secondary structure
may function as potent regulators of ARE-directed decay kinetics by modulating RBPs selectivity (23). For example, almost all mRNAs reported to be targets of the HuR RBP contain a 17-20 base-long motif rich in uracils that forms a stem-loop structure in the 3'UTR that is recognized by HuR. This specific secondary structure motif is conserved in more than 50% of human and mouse genes. A recent study by Chang et al. of mRNA decay in IDH4 and HeLa cells showed that p16 mRNA regulation is dependent on a stem-loop structure present in the 3'UTR of the transcript. This secondary structure is recognized by HuR, and simultaneous binding of HuR and AUF1 recruits the RNA-induced silencing complex (RISC) to initiate degradation of p16 mRNA (22). Similar to p16, TNFα mRNA also forms a stem-loop structure in the ARE region of the 3'UTR. However, the ability for TNFα ARE to fold into a hairpin secondary structure inhibits the binding of AUF1 RBP and thus stabilizes the mRNA (23). In S. cerevisiae, the presence of a large U-rich stem-loop within an AU-rich 3'UTR increased mRNA stability (t_{1/2} = 60 minutes); whereas a short AU-rich stem-loop in the 3'UTR decreased mRNA stability (t_{1/2} = 6 minutes). This dramatic difference in half-life between large and short stem-loops is due to the fact that a large U-rich stem loop is selectively bound by Poly(U)BP promoting mRNA stabilization, while a short AU-rich stem-loop is selectively bound by Puf RBP which targets the mRNA for rapid degradation (67).

In addition to the 3'UTR, secondary stem-loop structures are also found in the 5'UTR, and influence mRNA stability. For example, p21 mRNA contains a stem-loop structure in the 5'UTR. Competitive binding of RBPs CUG triplet repeat RNA binding protein 1 (CUGBP1) and Calreticulin (CRT) alters the fate of p21 translation. CUGBP1 promotes p21 translation, whereas CRT inhibits p21 translation by stabilizing the presence of the stem-loop in the 5'UTR (22). Structural motifs in the 5'UTR regulate mRNA localization, in addition to mRNA degradation. These motifs may be recognized and bound by RBPs involved in mRNA trafficking. Rabani et al. identified a stem-loop motif in the 5'UTR of 97 dendrite-localized mouse hippocampal mRNAs, suggesting that this structural motif may be involved in localization of these transcripts to dendrites. This may prove to be an important feature of dendritic mRNAs since localization of mRNAs is particularly important in neurons where the plasticity of synaptic connections requires local changes in gene expression (67).

Some secondary structural RNA motifs, such as Iron responsive element (IRE), are present in both the 3' and 5' UTR of mRNA transcripts involved in iron metabolism and transport. ferritin mRNA, which encodes an iron storage protein, contains one IRE in its 5'UTR. When iron concentration is low, RBPs Iron Response Proteins (IRPs) bind the IRE of ferritin mRNA leading to reduced translation rates; thus, inhibiting the further storage of iron. In contrast, binding of IRPs to multiple IREs present in the 3'UTR of transferrin mRNA, involved in iron acquisition, leads to increased mRNA stability and more iron production. The binding of RBPs to the stem-loop IRE affects the translation rate of multiple mRNAs, thereby coordinating the response of the transcriptome to changing levels of iron (67).

Although many RBPs recognize and bind to a wide variety of RNA motifs, a subset of RBPs preferentially recognize and bind higher order structures. For example, RBP heterogeneous nuclear ribonucleoprotein A2/B1 (HNRPA2B1) specifically binds stem-loop structures in many mRNA transcripts and promotes stabilization. HNRPA2B1
is a member of a larger family of A/B hnRNPs, which have established roles as mRNA stabilizers through binding terminal stem-loops (25).

Another well-characterized RBP that binds to mRNA secondary structures is Staufen (STAU1). Staufen is a double stranded RBP that recognizes, and binds with high affinity, to secondary structures within double stranded mRNA. Staufen plays a role in mRNA localization, translation, and turnover. For example, STAU1 RBP is responsible for efficient localization of *prospero* mRNA during neuroblast division. During *Drosophila* embryonic CNS development neural stem cells, called neuroblasts (NB), divide asymmetrically to produce another neuroblast and a ganglion mother cell (GMC), which terminally divides to produce two neurons. During neuroblast division transcription factor *prospero* is localized to the basal cortex and segregates exclusively into GMCs, where *prospero* translocates to the nucleus and establishes differential expression between NBs and GMCs. (46,80). In mammals STAU1 and STAU2 are present in dendrites and may facilitate mRNA transport for local translation at synaptic terminals, suggesting that Staufen plays an important role in neuron plasticity, learning, and memory. Staufen is an important regulatory component of P bodies and degrades transcripts by recruitment of NMD factor UPF1 (45,46). Staufen also has the ability to bind to single stranded RNA through associations with other RBPs. For example, STAU1 associates with RBPs TDP-43 and FMRP to regulate the expression of Sirtuin (Sirt1) mRNA. Sirt1 functions in double stranded DNA break repairs. Thus, depletion of the STAU1/FMRP/TDP-43 RBP complex causes neuronal cells to undergo DNA damage, and apoptosis; and may be an underlying mechanism of neurodegenerative disease. Single stranded RNA may aggregate to form secondary/tertiary structures allowing Staufen binding. For example, *Drosophila bicoid* mRNA contains a 3’UTR that dimerizes to form higher order mRNA structures that are bound by Staufen (45,46,47).

Regulation of mRNA decay through binding of RBPs to mRNA secondary structure has also been found to play an important role in mechanisms of disease. One profound example of this is in Human B-precursor leukemia. This leukemia is caused by a splicing defect that leads to the deletion of exon 12 in CD22. Aberrant CD22 mRNA gives rise to a truncating frameshift mutation that causes CD22 mRNA to fail to form proper secondary structures, which are target motifs for RBPs hnRNP-L, Polypyrimidine Tract Binding Protein (PTB), and Poly(C)-Binding Protein (PCBP). Dysregulation of mutant CD22 mRNA is found in infant leukemia cells and perturbs B-cell development in transgenic mice. Lack of proper regulation of CD22 mRNA also causes reduced expression of several tumor suppressor genes, suggesting that regulation of mRNAs through RBP binding to structural motifs is an important mechanism for proper control of gene expression, and protection against disease (24).

**CIS-ACTING ELEMENTS: SEQUENCE**

In contrast to the structural elements found in mRNAs, other cis-elements that regulate decay have little to no structural features, and appear to rely on their primary sequence, rather than their secondary structure, in determining their interaction with regulatory RBPs (2). Sequence elements that affect mRNA stability are numerous and vary in location, across the 5’ UTR, coding region, and 3’UTR. Some decay inducing elements are ubiquitously expressed, while others mediate mRNA or cell-type specific
degradation (39). Sequence elements in the 5’ and 3’ UTRs of mRNAs regulate export of mRNAs from the nucleus, translation efficiency, and subcellular localization, in addition to regulating mRNA turnover and decay kinetics (4). (Figure 2)

The most comprehensively characterized cis-acting element that controls mRNA decay is the AU-rich element (ARE) (2). Approximately 5-9% of mRNAs contain an ARE sequence, and 16% of human protein-coding genes contain an ARE in their 3’UTR (81). ARE-containing genes show 75% evolutionary conservation across organisms (4,5,27,48). AREs are found in the 3’UTR of many transcripts that encode cytokines, interleukins, proto-oncogenes, such as c-fos, c-jun, and c-myc, inflammatory factors like TNFα, IL-1β, and IL-3, growth factors including cyclins and Cdk inhibitors, and transcription factors. ARE-containing transcripts also encode proteins required for cell adhesion, growth and differentiation, apoptosis, immune responses, and intercellular signaling (1,15,26).

The first evidence that AREs function as potent mRNA destabilizing elements can from studies in which the 51 nucleotide ARE from Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) mRNA was inserted into the 3’UTR of β-globin mRNA, this caused otherwise stable β-globin mRNA to be rapidly decayed (26,39). This was followed up by the finding that deletion of the ARE from the c-fos 3’UTR results in the conversion of c-fos mRNA from a proto-oncogene to an oncogene, indicating that the ARE plays an important role in regulation of transcripts, as well as, protecting the transcriptome from mRNAs that lack regulation (26). Later studies proved that the presence of AREs alone can recruit the exosome to carry out rapid degradation of the ARE-containing transcript, further strengthening the model in which AREs directly modulate stability and translation of a large group of essential mRNA transcripts (1,5,31). A well-studied example of this is in Drosophila in which ARE-mediated decay regulates both temporal and spatial gene expression, and is critical for proper development (27).

AREs are characterized into three main classes based on the number and context of the AUUUA pentamer. Class I AREs contain 1-3 interspersed copies of the AUUUA motif in the context of a U-rich region. Class I AREs can be found in the 3’UTR of proto-oncogenes such as c-myc and c-fos. Class II AREs contain 5-8 tandem overlapping repeats of the (AUUUA),A sequence, and located in a variety of cytokine
mRNAs including GM-CSF and TNFα. c-jun proto-oncogene contains a class III ARE, which is characterized by the lack of the AUUUA pentamer motif, but rather, contains U-rich sequences in the 3'UTR (2,5,15,27). (Table 1)

<table>
<thead>
<tr>
<th>Class</th>
<th>Motif</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>WAUUAUW and a U-rich region</td>
<td>c-fos, c-myc</td>
</tr>
<tr>
<td>II - Group 1</td>
<td>AUUUAUUUAUUUAUUUAUUAUUA</td>
<td>GM-CSF, TNF-α</td>
</tr>
<tr>
<td>II - Group 2</td>
<td>AUUUAUUUAUUUAUUAUUA</td>
<td>Interferon-α</td>
</tr>
<tr>
<td>II - Group 3</td>
<td>WAUUAUUAUUUAUUAW</td>
<td>cx-2, IL-2, VEGF</td>
</tr>
<tr>
<td>II - Group 4</td>
<td>WWUAUUAUUUAUAW</td>
<td>FGF2</td>
</tr>
<tr>
<td>II - Group 5</td>
<td>WWWVAUUUAUWW</td>
<td>u-PA receptor</td>
</tr>
<tr>
<td>III</td>
<td>U-rich, non-AUUUA</td>
<td>c-jun</td>
</tr>
</tbody>
</table>

Table 1. AU-Rich Elements
AU-rich element classes I, II, and III. Class II consists of 5 subgroups differentiated based upon number of AUUUA tandem repeats. Examples of genes containing each AU-rich element motif.

No two AREs are identical. Even when ARE sequences are similar, the flanking sequences can influence mRNA stability. Spacing between AU-rich motifs also regulates in vivo decay kinetics since space between motifs modulates the binding of various sets of RBPs (1,26,27). Studies in Drosophila indicate that all three classes of AREs participate in degradation through poly(A) shortening and deadenylation. Other experiments, studying specific ARE-containing mRNAs, such as c-fos, propose a model in which AUUUA motifs (class I/II) facilitate degradation of the mRNA body, while the U-rich motifs (class III) promotes deadenylation which then enhances destabilization by the AUUUA motif (26,27). Although AREs are powerful determinants of destabilization, only 25-30% of ARE-containing transcripts exhibit rapid decay, suggesting that for 70-75% of transcripts other cis and trans factors are required for decay (2).

Although considerable insight into the mechanisms that coordinate mRNA degradation has originated from studies of AREs other cis-elements include GU-rich elements (GREs) found in the 3'UTR, internal ribosome entry sites (IRES), upstream open reading frames (uORFs), and 5' terminal oligopyrimidine (TOP) sequences in the 5'UTR; as well as a 249-nucleotide stretch in the coding region of transcripts known as the coding region instability determinant (CRD), (15,16,30).

TRANS-ACTING ELEMENTS: RNA-BINDING PROTEINS

RNA-binding proteins (RBPs) are proteins that bind to RNA recognition motifs (RRMs) of double or single stranded RNA and form ribonucleoprotein (RNP) complexes. RBPs are present in both the nucleus and cytoplasm. However, since mature mRNAs are quickly exported to the cytoplasm, most nuclear RBPs associate with pre-mRNA and
form complexes known as heterogeneous ribonucleoprotein particles (hnRNPs). Over the course of evolution the diversity of RBPs greatly expanded allowing eukaryotic cells to use different arrangements of RNA exons to produce distinct associations between RBPs and mRNA. Thus giving rise to unique sets of RNPs.

The critical role of RBPs is exemplified by the amount of the transcriptome dedicated to RBPs. For example 3-11% of bacteria and archaea transcriptomes represent RBPs (4). The Drosophila genome contains 259 RBPs compared to 694 transcription factors (71). Approximately 500 genes in eukaryotic cells encode diverse RBPs each with unique RNA-binding specificity and protein-protein interactions. RBPs associate with unique subsets of mRNAs, usually during a particular stage in the lifetime of the mRNA, to coordinate their localization, transport, translation, and/or degradation (5,35,66,71). Nuclear RBPs can also participate in splicing and polyadenylation of the mRNA transcript before being exported to the cytoplasm. These chemical modifications, or “mRNA imprinting,” that mRNAs undergo posttranscriptionally confers genetic information on transcripts. As mRNAs are exported, genetic information is conveyed from the nucleus to the cytoplasm, and can even transport information from one cell to the other (4). Although, RBPs play a critical role in posttranscriptional regulation of gene expression, relatively few RBPs have been extensively studied.

Typically one RBP can bind a large number of different mRNAs; and one mRNA can be bound by multiple RBPs. RBPs, like RPS4A/B, NF90, and TIAR, can even bind and regulate the stability of their own mRNA transcript, providing a mechanism to control the abundance of each protein (4,5,35,37). Multiple RBPs can bind a common mRNA target in a cooperative, competitive, or independent fashion. Studies in S. cerevisiae revealed that each distinct mRNA interacts with at least three RBPs; evidence of multidimensional regulatory networks in which combinations of RBPs bind to specific RRM s in mRNAs (35). For example, regulation of inducible nitric oxide synthase (iNOS) expression requires binding of five RBPs: AUF1, HuR, KSRP, PTB, and TIS11, which achieves highly tuned regulation of iNOS in response to cytokine signaling (5,35). Many RBPs bind mRNAs whose protein products share common location or function. For example, there is a relative overrepresentation of RBPs that associate with mRNAs encoding cell-wall components. This may highlight the particularly important role of RBPs in establishing and maintaining spatial organization through localized translation and/or mRNA decay, as well as, the need for extensive regulation of spatial and temporal assembly and remodeling of dynamic subcellular structures (35,43). It would be interesting to determine if this regulation is also extensively found in the nervous system where local translation and decay is particularly important for axon pathfinding, and dynamic remodeling is required for neural plasticity, learning, and memory.

Similar to AREs, AU-Rich binding proteins (AUBPs) are the most well characterized RBPs. (Figure 3A) AUBPs are evolutionarily conserved from S. cerevisiae to mice, and highly conserved within the Drosophila species (27). AUBPs can be strictly nuclear or cytoplasm, or have the ability to shuttle between both compartments (26). Numerous signal transduction pathways, such as p38 MAPK, ERK, and Wnt/β-catenin affect the abundance, localization, and activity of RBPs; and thus regulate ARE-mediated decay (5,27). For example, during muscle differentiation, phosphorylation of destabilizing RBP KSRP by p38 MAPK causes KSRP to dissociate from unstable p21 and myogenin mRNAs; which are then free to associate with HuR causing stabilization
of these muscle-specific transcripts (1,15,39). Stress-triggered signaling pathways also influence RBP activity. Heat shock increases ARE-mediated decay through AUF1, and suppresses HuR binding (15). Many AUBPs interact with the decay machinery to regulate ARE-mediated decay. KSRP binds to PARN and the exosome, while TIS11 interacts with decapping enzymes and CCR4-NOT (1,15,38).

Embryonic Lethal Abnormal Vision (ELAV) is a neuron-specific RBP expressed in the nuclei of all postmitotic neurons in Drosophila, and is required for proper development and maintenance of neurons, as well as differentiation. Since ELAV is a nuclear RBP it regulates alternative splicing of neuroglian, armadillo, and erect wing mRNA transcripts to produce neuron specific isoforms (37,71,72). ELAV contains three RRM. RRM1 and RRM2 bind AREs, while RRM3 recognizes and binds to secondary and tertiary structures in the poly(A)-tail; therefore ELAV has the ability to simultaneously binds both the ARE and poly(A)-tail in the 3'UTR of target mRNAs. Binding by ELAV inhibits endonucleolytic attack; stabilizing target mRNAs (36,39,71).

The ELAV-like protein 1, more commonly known as HuR, family of RBPs is related to the Drosophila ELAV RBPs and consists of four members: HuR (HuA), HuB, HuC, and HuD (5,11). HuR is ubiquitously expressed, while HuC and HuD are neural-specific, and HuB is expressed in both neurons and sex organs (39,40,41,72). Similar to ELAV, HuR simultaneously binds to AREs, through RRM 1 and RRM2, and the poly(A)-tail through RRM3. The function of the RRM3-poly(A)-tail interaction is not completely understood. Some models propose that RRM3 binding protects from deadenylation, while others suggest RRM3 actually aids in deadenylation, and still others propose that RRM3 plays no role in deadenylation, but rather helps delay the onset of decay following the deadenylation step (39,40,41). However, data has shown that the overall effect of binding by HuR stabilizes ARE-bearing transcripts in either the 5’ or 3’UTR; and overexpression of HuR stabilizes transcripts such as c-fos and β-globin (15,22,38).

Hu RBPs play a role in localization, translation, neuroblast to neuron differentiation, cell division and replicative senescence, immune cell activation, stress responses, and carcinogenesis. HuR has a wide range of ARE targets, such as proliferation associated mRNAs like cyclin A-E and c-fos, growth associated mRNAs like VEGF and TNFα, and cancer-related mRNAs such as c-myc, p21, and p53 (36,38,41).

### Figure 3A. Well-characterized ARE Binding Proteins (AUBPs).

<table>
<thead>
<tr>
<th>Destabilizing AUBPs</th>
<th>Stabilizing AUBPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUF1</td>
<td>ELAV / HuR (HuA)</td>
</tr>
<tr>
<td>KSRP</td>
<td>HuB - HuD</td>
</tr>
<tr>
<td>TIS11 / TTP</td>
<td></td>
</tr>
<tr>
<td>CUGBP1</td>
<td></td>
</tr>
<tr>
<td>TIAR</td>
<td></td>
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</tbody>
</table>

Left column contains AUBPs known to destabilize ARE-containing mRNAs. Right column contains AUBPs known to stabilize ARE-containing mRNAs.
HuR is part of a subset of RBPs that are nucleocytoplasmic due to their ability to shuttle between the nucleus and cytoplasm. For example, during cellular stress, such as heat shock or UV light, and during T cell receptor stimulation in human T lymphocytes, the abundance of HuR in the cytoplasm rapidly increases, which implicates HuR binding as a mechanism for stabilizing mRNAs (2,39). The current model of HuR binding is that HuR may bind to newly synthesized ARE-mRNAs in the nucleus and travel with them to cytoplasm, where it protects the transcript from degradation. HuR may be required for efficient nuclear transport of mRNAs. Once in the cytoplasm HuR may participate in localization, translation, and turnover of the mRNA transcript before shuttling back to the nucleus. Dissociation of HuR from mRNA allows binding of decay factors and degradation of mRNA (2,38,40,41).

Stabilizing RBPs, like HuR and ELAV, may function by removing mRNAs from sites of decay or by competing for binding sites of destabilizing factors. Binding of HuR has been shown to relocate mRNAs from P-bodies to active polysomes. HuR has also been shown to compete for binding sites with destabilizing RBPs such as AUF1, KSRP, and TIS11 (1). Stabilizing RBPs may also function by strengthening the interaction between PABP and the poly(A)-tail to prevent deadenylation. RBPs may directly interact with and sequester decay machinery, therefore inhibiting the assembly of degradation complexes (39,40). Alternatively, increased abundance of stabilizing RBPs may not inhibit, but rather slow, decay of target mRNAs by creating an imbalance between stabilizing and destabilizing RBPs. For example, overexpression of HuB in mouse adipocytes slows the decay of ARE-bearing glucose transporter (GLUT1) mRNA (40,41).

AU-rich Factor 1 (AUF1), also known as hnRNP D, was the first ARE-BP identified and cloned. AUF1 binds to AU-rich sequences capable of forming stem-loop structures (42). Similar to Hu/ELAV RBPs, AUF1 participates in mRNA splicing and transport and is nucleocytoplasmic (42). AUF1 also regulates mRNAs that function in cell cycle progression, immune and inflammatory responses, and carcinogenesis; suggesting that AUF1 and HuR may regulate the same subset of mRNAs. AUF1 is a unique RBP in that it has dual roles in ARE-mediated decay as both a stabilizing or destabilizing factor depending on the mRNA target. For example, AUF1 p37 and p42 isoforms stabilized reporter mRNAs, while AUF1 p40 and p45 isoforms promoted mRNA degradation (38,42). Immunoprecipitation of AUF1 reveals its association with eIF4G, PABP1, Hsp70, and lactate dehydrogenase (LDH). This data suggests that AUF1, as well as other RBPs, may be components of a multi-subunit mRNA degradation complex (15).

TPA-inducible sequence 11 (TIS11) is a potent destabilizing ARE-BP. TIS11 contains two zinc finger motifs that interact with AREs to promote deadenylation, which is most strongly induced with the presence of two or more ARE sequences. TIS11 co-immunoprecipitates with decapping enzymes DCP1/DCP2, XRN1, CCR4-NOT, and exosome component RRP4; supporting the mechanism that destabilizing RBPs recruit and interact with decay machinery to promote degradation. TIS11 has also been shown to localize mRNAs to P-bodies for silencing and/or degradation. TIS11 regulates the degradation of transcription factors, cell cycle regulators, and cell survival mRNAs, such as Bcl2, which contains a class II ARE in its 3'UTR; implicating TIS11 as a potential tumor suppressor. In support of this, overexpression of TIS11 delayed tumor formation
by four weeks in mast cells transfected with v-H-Ras. In head and neck squamous cell carcinoma cell lines, tumor cells expressing TIS11b underwent apoptosis following drug exposure, while tumor cells with low/absent levels of TIS11b were resistant to apoptosis and promoted tumor formation. TIS11 also interacts with miRNA pathways, requiring both miR16 and Dicer to degrade TNFα mRNA, indicating multidimensional regulatory networks and an important point of connection between ARE-mediated decay and miRNA pathways (48). Interestingly, tis11 mRNA is exhibits average stability, neither falling into the low or high stability decay classes. This suggests, that like other ARE-bearing mRNAs, tis11 may be targeted by multiple AUBPs. TIS11, like ELAV and FNE, also autoregulates its own mRNA in a destabilizing fashion. Therefore, tis11 mRNA may be regulated through competitive binding between destabilizing TIS11 RBP and stabilizing ARE RBP, such as ELAV; producing a transcript with average stability.

Destabilizing AUBPs, such as AUF1, TTP, TIS11, and KSRP, have been better characterized than stabilizing AUBPs. To date the only known stabilizing AUBPs belong to the Hu/ELAV family of RBPs, and include HuA – HuD, RBP9, and FNE. Although, RBP9 has been shown to destabilize mRNAs, the function of FNE, remains largely unknown. What is known of FNE is that fne mRNA is present throughout development, but is restricted to neurons in CNS and PNS. fne is required for the proper development of the adult mushroom body, indicating that fne may have a functional role in developing complex behaviors, such as courtship behavior which is altered when fne is knocked out. FNE RBP is present in neuronal cytoplasm suggesting that, similar to other cytoplasmic RBPs, it has a role in mRNA posttranscriptional regulation. FNE can also bind to the same site that ELAV RBP binds in the 3’UTR of elav and Nrg mRNAs. This indicates that FNE and ELAV may form multiprotein complexes to regulate mRNA stability. For example, some transcripts may require binding of both FNE and ELAV to be effectively stabilized (70,71,72). FNE has also been shown to bind to its own mRNA transcript, as well as to elav mRNA as a mechanism to maintain proper levels of FNE and ELAV RBPs. Overexpression of FNE leads to the reduction of endogenous fne and elav mRNA. Thus, in addition to an autoregulatory connection between FNE, ELAV, and their respective mRNAs, a destabilizing regulatory connection can also be established between FNE and elav mRNA. Overall this data strongly suggests that FNE is a neural-specific RBP and may function by binding to, and stabilizing, neural-specific mRNAs, similar to RBPs of the Hu/ELAV family; making FNE a choice candidate for studying ARE-mediated decay.

CUG-repeat binding protein 1 (CUGBP1) is a member of the CELF (CUGBP1 and embryonic lethal abnormal vision-like factor) family of RBPs and was shown to bind and deadenylate mRNAs containing CUG and GU-rich (GREs) sequences in their 3’UTRs by recruitment of PARN (5,28). In addition to binding GREs CUGBP1 also has an affinity for binding ARE such as TNFα (29). Knockdown of CUGBP1 leads to stabilization of GRE-containing mRNAs, indicating that CUGBP1 is required for GRE-mediated decay. Specificity of CUGBP1 binding and function have been conserved over evolution. In Xenopus, the CUGBP1 orthologue EDEN-BP, binds to the GU-rich EDEN element and functions as a deadenylation signal and translational activator. In Drosophila, the CUGBP1 orthologue, Bruno-3 (Bru-3) binds specifically to (UG)15 and regulates translation of embryogenesis and organogenesis (28). Bru-1, the Zebrafish orthologue of CUGBP1, also binds GREs and regulates development.
mRNA targets of CUGBP1 are involved in cell cycle, cell growth, mobility (migration/intracellular transport), and apoptosis. The most notable function of CUGBP1 is during muscle differentiation. CUGBP1 promotes myoblast growth and differentiation in mice by binding transcription factors Myod1 and Myog (28,29). However, a genetic screen in mice also found CUGBP1 to be one of the top ten genes driving tumorigenesis if mutated and/or dysregulated. In HeLa cells mRNA targets of CUGBP1 play a role in processes important in cancer development, such as cell growth, migration, and apoptosis. For example, G-protein signaling pathway and G-protein coupled receptor ligands which either activate or repress cell-cell interactions, cell migration, and cell invasion; playing an important role in cancer development and metastasis, are targets of CUGBP1 (30).

Although the work has primarily focused on ARE RBPs, many other RBPs have been shown to regulate mRNA stability and translation. For example, in Drosophila the two most well studied non-ARE RBPs are Smaug and Pumilio. Smaug, a highly conserved RBP, targets 67% of maternal transcripts for degradation, through recruitment of CCR4-NOT/PARN, followed by 5'→3' decay by XRN1, or 3'→5' decay by the exosome. Right pathway shows binding by stabilizing AUBP (HuR). Binding by HuR stabilizes mRNA or slows mRNA decay.

Although the work has primarily focused on ARE RBPs, many other RBPs have been shown to regulate mRNA stability and translation. For example, in Drosophila the two most well studied non-ARE RBPs are Smaug and Pumilio. Smaug, a highly conserved RBP, targets 67% of maternal transcripts for degradation, through recruitment of CCR4-NOT/PARN, followed by 5'→3' decay by XRN1, or 3'→5' decay by the exosome. Right pathway shows binding by stabilizing AUBP (HuR). Binding by HuR stabilizes mRNA or slows mRNA decay.

![Figure 3B. Recruitment of AUBPs by ARE-containing mRNAs.](image)

Left pathway shows binding by destabilizing AUBP (AUF1). Binding by AUF1 induces deadenylation dependent decay through recruitment of CCR4-NOT/PARN, followed by 5'→3' decay by XRN1, or 3'→5' decay by the exosome. Right pathway shows binding by stabilizing AUBP (HuR). Binding by HuR stabilizes mRNA or slows mRNA decay.
asymmetric division of germ line stem cells, morphogenesis of sensory neurons, and long-term memory in adult flies (43,44).

**TRANS-ACTING ELEMENTS: MICRO-RNAs**

Regulation of gene expression is also controlled by microRNAs (miRNAs). miRNAs are derived from long nuclear RNA precursors that are processed, exported to the cytoplasm, and incorporated into the miRNA-loaded RISC complex. miRNAs direct RISC to specific mRNA targets by pairing to the complementary seed region in the 3'UTR. Partial pairing of miRNA with mRNA target yields translation inhibition, stalling of translation elongation, or stimulation of polypeptide proteolysis. Perfect, or near perfect, pairing of miRNA induces degradation of the target mRNA by the Argonaute (AGO) containing RISC complex. miRNA binding can also induce deadenylation of target mRNA by recruitment of PAN2-PAN3 and CCR4-NOT, followed by decapping by DCP1/DCP2 (4,5).

These 21-25 nucleotide RNAs comprise about 3% of all human genes, and regulate the expression of 20-30% of genes, and 60% of protein-coding genes, by controlling translation inhibition or degradation of target mRNAs (32,81,82,90). Signaling molecules and transcription factors are particularly sensitive to changes in concentration, making them prominent target of miRNAs (91). miRNAs function by “fine-tuning” the expression of target genes to an optimal level, rather than an on-off switch. In humans, miRNAs regulate diverse cellular processes, such as proliferation, differentiation, and apoptosis (89,90). 3'UTRs of mRNAs are not under the control of a single miRNA species, although a single miRNA may dominant under specific conditions (87). Expression of miRNAs is often tissue or stage specific, and correlations between miRNA expression patterns and miRNA functions have been observed (82,83). Many miRNAs have been shown to be enriched in the nervous system, suggesting the need of complex regulation to produce proper neural cell identities. For example, miR-124 and K-box class miRNAs (miR-13b-1/13b-2/2c) are specifically expressed in the CNS (83). miR-315 and miR-92a/7 are expressed in the brain and ventral cord.

During *Drosophila* development miRNA expression is diverse and dynamic, and has been shown to regulate cell identity and early tissue specification. Some miRNAs exhibit expression along the anterior-posterior or dorsal-ventral axes, while others are expressed in specific germ layers, organs, or differentiating cell types. These miRNA expression patterns parallel vertebrate counterparts, suggesting that miRNAs may have ancient roles in animal patterning (83). Many unstable short-lived mRNAs in *Drosophila* and mammalian cells contain motifs targeted by miRNAs (4,50). Most notably in *Drosophila*, miRNAs have been shown to target genes involved in cytokine signaling pathways such as Notch, TGFβ, Wnt/Wingless, Hedgehog, and Receptor Tyrosine Kinase. miRNAs have been shown to potently inhibit cytokine mRNAs by targeting regulators of cytokine gene expression (87). For example, miR-315, which possesses one of the largest libraries of conserved targets of all *Drosophila* genes, has also been shown to regulate the Wnt/Wg signaling pathway (84,88). miR-315 activates Wg signaling through repression of two negative regulators, Axin and Notum. Hyperactivated Wnt/Wg signaling underlies developmental disorders and diseases, including liver, colorectal, breast and skin cancer, suggesting the need to maintain tight control of this signaling pathway through miRNAs (84).
ARE-mRNAs: AN INTERPLAY BETWEEN AUBPs and miRNAs

Evidence of coordinated regulation of mRNAs by both cis and trans elements has become an emerging trend in posttranscriptional regulation. Genome wide regulation of miRNA targeting may be modulated by the presence or absence of sequence elements, such as binding sites for the hundreds of RBPs expressed in any cell (69,87). Proteins that interact with the 3’UTRs of mRNAs, specifically AUBPs, generally act as modifiers that alter the potential of miRNAs to repress gene expression (82,90). Imperfect matching between miRNA seed sequence and mRNA target results in translational repression and frequently results in degradation of the mRNA by non-RNAi mechanisms, such as AUBPs that can recruit decay machinery (82). miRNAs and RBPs have been shown to interact through three main mechanisms: 1) mRNA decay and translation inhibition due to miRNA and RBP cooperation; 2) mRNA stabilization or slowed decay rate due to competition between miRNAs and RBPs; and 3) environmental effects on mRNA stability, mediated through miRNAs and RBPs (69,89).

The most notable example of posttranscriptional regulation mediated by interactions between cis and trans factors is regulation of ARE containing mRNAs by both miRNAs and AUBPs. Hundreds of mRNAs are down regulated upon miRNA transfection and up regulated following miRNA inhibition. For example, transfection of miR-1 and miR-124 in HeLa cells correlated with changes in expression of ARE-bearing mRNAs (69). This is presumably due to enhanced activity, or lack of activity, by miRNAs. However, a substantial portion of changes in mRNA expression is not accounted for by miRNA targeting activity. A large group of genes that contain a predicted miRNA binding sites do not display detectable down regulation after miRNA transfection; and it has been reported that only 30-40% of mRNAs that are up regulated after miRNA inhibition contain predicted miRNA binding sites. These findings suggest that other signals, such as RBPs, may modulate miRNA targeting (69). Interestingly, mRNAs that are up or down regulated by miRNA activity are most significantly enriched for ARE motifs (69). Two novel U-rich motifs (URMs), recently discovered as binding sites for HuD RBP, are overrepresented in down-regulated mRNAs; while the ARE pentamer is overrepresented in up-regulated mRNAs. Also, both ARE and URM motifs were found to associated with endogenous miRNA binding sites in mRNAs bound to Argonaute proteins, further confirming that AREs, and their interactions with AUBPs, function to modulate miRNA activity during posttranscriptional regulation.

Machinery involved in miRNA degradation pathways has also been shown to mediate regulation of ARE-mRNAs, further connecting the miRNA and ARE regulatory networks. miRNAs with appropriate sequence complementarity have the ability to recruit RISC to AREs causing repression of ARE-mRNAs (86). In Drosophila, knockdown of Dicer1, which encodes an RNase enzyme that cleaves pre-miRNA in the cytoplasm, led to stabilization of ARE-mRNA reporters; and knockout of AGO1 in Drosophila S2 cells also leads to an upregulation of ARE-mRNAs (31,69). Knockout of AGO1 and AGO2, components of the RISC complex and involved in miRNA processing, have also been shown to be required for ARE-mediated decay in Drosophila and HeLa cells. Other components of decay pathways such as CCR4-NOT and DCP1/DCP2 have been shown to associate with TTP AUBP, and are required for miRNA mediated decay (19). RBPs
have also been shown to regulate the biogenesis of miRNAs. For example, HuR interaction with H19 mRNA suppresses generation of miR-675 (93). HuR binding to first intron and flanking exons of Tll10-001 mRNA may directly influence the efficiency of miR-200b generation. Also, both HuR and Dicer have been shown to play a key role in miRNA biogenesis (19).

miRNAs and AUBPs have been shown to compete for binding of AREs in 3'UTRs of many mRNAs. One of the best-studied examples of this is regulation of cationic amino acid transporter 1 (cat1) mRNA by both miR-122 and HuR (82). cat1 mRNA, which facilitates the uptake of arginine and lysine in mammalian cells, bears an ARE in its 3'UTR. cat1 is negatively regulated by miR-122. However, miRNA-mediated inhibition is relieved in hepatocarcinoma cells subject to stress conditions, and causes release of cat1 mRNA from cytoplasmic P bodies and recruitment to polysomes for active translation (82,86). Derepression has been shown to require binding of HuR AUBP to the 3'UTR of cat1. In response to cellular stress, HuR is known to relocate from the nucleus to the cytoplasm, where it can modulate the translation and/or stability of ARE-mRNAs. Upon amino acid starvation, HuR is thought to bind to the ARE in the 3'UTR of cat1 mRNA downstream from the miR-122 binding site (19,86,89,93). Recruitment of HuR may either lead to dissociation of miR-122 or inhibit miR-122 binding leading to stabilization and translation of cat1 mRNA. Although HuR binding to cat1 relieves miRNA-mediated repression, HuR binding to ARE in c-Myc represses expression by recruiting let-7 miRNA and RISC to an adjacent binding sites in the c-Myc 3'UTR (89,93). Later experimentation revealed that HuR does not interact with let-7/RISC suggesting that HuR binding may unmask the let-7 binding site leading to reduction in c-Myc mRNA levels as well as translation inhibition.

Posttranscriptional regulation has also been shown to play a vital role in controlling the expression of cytokines by modulating mRNA stability. Many cytokine mRNAs contain AREs and have been shown to be regulated by both AUBPs and miRNAs (89) (Figure 4A, Table 2) Regulation of cytokine genes via the destabilizing activity of AREs and miRNAs is required for regulation of mRNA HL and achievement of proper temporal and spatial distribution of cytokine expression (90). Regulation of inflammatory cytokines is critical for many cellular processes, such as proliferation and angiogenesis; and aberrant expression of cytokine mRNAs has been correlated with inflammatory disease, autoimmune disorders, and cancer (89).

<table>
<thead>
<tr>
<th>miRNA sites</th>
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<tr>
<td>IL-1A</td>
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Figure 4A. Cytokine mRNAs with miRNA and ARE sites.
Left column lists cytokine mRNAs with miRNA binding site in 3'UTR. Right column lists cytokine mRNAs with ARE in 3'UTR. Cytokine mRNAs highlighted in red in right column contain both miRNA and ARE sites in 3'UTR.
Figure adapted from Palanisamy et al. Journal of Dental Research (2012)
miRNAs have been shown to regulate cytokine expression through several mechanisms. These include direct targeting of cytokine mRNAs by miRNAs, miRNA regulation of cytokine signaling, and miRNA-mediated regulation cytokine mRNAs through association with RBPs (89) (Figure 4B) Although direct miRNA targeting of cytokine mRNAs and signaling pathways, plays a major role in regulation of cytokine expression; this paper will solely focus on miRNA-mediated regulation of ARE-containing mRNAs via their association with RBPs, specifically AUBPs. For example, miR-4661, which contains a seed region that is complementary to ARE sequences, was demonstrated to upregulate IL-10 mRNA by competing with TTP. Binding of miR-4661 to the ARE blocks TTP binding, protecting IL-10 mRNA from TTP-mediated degradation (89). Although computational analysis indicates that the 3’UTRs of most cytokines lack miRNA target sites, miRNA may regulate cytokine expression through targeting of AUBPs. For example, both TTP and AUF-1 are predicted targets of miR-146; and ARE-mediated decay machinery components are also heavily predicted targets of miRNAs (90).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>miRNA(s)</th>
<th>RBP(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>miR-16</td>
<td>TTP</td>
</tr>
<tr>
<td>TNF-α</td>
<td>miR-125b, miR-221, miR-579</td>
<td>TTP, TIAR</td>
</tr>
<tr>
<td>IL-10</td>
<td>miR-4661</td>
<td>TTP</td>
</tr>
<tr>
<td>TNF-α</td>
<td>miR-369-3</td>
<td>FXR1, AGO2</td>
</tr>
<tr>
<td>VEGFA</td>
<td>miR-297, miR-299</td>
<td>hnRNP L</td>
</tr>
</tbody>
</table>

**Table 2. Regulation by miRNAs and AUBPs.**
Examples of cytokine mRNAs that are regulated by cooperative or competitive binding of miRNAs and AUBPs. Cytokine mRNAs listed contain both miRNA and ARE binding sites in 3’UTR.
Figure adapted from Palanisamy et al. *Journal of Dental Research* (2012).
One of the most noted examples of cooperative posttranscriptional regulation by AREs and miRNAs, is regulation of cytokine TNFα mRNA. In Drosophila S2 cells, miR-16, which has a partial sequence match to ARE, binds to the ARE in 3'UTR of TNFα. Binding of miR-16 requires the presence of TIS11. miR-16 bound RISC assists TIS11 binding to the ARE, which subsequently induces mRNA degradation by recruiting decay machinery (19,89,90). miR-125b, miR-221, and miR-579 have also been shown to regulate TNFα mRNA through association with either TIS11 to accelerate decay or by blocking TNFα translation by recruitment of translational inhibitor TIAR (89). Cooperative between miRNA and AUBP is thought to facilitate a more stable interaction with TNFα mRNA, allow efficient recruitment of degradation machinery. TNFα mRNA has also been shown to activate translation in response to quiescence via miRNA targets sites in the TNFα ARE (86,87). miR-369-3 binds directly within a region of the TNFα ARE and activates translation in quiescent cells through recruitment of FXR1 and AGO2, during serum starvation (89,19). This demonstrates the role of both the cellular environment and interplay between miRNAs and AUBPs in regulation ARE-mRNA gene expression.

Although much evidence points to the interactions between miRNAs and AUBPs in regulation of ARE-containing mRNAs, other studies have shown that there is no requirement of miRNAs in regulation of ARE-mRNAs. Studies by Helfer et al. demonstrated that ie3 mRNA, which is known to harbor a functional ARE in its 3'UTR, was rapidly degraded at equivalent rates in both wild type and dicer knock out mouse embryonic fibroblasts (86). This study also showed that in Drosophila S2 cells, ceca1 ARE-mRNA is repressed by TIS11, but this repression is not dependent on miRNA factors, since direct inhibition or overexpression of miRNAs with potential sequence complementarity to the ceca1 ARE do not alter reporter gene expression. Moreover, Drosophila miR-289, which was previously recognized to recognize AREs was not detectably expressed in flies or S2 cells. Altogether, Helfer et al. concluded that AREs do not need to be recognized by miRNAs to exert their destabilizing function. However, other experimental evidence, has demonstrated that target sites of miRNAs are more efficient when they occur in 3'UTRs that contain signature motifs, such as AREs and URM (69). This may indicate that although AREs and AUBPs are not required for
miRNA-mediated regulation, they may however increase the specificity and efficiency of mRNA decay through coordinated regulation of cis and trans elements (31).

**IMPORTANCE OF mRNA DECAY**

Gene expression is canonically divided into five major stages: mRNA synthesis, mRNA processing, export, translation, and decay. However, a more accurate representation of gene expression mechanistically links all five stages, which work together to form a single cohesive system. Thus, gene expression is not linear, but rather circular. One of the most important features of this circular system is that mRNA decay is directly linked to transcription. Recent studies by Haimovich et al. reveal that mRNA decay factors promote mRNA synthesis, specifically transcription initiation and elongation (76).

In *S. cerevisiae*, the majority of transcripts are degraded through components of the 5′ – 3′ pathway collectively known as the decaysome. Many components of the decaysome have been found to play a role in transcription. For example, defects in XRN1 and CCR4-NOT cause transcriptional down regulation in many mRNAs, although increases in stability were observed. Knockout of XRN1 decreased the amount of active RNA Polymerase II, indicating that mRNA synthesis is significantly dependent on the presence of XRN1. Also, decay factors XRN1, DCP2, and LSM1 have been shown to associate with chromatin by binding approximately 30 base pairs upstream of the transcription start site; and directly stimulating transcription initiation and elongation (76).

mRNA decay not only plays a role in genome wide transcription initiation and elongation, but also in muscle differentiation, and pathogenesis (29). Approximately 80% of the human genome is transcribed into RNA, underscoring the importance of RNA regulatory networks in establishing precise spatial and temporal gene expression; which is exemplified in the patterning and development of the nervous system by RBPs, NMD, and miRNAs (17).

**mRNA DECAY IN NERVOUS SYSTEM DEVELOPMENT**

Maturation and plasticity of the nervous system is a dynamic balance between the transcriptome and the environment. The nervous system is comprised of a wide range of cell types and connections. Changes in the strength of synaptic connections are thought to underline long-term memory and neural plasticity. The maturing nervous system involves numerous developmental transitions: neural tube patterning, neural stem cell proliferation and maintenance, differentiation and lineage restriction, migration, neuronal and glial subtype specification, dendritogenesis, axon pathfinding, synaptogenesis, and formation of neural networks (14). RBPs and non-coding RNAs that act through recognition of RNA and DNA motifs promote these diverse developmental transitions through regulation of splicing, export, transcription, translation, and decay. Several classes of RBPs and miRNAs are over represented in the central and peripheral nervous system, and play a key role in nervous system development, maturation, and dysfunction (14). Thus, nervous system development, plasticity, and diseases are linked to, and may derive from, complex RNA regulatory networks.
RBPs IN NERVOUS SYSTEM DEVELOPMENT

In *Drosophila*, ARE-mediated decay of Glial cells missing (*gcm*) mRNA ensures tight regulation of Gcm, and therefore proper glial development in the nervous system. ARE mutagenesis leads to increased stability of *gcm* and enhances the production of glia at the expense of neurons, thus disrupting the balance between these cell types (11). HuR RBPs have been shown to have an important role in neural plasticity, through transcription stabilization, reflected by their accumulation during acquisition of spatial memory in hippocampal neurons (72).

Perhaps the most important role of mRNA decay in nervous system plasticity and memory formation is through regulation of mRNA transport and local protein synthesis at synaptic terminals. The ability to supply specific gene products to distant synaptic terminals is crucial for neuronal functions. Although proteins can be made in the cell body and trafficked to synapses, local protein synthesis of dendritic mRNAs provides a more efficient mechanism for protein delivery. The discovery of synapse-associated polyribosome complexes (SPRCs) evidenced that mRNAs were in fact being transported to dendrites and locally translated near the synapse (74).

In *Aplysia* neurons, inhibitors of local protein synthesis and deletion of the dendritic targeting element in *CAMKIIα* mRNA, impairs synaptic plasticity and memory consolidation, indicating that mRNA transport and local protein synthesis are required for long-term memory and synaptic plasticity. In rat hippocampal CA1 neurons 154 dendrite-specific mRNAs identified categories of mRNAs involved in trafficking, protein synthesis, posttranscriptional modification, and degradation, among others. Of the 154 dendrite-specific mRNAs, seven fell into the RNA-binding category including PUM, QKI-6, and Poly(C) binding proteins (74). Implicating these, and possibly other RBPs, as having an important role in synaptic plasticity and memory through regulation of transport, translation, and degradation.

miRNAs IN NERVOUS SYSTEM DEVELOPMENT

Neural transcription factors appear to be targets of miRNAs (14). miRNAs may modulate the behavior of thousands of dendritic synapses through regulation of local translation. In mammalian cells, miRNAs are significantly localized to neuronal cell types, with the highest concentration found in the cerebral cortex and cerebellum (14). Also, in *Drosophila*, many miRNAs, such as miR-315, miR-92a, miR-7, and miR-124, are specifically transcribed in the embryonic brain, and their expression is often restricted to the developing CNS. The largest group of predicted miRNA targets includes mRNAs encoding synapse-associated proteins, such as Synapsin 1, Synaptotagmin, and Fragile-X mental retardation protein (FMRP); suggesting that miRNAs may be involved in synaptic input during memory formation, and may interact with cellular machinery in dendritic spines to produce long-term changes in synaptic function (15,32).

miRNAs also play a crucial role in development of the nervous system. In mice, approximately 20% of miRNAs display regulated expression during neural development.
For example, miR-23 enhances Notch transcription leading to preservation of self-renewing neural stem cell fate (14). Zebrafish mutations in dicer, causing inhibition of the miRNA pathways, leads to defects in neural induction, neural plate and neural tube formation, segmental morphogenesis, neural lineage restriction, and axon pathfinding. These defects were largely rescued by expression of miR-430. In Drosophila, Zinc-finger transcription factor Nerfin-1, which is required for axon pathfinding, is spatially restricted in the CNS, and temporally restricted in the PNS by miRNAs during nervous system development. Nerfin-1 mRNA contains multiple highly conserved sequences that harbor 21 predicted binding sites of 18 different miRNAs (12). In mammalian cells, miR-128 levels are dramatically increased in differentiating neural cells during brain development. Increased miR-128 levels repressed NMD, stabilizing NMD-targeted mRNAs that encode proteins that control neuron development and function; linking miRNA and NMD pathways to nervous system development (9).

miRNAs function in differentiation and maintenance of tissue identity by creating sharp boundaries between cellular identity states. A well-characterized example of this is miR-124. During neurogenesis, in both mice and zebrafish, miR-124 enhances neural lineage expansion by promoting neurogenesis while inhibiting gliogenesis (14,32). miR-124 is conserved in sequence and nervous system expression across all metazoans. In Drosophila, miR-124 targets retrograde BMP signaling which leads to incomplete transitions from neuroblast to neurons (33). Also, in embryonic mammalian brains, miR-124 regulates neural differentiation. miR-124 has been shown to target polypyrimidine tract binding protein 1 (PTBP1) mRNA. PTBP1 is a global repressor of alternative splicing in non-neural cell types. During neural development miR-124 also directly targets transcription factor Sox9. Sox9 maintains neural progenitors known as transit-amplifying cells, and is down regulated during neural differentiation by miR-124. Baf53a, a neural progenitor specific chromatin regulator that must be exchanged for its neural specific homolog to secure neural fate, is targeted by miR-124 during neural differentiation (33). Interestingly, organisms tolerate broad misregulation of miR-124. For example, although miR-124 is not required for proper dendrite morphogenesis in C. elegans, lack of miR-124 causes a broader distribution of dendrites on neurons (32). However these findings are consistent with the current model that miRNAs are used as a mode of fine-tuning, and lack of miRNAs leads to problems in robustness during neural development.

miRNAs are also expressed at high levels in the mature brain in order to maintain adult neural traits. Repression of genes involved in maintaining undifferentiated neural states by miRNAs, promotes the presence of specific differentiated neural phenotypes. For example, expression of miR-134 increases as the brain matures. miR-134 is localized to the dendritic synapses of rat hippocampal neurons. One major target of miR-134 is LIM-domain kinase 1 (Limk1) mRNA, which encodes a kinase that affects spine structure through regulation of actin filament dynamics. miR-134 negatively regulates the size of dendritic spines through translational inhibition of Limk1. Limk1 is transported in a dormant state to synaptic terminals through its association with miR-134. Lack of synaptic activity causes miR-134 to inhibit translation of Limk1. However, increased synaptic development and plasticity, which promotes expression of Brain-derived neurotrophic factor (BDNF), relieves inhibition of Limk1 (14,32,90). Thus, miR-134 may have an important role in the storage of memories and tasks through inhibiting Limk1 as the brain matures.
Nonsense mediated decay (NMD), although not a main concentration of this current work, has been shown to play a critical role in brain development and neurological function. Recent studies of NMD have revealed that the NMD pathway is not solely reserved for the decay of aberrant mRNA transcripts; but has an essential role in regulating the gene expression of normal transcripts. NMD regulates approximately 10-20% of normal mRNAs, and 10% of the whole transcriptome in *S. cerevisiae*, *Drosophila*, and human cells (5,10). Knockout of NMD factors in zebrafish and mice cause disruptions in brain patterning, reduced brain size, and cortical defects (9). NMD is also important for regulating the function of mature neurons and axon guidance and positioning (20,75).

**EXTENDED 3'UTRs IN NERVOUS SYSTEM DEVELOPMENT**

Alternative cleavage and polyadenylation (APA), also known as alternative 3' end formation, is the usage of different polyadenylation sites during mRNA synthesis. APA results in the synthesis of multiple mRNA isoforms that only differ by the length of their 3'UTR (19). Thus APA has a major impact on transcript diversity and function; and is an important mechanism for correct temporal and spatial control of gene expression during development. Approximately 50% of all expressed genes are though to be subject to APA in humans, mice, and Drosophila; often in a tissue-specific manner (7,19). Recent studies of APA have identified several trends across various cell types. For example, proliferating cells express shorter 3'UTRs upon T cell activation. Cell transformation, reprogramming of somatic cells to iPS cells, and *C. elegans* development may also be correlated with 3'UTR shortening (7). On the other hand, 3’UTR lengthening has been shown to occur during mouse embryonic development and differentiation of C2C12 myocytes. In mammals 3'UTRs exhibit a trend for lengthening in neurons and is correlated with loss of cell proliferation and the onset of differentiation (7,8). 3'UTRs harbor the majority of cis-regulatory information required for posttranscriptional regulation, including binding sites for RBPs and miRNAs. These trans-factors can either positively or negatively regulate transcript stability, translation efficiency, and localization. Consequently increasing or decreasing the length of 3'UTRs can substantially alter gene function. For example, loss of distal 3'UTR sequences allows certain oncogenes to escape repression by miRNAs, strengthening their potency (7,19).

Recent studies in *Drosophila* have shown that hundreds of genes undergo synchronous APA-mediated 3’UTR extensions during development, resulting in UTRs up to 20-fold longer than typical mRNA transcripts (8,19). Extended mRNA isoforms are specifically enriched in neural tissues, which are a known breeding ground for posttranscriptional regulation by miRNAs, and differential splicing by RBPs (7,8). Many of these genes exhibit differential 3'UTR lengths with intermediate mRNA isoforms appearing during early stages of neurogenesis, and longer mRNA isoforms appearing at later stages (19). Extended mRNA isoforms emerge shortly after the onset of zygotic transcription and contain putative recognition motifs for miRNAs and RBPs. (8).

Binding sites for neural miRNAs and RBPs are among the most highly conserved motifs within 3’UTR extensions. miRNA-mediated regulation may serve to restrict transcript function to areas of local translation or in response to environmental cues and neural activity. miR-190, K box miRNAs (miR-2/11/13), and Brd box miRNAs (miR-4/79)
were among the most conserved motifs found in 3'UTR extensions. Specifically, recognition sequences for neural miRNAs miR-315/317/190, were found 5-10 fold more often in 3'UTR extensions compared to shorter mRNA isoforms (7,8). Additionally, highly conserved motifs corresponding to the binding sites for Pumilio, and potentially ELAV were also found. The PUM motif was found 9-16 times in the extended isoforms of neural mRNAs *pum, elav, brat*, and *imp*; whereas it is absent or found only once in the respective short isoforms (8,19). Interestingly, PUM is required for dendrite morphogenesis during *Drosophila* nervous system development.

*Drosophila* genes subject to 3'UTR extension in the nervous system were preferentially enriched for mRNAs encoding RBPs, including AGO1, Brat, PUM, IMP, and ELAV (7,8). Furthermore, several of the RBPs that exhibit extraordinary 3'UTR lengthening, such as *elav, pumilio*, and *ago1*, are involved in 3'UTR interaction and/or miRNA-mediated regulation (7). This phenomenon may imply the need to uniquely control key regulatory molecules in the nervous system; as well as a complex network of auto-regulation and cross-regulation of posttranscriptional regulatory factors in the CNS.

Although the function of 3'UTR extensions is currently unknown current observations suggest that in the *Drosophila* neural-specific 3'UTR extensions render mRNAs susceptible to complex regulation by neural-specific posttranscriptional regulatory machinery. Proximal poly(A) sites are thought to enhance gene expression by minimizing the presence of destabilizing elements that likely occur in extended 3'UTRs (19). By the same logic, extended 3'UTRs may regulate associated mRNAs by adding recognition sequences for miRNA and RBPs. However, it remains unclear why coordinate lengthening of 3'UTR is restricted to neural tissues and enriched for mRNAs that function in RNA binding and processing. It is possible that genes with extended 3'UTR are subject to a unique mode of regulation in neural tissues. Interestingly, many of the genes subject to neural-specific APA are required for nervous system function. For example, miR-190 and Brd box miRNAs are known to regulate nervous system development, and PUM is also known to regulate neural mRNAs by repressing their expression (7,19). Neural 3'UTR extensions may not only facilitate down regulation of mRNAs. Recent studies identified distal APA variant of mammalian brain-derived neurotrophic factor (BDNF), but not the proximal variant, localize to dendrites and play a role in long-term potentiation (7). These findings suggest that 3'UTR extensions may serve to mediate specific functions within neurons, such as mRNA transport along axons or localized translation, through their interaction with miRNAs and RBPs. *Drosophila* Hox genes may also be regulated by a similar mechanism. Although 3'UTR extension is less dramatic, *Ubx, abd-A, Abd-B*, and *Antp* mRNAs contain short 3'UTRs during early development but exhibit longer 3'UTRs at later stages. These 3'UTR extensions are thought to be primary targets of miRNAs (8).

Shortly after 3'UTR lengthening was identified in *Drosophila* neural tissues, ELAV RBP was identified as a key mediator of neural-specific extensions. ELAV had previously been known to regulate APA of multiple mRNAs in the developing nervous system, as well as regulate 3'UTR extension during neuronal differentiation. However, it was not known whether ELAV was necessary and sufficient to mediate 3'UTR lengthening in neural tissues. Further study identified that ELAV protein is present in newborn neurons at the onset of embryonic stage 10. Extended 3'UTR isoforms were also found to first be synthesized 4-6hr after egg laying and specifically localize to the
nervous system (19). This indicates that extended mRNA isoforms are expressed in cells where ELAV protein is present. This study also found that elav mutants exhibit a dramatic reduction of extended 3'UTR mRNA isoforms, and overexpression of elav in nonneural embryonic tissues induced ectopic 3'UTR extension. Immunoprecipitation assays identified binding by ELAV near the proximal poly(A) signal, and 3'UTRs of extended mRNA isoforms. Also, tethering ELAV to exogenous mRNAs causes transcriptional read-through several kilobases beyond a strong poly(A) signal, suggesting that ELAV may function by repressing the proximal poly(A) signal, allowing read-through of elongated polymerase II complexes (19).

Overall, ELAV functions to foster 3'UTR extensions in a number of Drosophila genes. Although ELAV has the ability to induce 3'UTR extensions in a variety of cell types its activity is tightly restricted spatially, being present exclusively in neural tissues; and temporally, at the onset of stage 10 of embryogenesis (19). Interestingly, elav mutants are embryonic lethal and although neurons are generated they do not differentiate normally and display numerous axonal defects. elav mutants express only short isoforms of target mRNA, suggesting that low levels of ELAV may produce insufficient amounts of mRNAs with extended 3'UTRs, or mRNAs that fail to full elongate, potentially contributing to the mutant phenotype. Mammalian homolog of ELAV, HuR RBPs, also promote APA and generate extended 3'UTRs in neural tissues, and may regulate neural mRNAs in a similar fashion to Drosophila.

**BIOLOGICAL SIGNIFICANCE OF mRNA DECAY**

mRNA decay and NMD play a critical role in nervous system development and functionality. Sequence elements, RBPs, miRNAs, and NMD factors all work in unison to create proper mRNA levels that regulate highly restrictive spatial and temporal gene expression networks. Abnormally high mRNA concentrations can be toxic to neural development since it can affect chromatin structure, translation, and mRNA turnover. Perturbations in any component of this system, such as mutations that cause inappropriate sequestration of RBPs or elimination of RBP and miRNA recognition elements, lead to dysregulation and often neurodevelopmental and neurodegenerative disease (4,13,14).

**RBPs IN HEALTH AND DISEASE**

Alzheimer’s disease (AD) is characterized by the accumulation of plaques and neurofibrillary tangles in regions of the cortex and hippocampus progressively resulting in neurodegeneration and dementia. A major component of plaques are different sized β-amyloid precursor protein (APP). Overexpression of APP mRNA is linked to the development of AD and Down Syndrome (DS), and is typically increased by 1.5-2 fold. Many studies have suggested that elevated levels of APP mRNA are due to dysregulation of transcription and/or mRNA decay. APP contains four ARE motifs in its 3'UTR, suggesting that it is most likely regulated by AUBPs. A decrease in APP mRNA stability has the capability of reducing APP synthesis leading to a reduction in β-amyloid plaques. One current technique to accomplish this is to present transcriptome with decoy
mRNA engineered to bear the APP mRNA 3'UTR. Overexpression of decoy mRNA causes sequestration of AUBPs, which normally bind to endogenous APP mRNA; thus endogenous APP synthesis and β-amyloid plaques are reduced, providing a new approach to potential AD and DS therapeutics (16).

The function of CUGBP1 is dramatically altered in neuromuscular disease like myotonic dystrophy (DM) and Fragile X-associated tremor/ataxia syndrome (FXTAS). CUGBP1 is sequestered by pathogenic mRNA in FXTAS; and changes in mRNA decay rates in muscle cells, potentially due to abnormal sequestration of RBPs, is thought to be responsible for aspects of pathogenesis in DM (29). FXTAS is characterized by the onset of tremors, cerebellar ataxia, Parkinson’s disease, dementia, muscle weakness, autonomic system dysfunction, and multisystem atrophy. FXTAS is caused by small CGG repeats expansions in Fmr1 gene, known as premutations. These CGG expansions lead to an increase in Fmr1 transcripts, but have reduced translation efficiency, and thus sequester and misfold RBPs, like CUGBP1. Similar to FXTAS, Fragile X syndrome results from the dramatic expansion of CGG repeats in the 5’UTR of the Fmr1 gene. However, unlike DM and FXTAS, CGG repeats leads to loss of function of Fmr1 due to complete transcriptional silencing. Fragile X Mental Retardation Protein (FMRP), encoded by Fmr1, is an RBP that functions in synaptic plasticity and protein transport through suppressing the translation of a select group of mRNAs (14,32). Loss of FMRP leads to mental retardation, and has been found to be the most widespread single-gene cause of autism. Interestingly, double stranded RBP Staufen has been shown to bind CUG repeats in pathogenic mRNAs, through recognition of extensive secondary structures, and promote degradation (45). Thus, Staufen may play an important role in helping treat DM, FXTAS, and Fragile X syndrome.

RBPs have also been shown to play a role in other major diseases. Genetic variants of ELAV, Drosophila-like 4 (ELAVL4) have been reported to be associated with the onset age and risk of Parkinson’s disease (PD) in Caucasian populations. ELAV functions in neuronal differentiation and maintenance, and ELAVL4 binds to microtubule-associated protein, tau, which has previously been implicated in Parkinson’s disease (18). Taken together, ELAVL4 may be an important tau regulator, thus playing a critical role in the mechanism of Parkinson’s disease. Let-7 mRNA, which is regulated by RBP KSRP, exhibits reduced levels in lung carcinoma tissue, and overexpression of let-7 inhibits lung tumor growth (15). This suggest that let-7, in combination with KSRP, may have a role in tumor suppression.

**miRNAs IN HEALTH AND DISEASE**

miRNAs play a critical role in many diseases, specifically neurological and neurodegenerative diseases, cancers, and HIV. miR128 levels are dysregulated in many neurological diseases such as autism, prion-induced neurodegeneration, Huntington’s disease, PD, and AD (9). miR-9, miR-125b, miR-131, and miR-146 were found to be significantly upregulated in the temporal lobe neocortex in AD patients. Alterations of miR-175 are found in early-onset PD and X-linked mental retardation (14,17). In mammals a behavioral syndrome characterized by excessive grooming leading to hair removal and lesions, similar to obsessive compulsive disorder (OCD) in humans, has been attributed to Hoxb8 gene, which is regulated by miR-196. Tourette’s syndrome has
also been linked to sequence variations that disrupt the docking sites for miR-189 (32). In glioblastoma multiforme, a highly malignant glia cell-associated brain tumor, there is a significant overexpression of miR-21. Overexpression of miR-21 specifically blocks production of gene products necessary for glia cell differentiation and apoptosis, thereby leading to tumorigenesis (14). Mutations in human genes encoding proteins that are subunits of RISC, hAGO1, hAGO3, and hAGO4, are lost in patients that develop kidney tumors; and in Drosophila mutations in AGO1 and AGO2 lead to severe reductions in all types of neurons and glia (15,32).

REGULATION OF ARE-mRNAs IN HEALTH AND DISEASE

Regulation of ARE-containing mRNAs by miRNAs plays a crucial role in proper development. Dysregulation of these miRNAs may contribute to inflammatory diseases and cancers by altering the interaction between miRNAs and AUBPs with shared target ARE-mRNAs, specifically cytokine transcripts, affecting their expression levels (89). Also, a better understanding of how miRNAs function together with AUBPs to regulate gene expressed may provide new approaches and avenues for improved therapies and potentially prevention of cancer and inflammatory diseases.

One example of dual posttranscriptional regulation by miRNAs and AUBPs is in the synthesis of prostaglandins. Many transcripts involved in prostaglandin pathways, such as cPLA2, PLC, COX-1/2, mPGES-1/2, cPGES, and EP1-4, are potent targets of both miRNAs and AUBPs (81). Prostaglandins play an important role in cancer, and enhance synthesis of prostaglandin E2 (PGE2) is often observed in human malignancies and is associated with poor prognosis. PGE2 can promote tumor growth through binding of four prostaglandin receptors to activate signaling pathways that control proliferation, migration, apoptosis, and angiogenesis. Interestingly, cancer cells often harbor defects in function and/or expression of miRNAs and AUBPs, which may be responsible for increased levels of PGE2 leading to tumor development (81). For example, members of the miR-200 family, which are regulators of prostaglandins, are often deleted in aggressive tumors found in breast, prostate, and non-small cell lung cancer, as well as meningioma brain neoplasms (81). Therefore, understanding regulation of PGE2 by miRNAs and AUBPs may provide new therapeutic approaches to combat tumor growth and metastasis. (Figure 5)
Other cytokine mRNAs, regulated by miRNAs and AUBPs, have also been shown to play important roles in health and disease. For example, in mice, knockdown of miR-155, which regulates IL-4 expression, results in multiple defects in adaptive immunity; and IL-6, which is targeted for degradation by let-7 and AUFB1, is elevated in patients with endometrial cancer, non-small cell lung carcinoma, colorectal cancer, renal cell carcinoma, and breast and ovarian cancer (90). Also, mice with a germline deletion in the 3’UTR of TNFα caused a 3-10 fold increase in TNFα mRNA and protein levels. This increase of mutated TNFα mRNA resulted in mice with conditions similar to rheumatoid arthritis and Crohn’s disease (90). Cytokine mRNAs, which are important in innate immunity and inflammatory responses, are also known to play an important role in viral infections. Viral infections, such as influenza, have been shown to strongly induce the expression of miRNAs, which target cytokine mRNAs, thus regulating the cellular response to infection (91). For example, IL-6 and TNFα, which are targets of both miR-451 and TIS11, play critical roles in the immune system by driving acute inflammatory responses during sepsis, and in chronic inflammatory diseases such as rheumatoid arthritis, lupus, and type I diabetes (91). Viral infection induces the expression of miR-451, which inhibits 14-3-3ζ protein levels. 14-3-3ζ sequesters destabilizing AUBP TIS11 from binding and degrading cytokine mRNAs. Thus during viral infection, reduction of 14-3-3ζ, by miR-451, allows increased expression of TIS11 and rapid degradation of cytokine mRNAs, therefore minimizing an inflammatory response and promoting viral infection (91). Interestingly, 14-3-3ζ mRNA also contains a function ARE, suggesting that both miRNAs and AUBPs may regulate 14-3-3ζ expression levels in a competitive manner under various conditions. For example, under normal conditions 14-3-3ζ mRNA may be stabilized by an AUBP, promoting the repression of TIS11 and expression of cytokine transcripts.
Aberrant mRNA stability has been shown to contribute to oncogenesis and the invasive phenotype of tumor cells (94). mRNA stabilization may lead to an overproduction of growth factors and other mediators of cancer development. Inappropriate mRNA stabilization can be caused by increased activity of mRNA stabilizing proteins, like HuR, or reduced activity of mRNA decay-promoting proteins, such as TIS11. HuR has been proposed to play a critical role not only in stress response, but also in cell proliferation, differentiation, tumorigenesis, and apoptosis (82). In normal tissues, HuR is expressed at low levels and is localized to the nucleus; however HuR overexpression and increased cytoplasmic localization has been demonstrated in many tumor types, such as colon, breast, gastric, glioma, lung, ovary, and prostate (81,94). It is proposed that these malignancies may be due to HuR-induced dysregulation of miRNAs. HuR has also been shown to play an important role in tumor angiogenesis. In macrophages HuR and miR-200b, which were found to have overlapping binding sites in the 3'UTR, antagonistically regulate expression of VegfA and tumor angiogenesis in mice (93). Tumor growth, angiogenesis, vascular sprouting, and branching were significantly reduced in HuR knockout mice, suggesting that HuR regulates the expression of myeloid-derived factors that modulate tumor angiogenesis.

Interactions between TIS11 and miR-29a have also been shown to play a role in cancer pathogenesis. TIS11, targets many cytokine mRNAs that play an important role in cancer, and are often overexpressed in several cancers and promote cellular growth and resistance to apoptosis. Expression of TIS11 is reduced in a number of tumors, including thyroid, lung, colon, ovary, uterus, cervix, brain, head and neck, and breast (94). This suggests that TIS11 may function as a tumor suppressor. In combination with decreased levels of TIS11, miR-29a expression is upregulated in metastatic cells, such as the highly invasive breast cancer cell line MDA-MB-231 compared to other non-invasive and normal breast cell line. miR-29a negatively regulates TIS11 by binding to a target seed sequence in the 3'UTR of tis11 mRNA. Interestingly, TIS11 also regulates expression of HuR mRNA. Thus, repression of TIS11 by miR-29a causes overexpression of HuR leading to an invasive cancer phenotype. Exogenous inhibition of miR-29a alleviates miR-29a-mediated repression of TIS11. This restores the balance between TIS11 and HuR protein levels normalizing the stability of ARE-containing mRNAs that are key genes involved in invasion and metastasis of breast cancer (94). Normalization of aberrant ARE-mRNAs through inhibition of miR-29a caused significant reduction in actin polymerization and pseudopodia formation that assist in breast cancer metastasis and invasion.

In chronic lymphocytic leukemia (CLL), the most common adult human leukemia, miR-15a and miR-16-1 are lost or down regulated in the majority of cases, indicating that these miRNAs may function as tumor suppressors (85, 92). miR-15a and miR-16-1 target antiapoptotic protein BCL2, which is overexpressed in malignant B cells of CLL. Loss of miR-15a and miR-16-1, which are involved in ARE-mediated mRNA instability, leads to upregulation of mRNAs, 13.6% of which contain AREs, and down regulation of mRNAs, 20.1% of which also contain AREs (85). Gene ontology of these ARE-mRNAs show enrichment of cancer related genes, such as mcl1, bcl2, ets1, and jun, which are directly and/or indirectly involved in apoptosis and regulation of cell cycle checkpoints. Restoration of miR-15a and 16-1 induces apoptosis of MEG-01, a cell line derived from acute megakaryocytic leukemia, further implicating these miRNAs as tumor suppressors and potential targets of cancer therapeutics. Members of the let-7 miRNA family have
also been suggested to serve as tumor suppressors by directly inhibiting the expression of Hmg2A and RAS protooncogenes (84). In mantle cell lymphoma (MCL), overexpression of cyclin D1 leads to malignant transformation. Deletions or point mutations leading to premature truncation of the cyclin D1 3’UTR, which harbors both an ARE and miRNA target sites, has been reported in several MCL patients and cell lines (92); indicating the importance of ARE- and miRNA-mediated regulation ARE-mRNAs in cancer pathogenesis.

PREVIOUS STUDIES OF mRNA DECAY KINETICS

Three types of molecular assays are commonly used to determine mRNA decay kinetics: Northern blots, real time reverse transcription quantitative polymerase chain reaction (qRT-PCR), and Nuclear run-on assays. Northern blots resolve RNAs according to length by denaturing RNAs through gel electrophoresis. qRT-PCR resolves RNA through reverse transcription and simultaneous amplification and detection of target DNA. However, each method lacks the ability to detect one or more of the following features: rare transcripts, small amounts of RNA, multiple isoforms of an RNA species, and/or spatial information (65). Measuring transcriptional activity and turnover can be accomplished by nuclear run-on assays, however this technique is technically difficult, requires isolation of nuclei, which is very difficult for specific cell types, and can only be performed in vitro (63). Due to these technical limitations, more recently, high throughput microarray analysis has been used to more accurately measure mRNA decay kinetics.

In 2003 Yang et al. used microarray analysis to study the relationship between functional class and decay rates. Using two human cell lines, HepG2 hepatocytes and Bud8 fibroblasts, and the existing gene ontology hierarchy of biological processes mRNAs were assigned to functional classes and then decay rates between classes were compared. Microarray results indicated statistically significant organization of decay rates among functional classes. Specifically, transcription factors were enriched in the “fast-decaying” mRNA class, having HLs less than 2 hours; while mRNAs that encoded biosynthetic proteins were depleted from the “fast-decaying” class, and had increased average HL. These “fast-decaying” mRNAs were also found to be induced more rapidly, allowing for quick and dramatic changes based on cellular signals. Decay rates were found to most strongly correlate with motifs in the 3’UTR, with a longer 3’UTR induced faster decay. The median HL for all human mRNAs was estimated to be 10 hours, with only 5% of mRNAs exhibiting rapid decay. Yang et al. utilized actinomycin D (actD) to inhibit transcription, followed by RNA extraction and microarray analysis to measure decay rates (56).

Three years later, using the same experimental techniques, Sharova et al. measured the decay rates of 19,997 non-redundant genes in mouse embryonic stem cells. Similar to Yang et al., Sharova et al. found that mRNA transcripts with short HL were enriched among genes with regulatory functions in transcription, cell cycle, apoptosis, signal transduction, and development; whereas mRNAs with long HL were enriched in genes related to metabolism and cellular structure. mRNA stability most significantly correlated with structural features of genes, rather than function. Increased mRNA stability showed a strong positive correlation with the number of EJCs per open
reading frame length, and negatively correlated with the presence of PUF and ARE-binding motifs in the 3'UTR (53).

Shortly after, Thomsen et al. performed genome-wide analysis of decay kinetics in all mRNAs during early *Drosophila* development using early pre-zygotic transcription embryos and unfertilized egg collections followed by RNA extraction and microarray analysis. Thomsen et al. set out to elucidate the contributions of maternally and zygotically encoded factors to mRNA degradation; as well as how decay profiles relate to gene function, localization, translation, and turnover. Microarray analysis identified trans-acting factors, such as RBPs and miRNAs that serve as regulators of mRNA decay during early development. Unstable mRNAs were found to function in cell cycle progression, transcription, and mRNA processing; while stable mRNAs functioned in protein synthesis. Specifically, during early development low stability mRNAs often encode proteins whose expression profiles are dramatically altered during the MTZ, while high stability mRNAs encode proteins that are required throughout early development (50).

In 2012, using actD treatment followed by microarray analysis, Neff et al. assessed the decay rates of 5,481 mRNAs present in both iPS cells and the HFFs from which they were derived. Comparison of decay kinetics between iPS and HFF cells identified three major independent regulatory mechanisms: 1) increased stability of histone mRNAs in iPS cells, 2) stabilization of zinc finger protein mRNAs, through reduced levels of the miRNAs that target them, in iPS cell, and 3) C-rich sequence elements in 3'UTR of transcription factors mRNAs are significantly less stable in iPS cells. All three mechanisms may be key regulators of iPS differentiation, and genes differentially expressed between iPS and HFF cells may be factors that regulate, establish and/or maintain pluripotency. The histone: DNA ratio may be higher in iPS cells to increase their ability to rapidly respond to differentiation cues by altering chromatin structure. Interestingly, zinc finger mRNAs have been previously linked to pluripotency, and mRNAs containing C-rich elements have been found to encode transcription factors and proteins required for embryonic development and differentiation (52).

Using metabolic labeling, Dolken et al. studied short-term RNA decay in response to interferon activation in Murine NIH-3T3 fibroblast. Fibroblast cells were treated with actD and 4-thiouridine (4sUd), to inhibit transcription while simultaneously labeling nascent RNA. Following RNA extraction, samples were biotin labeled, and purified to separate 4sUd-labeled RNA from total RNA. This experiment identified novel short-lived mRNAs, encoding proteins involved in cell cycle and apoptosis are coordinately down regulated by interferon activation.

Each of these previous experiments provided valuable insight into mRNA decay kinetics. Many identified major principles that govern mRNA decay, such as mRNAs with short HL are generally involved in transcription and regulation, while mRNAs with long HL encode proteins that function in protein synthesis, metabolism, and maintenance of structural integrity. However, each of these experiments exhibited technical limitations that alter measurements of mRNA decay kinetics. Two of the most common technical limitations found in most mRNA decay studies, and seen in the studies above, are the use of transcriptional inhibitors, as well as lack of cell-type specific decay measurements, specifically *in vivo*.
LIMITATIONS: USE OF TRANSCRIPTIONAL INHIBITOR

Blocking transcription has proved to be a useful technique for studying mRNA decay. Once transcription is inhibited only decay machinery is active, thus mRNA concentration progressively reduces at a rate that directly depends on $k_d$ (4). Transcription can be inhibited by either the use of drugs, such as actD, or conditional mutants, like Y262 in yeast. Actinomycin D inhibits transcription by binding to single stranded DNA at the transcription initiation complex and preventing elongation of mRNA by RNA polymerase II (53). Yeast strain, Y262, contains a temperature sensitive mutation in the major RNA polymerase II subunit, RPB1, which inhibits mRNA synthesis upon a shift to 37°C (56). Although transcriptional inhibition has been widely used to measure decay rates, transcriptional shutoff is often incomplete and may affect cellular physiology, thus affecting HL measurements.

Transcriptional inhibitors may cause non-specific effects and development arrests, fail to inhibit transcription uniformly across all cell-types, and may affect mRNA degradation by eliminating regulatory elements (50). Many genes have been shown to be significantly activated or repressed during transcriptional shutoff. In S. cerevisiae the initial phase of cellular shock causes transcription to be globally slowed, and mRNAs to be stabilized. Osmotic stress also provokes mRNA stabilization and sequestration into P-bodies. In NIH-3T3 cells, actD leads to complete stabilization $\beta$-globin, c-fos, and GM-CSF mRNAs (26). Mechanisms that control mRNA stability, such as miRNA pathways, are activated during cellular stress responses and can also lead to transcript stabilization (49,63). Transcriptional inhibition cause repressed mRNAs to be stabilized, while induced mRNAs are destabilized (55).

An extensive bias in mRNA decay measurements has been demonstrated due to stress shock caused by transcriptional shut off. For example, in S. cerevisiae, the HLs of ribosomal protein genes have been significantly undervalued since transcriptional shutoff causes destabilization of these mRNAs (4). HL measurements can also be significantly overestimated because most RNA Polymerase II inhibitors do not produce immediate transcriptional shut off. Overall, HL measurements based on mRNA decay after blocking transcription are inherently imprecise for transcripts, specifically medium and long-lived mRNAs (73).

LIMITATIONS: LACK OF CELL-TYPE SPECIFICITY

One of the most recent technical challenges when studying mRNA decay kinetics is lack of cell-type specific decay measurements. Over time it has become more evident that in order to gain an accurate representation of decay kinetics, mRNA cell-type must be taken into account. A defining feature of multicellular organisms is their ability to differentially utilize genomic information to generate morphologically and functionally specialized cell types during development. For example, 35% of Drosophila embryonic transcripts are tissue specific, or exhibit restriction in temporal or spatial gene expression. Regulation of differential gene expression, both temporally and spatially, is a driving force of the developmental process. During nervous system development distinct
neural cell types obtain and preserve their identity through differential gene expression. Neurons, with their huge axonal and dendritic arbors, and high levels of transport and secretion, are among the most transcriptionally active cells in the body. Thus, brain tissues often have heterogeneous mRNA composition (51,60).

Gene expression profiling analyzing cellular responses to different stimuli or conditions is routinely performed on total cellular RNA. Analysis of heterogeneous mRNA pools results in lack of cell-type specificity, temporal and spatial resolution, and often the inability to distinguish changes in transcription versus change in decay. For example, experiments by Thomsen et al., although they did not require the use of transcriptional inhibition, lack cell-type specific decay measurements and may provide an imprecise representation of true mRNA decay kinetics during early Drosophila development.

Transcript stability significantly contributes to differential expression. Consequently, mRNAs from specific cell types exhibit differential decay kinetics (64). Decay rates have been shown to significantly vary between whole organisms and specific cell types. For example, in Drosophila embryos Hsp83, hunchback, and cyclin B mRNAs are identified as eliminated when total RNA levels are analyzes, but in situ hybridization shows differential spatial stability (65). Drosophila embryonic mRNAs also exhibit differential stability in different regions of the cytoplasm.

The factors that influence mRNA decay vary between cell types due to the variation in cis- and trans-elements between different cell types. In differentiated C2C12 myoblasts GREs have a more significant impact on decay than AREs; whereas AREs contribute more significantly to decay in embryonic stem cells. GREs may be more likely to be present in muscle-specific mRNAs and/or GRE binding factors, like CUGBP1, may be more active in myoblasts (29). Also, most miRNAs in Drosophila are tissue or cell-type specific, and inclusion of irrelevant cell types during analysis may mask the action of miRNAs (33). These findings support the concept that cis and trans regulatory elements affect mRNA stability in a cell-type specific manner. Analysis of cell-type specific transcripts and regulatory factors will provide important insight into the mechanism used to generate cellular diversity (62,63,65).

The powerful new tool of genome wide microarrays of purified cell types has enabled the identification of cell-type specific transcriptional signatures. For example, Berger et al. used functional genomics to identify a small set of genes differentially expressed between neuroblasts and differentiating neurons in Drosophila. Cell type specific purification identified 28 neuroblast-specific genes that may play a key role in maintenance of neuroblast fate, and may provide insight into stem cell self-renewal and differentiation (34). These genes are pertinent candidates as regulators of neural progenitor self-renewal in Drosophila and mammals, and highlight the importance of cell-type specific profiling and analysis. Genome wide microarrays of purified cell-types have also led to the identification of transcriptional signatures in pyramidal neurons and interneurons in the neocortex, dopaminergic and serotonergic neurons, and striatal neurons in the basal ganglia (60).
**TU-TAGGING: A METHOD FOR MEASURING CELL-TYPE SPECIFIC mRNA DECAY**

Lack of high quality mRNA decay data and mapping of decay motifs indicates the need for genome wide cell-type specific decay measurements. For example, only a small handful of trans-acting factors are known in *Drosophila*, such as Pumilio, Smaug, and miR-390, and not much is known about the common features across protein regulators. There is a need for systems biology and bioinformatics to attain a comprehensive understanding of global mRNA decay regulatory processes. Larger datasets would allow the systematic search for common features in transcripts with similar decay patterns, and establish whether functionally related genes share common decay kinetics. There is also a necessity of unperturbed in vivo measurements of mRNA decay kinetics. *In vivo* cell-type specific decay studies will provide a more accurate representation of regulatory networks that govern mRNA stability (32,50,55,56).

Standard microarrays measure mRNA abundance, but cannot distinguish between changes in mRNA abundance as due to synthesis or decay, and thus does not provide significant insight into the mechanisms of mRNA regulation (49,61,64). However, pulse-chase experiments allow us to measure mRNA decay kinetics by labeling transcripts and measuring their decay over time. Pulse-chase experiments together with TU-Tagging provide the most effective and dynamic method for studying mRNA regulation. For example, Munchel et al. determined the decay rates of all mRNAs in *S. cerevisiae* under normal as well as various growth and stress conditions using TU-Tagging. This study revealed that mRNA turnover is highly regulated both at the system-wide level and cell-type specific level (54).

TU-Tagging overcomes the technical limitations of traditional methods for studying mRNA decay. TU-tagging does not require the use of transcriptional inhibitors. TU-Tagging can be used to measure both genome wide and cell-type specific decay rates, which is useful for when isolation and/or separation of mRNA from specific cell types is difficult. It also provides unperturbed in vivo labeling which can be used in dynamic experimental conditions, such as stress response in yeast or the response of mouse dendritic cells to glucose and galactose (4,25). Technical challenges, such as poor temporal and spatial resolution of decay and inability to distinguish transcription from decay, can be overcome using TU-Tagging. Precise measurement of synthesis and decay kinetics using nascent, unlabelled, and total RNA pools provides more insight into regulation of differentially expressed mRNAs.

Thio-group containing nucleosides, such as 4sUd and 4-thiouracil (4TU), can be introduced into nucleoside salvage pathways in eukaryotic cells allowing for nondisruptive metabolic labeling of nascent mRNA (49). All metabolic labeling can be attributed to 4sUd or 4TU, as eukaryotic mRNAs normally do not contain thio-groups. *Toxoplasma gondii* nucleotide salvage enzyme uracil phosphoribosyltransferase (UPRT) can be used to biosynthetically label newly synthesized RNA *in vivo*. When a modified form of uracil, 4TU, is provided as a substrate to UPRT, the resulting product, 4-thiouridine-monophosphate (4UMP), is incorporated into nascent RNA. This labeling process is known as TU-Tagging. TU-Tagging achieves both spatial (when UPRT is placed under a cell-type specific promoter) and temporal (when 4TU is applied at a specific time) resolution. Nascent thio-labeled RNA can be tagged and purified allowing newly synthesized to be separated from total RNA. The innovation of TU-Tagging can be
used to measure mRNA synthesis and decay rates, as well as identify cell-type specific transcripts (62,64). (Figure 6A, Figure 6B)

TU-tagging studies in Toxoplasma showed that 4TU is incorporated one in every 26 uridines and after only one hour of labeling nascent thio-labeled RNA is approximately 5-10% of the total RNA pool (49). Exposure to 4TU or 4sUd does not affect gene expression or protein synthesis. Cells can be cultured in the presence of thio-containing nucleosides for more than 24 hours without gross toxic effects (61,63,64).

RNA tagging using 4sUd, the nucleoside form of uracil, relies on the activity of uridine kinase to convert 4sUd into 4sUd-monophosphate. TU-Tagging using 4sUd does not require the presence of UPRT and lacks cell-type specificity (64).

**RESEARCH AIMS**

Microarray experiments in *Drosophila* embryos have established that major developmental transitions, such as gastrulation and imaginal disc formation, are mirrored by global changes in gene expression (50). Also processes that govern early embryonic body patterning in *Drosophila* are the best-understood examples of complex transcriptional regulatory networks during development (51). These two features make *Drosophila* embryos an ideal model organism for studying both genome wide and nervous system-specific mRNA decay kinetics.

The overall goal of this research project is to study the dynamic regulation of mRNA decay during *Drosophila* neural development. This will be achieved through three research aims: 1) identification of global decay kinetics and validation of TU-Tagging as a method to measure mRNA decay, 2) identification of nervous system specific decay kinetics, and 3) identification of candidate RBPs and miRNAs that control neural mRNA decay.
CHAPTER 2: METHODS

FLY STOCKS

cDNA encoding TgUPRT has been cloned into pUAST construct for generation of transgenic *Drosophila*. Sp/Cyo; UAS-HA-UPRT flies served as an experimental control in the absence of a GAL4 line. Prospero-GAL4 (Pros-GAL4) transgenic line was used to drive nervous system specific GAL4 expression, as *prospero* is a pan-neuronal DNA binding transcription factor present throughout the nervous system. By crossing the Pros-GAL4 line to the Sp/Cyo;UAS-HA-UPRT nervous system TU-Tagging was achieved.

UAS-FNE flies were obtained from the Bloomington *Drosophila* Stock Center and were used to generate UAS-FNE; UAS-HA-UPRT. These flies were then crossed to Pros-GAL4 to achieve overexpression of FNE in the nervous system.

4sUd / 4TU TREATMENT AND RNA EXTRACTION

Embryos were collected at room temperature for approximately 3 hours, then incubated for 14 hours at 18°C. Development at 18°C is slowed by about 1.5-fold compared to 25°C. 14 hours at 18°C is equivalent to 9.3 hours at 25°C; making embryos range from 9.3 – 12.3 hours old. This corresponds to stages 12-15 of embryonic development that cover the major stages of nervous system development.

To treat embryos with 4sUd / 4TU embryos and yeast paste from apple caps were gently scraped and washed into a small mesh basket using a squirt bottle filled with ddH₂O. Embryos were then soaked in 50% bleach for 3 minutes to remove outer chorion layer, rinsed lightly with ddH₂O, and then dipped in isopropanol for approximately 5 seconds. (Embryos should immediately drop to the bottom of the mesh basket indicating that the isopropanol rinse is complete). Embryos were subsequently air dried for 30 seconds by first blotting mesh with a KimWipe then carefully inverting mesh basket tube to allow excess isopropanol to flow out. Embryos were submerged in octane for 3 minutes, which permeabilizes the embryonic membrane allowing 4sUd / 4TU to enter. Embryos were then air-dried for 3 minutes by carefully unscrewing mesh basket tube and removing mesh which is laid flat on a KimWipe to dry, while tube is cleaned with a KimWipe to remove any excess octane. Embryos were then transferred to *Drosophila* 22 insect media (D22) containing either 400mM 4sUd or 4TU (Sigma-Aldrich) for 1 hour at 25°C (30°C when Pros-GAL4 is present). If a chase period is desired, embryos were immediately transferred to D22 media containing 0.5M Uridine for either 1 hour or 3 hours at 25°C (30°C when Pros-GAL4 is present). (Actinomycin D treatments required embryos to be placed in D22 media containing 0.4M ActD during 1-hour pulse).

Mesh basket containing embryos was removed from D22 media and blotted dry on a paper towel. Mesh was then removed from mesh basket and carefully rolled vertically, to form a column like tube, and place into a 1.5mL RNase-Free microcentrifuge tube containing 1mL of 1X PBS. Embryos were centrifuged at 16,000g
for 1 minute; mesh was gently removed, and then centrifuged again at 16,000g for 1 minute. 1X PBS was removed and the embryos were homogenized in 100-200µL of Trizol and stored at -80°C until RNA extraction.

TU-Tagging homogenized samples from each of the three time points were combined before RNA extraction. Total RNA was extracted from Trizol using standard methods with the following additional step: Phase Lock Gel Heavy 2mL tubes (5-Prime) were used to effectively separate RNA from DNA, lipids, and proteins.

**PURIFICATION OF TU-TAGGED mRNA**

Biotinylation of 4sUd- and 4TU-labeled RNA was performed using EZ-Link Biotin –HPDP (Pierce) dissolve in dimethylformamide (DMF) at a concentration of 1mg/mL and stored at -20°C. Biotinylation was carried out in 10mM Tris (pH 7.4), 1mM EDTA, and 2uL Biotin-HPDP per 1µg of RNA for 1.5-3 hours at 25°C-28°C protected from light. Approximately 50µg of total RNA is used for each biotin-labeling reaction. Biotinylated RNA is resuspended in 50-100µL of RNAse-free water.

Biotinylated RNA was denatured at 65°C for 10 minutes followed by immediate cooling on ice for 5 minutes. Biotinylated RNA was captured using μMACS streptavidin beads and columns (Miltenyi Biotech). 1µL of μMACS streptavidin beads was added per 1µg of RNA (up to 100µg) and incubated with rotation for 15 minutes at room temperature. RNA-bead mixture was then applied to equilibrated μMACS magnetic columns. To recover the unlabelled preexisting RNA the flow-through of the RNA-bead mixture is captured and kept at -80°C until RNA is precipitated. Columns are washed three times with 0.9mL 65°C washing buffer (100mM Tris, pH 7.5, 10mM EDTA, 1M NaCl, 0.1% Tween20) followed by three washes with room temperature washing buffer. The last wash of room temperature washing buffer is captured and kept at -80°C until RNA is precipitated, to test efficiency of purification. 4sUd- or 4TU-labeled RNA was eluted by the addition of 100µL of freshly prepared 5% β-mercaptoethanol (BME) solution, followed by a second elution 3 minutes later. Labeled RNA was extracted from BME elution using RNasy MinElute Spin columns (Qiagen). RNA from flow-through and last-wash fractions was precipitated using NaCl, isopropanol, and linear acrylimide (49,54,62,64,77).

Purified RNA from each time point was combined and precipitated using 1/10 volume of 5M NaCl, equal volume of isopropanol, and 1µL of linear acrylimide. 0.5µL of purified RNA was combined with 0.5µL of RNase-Free H2O for measuring 1:2 concentration of RNA using a Nanodrop.

**MICROARRAY ANALYSIS**

**cRNA SYNTHESIS, CY DYE LABELING, AND HYBRIDIZATION**

Between 75ng–200ng of purified RNA was amplified and labeled using the Agilent Low Input Quick Amp Labeling Kit according to the manufacturer’s recommendations. The amplified and fragmented biotinylated cRNA was hybridized to
an Agilent 4x 44,000 array using standard procedures. The microarray experiments were performed according to Agilent’s protocol (Version 6.5, May 2010) and scanned using Axon Instruments GenePix 4000B. Fluorescent ratios for each microarray element were recovered and normalized using GenePix Pro 6.1 (62).

**SPIKE-IN CONTROLS, NORMALIZATION, AND QUALITY CONTROL**

Normalization of the fluorescent intensities measured in pulse-chase microarrays cannot rely on the common normalization approach where signal intensities are adjusted to make the average mean fluorescence minus background (Mean532-B532) equal to 1.0 across all time points. (532 corresponds to the scanning channel used to read Cy3 dye intensity). This is because there are often an increasingly small number of spots with detectable signal at each subsequent chase time point. Therefore it cannot be assumed that the average Mean532-B532 is equal to 1.0. However normalization is still necessary to correct for variability in cRNA synthesis and Cy labeling. Therefore 1P, 1C, and 3C microarrays were normalized using spike-in controls from the Agilent One Color RNA Spike-In Kit.

The Spike-In Kit contains a mixture of 10 in vitro synthesized, polyadenylated transcripts derived from the Adenovirus E1A gene. Spike-In transcripts are premixed at concentrations that span six logs and differ by one log or half log increments. Each microarray contains 22 different spots for each of the 10 (+) E1A transcripts. For each (+) E1A transcript the (MeanF532-B532) value was average across all 22 spots. These values can then be plotted against the log of the concentration for each time point, producing a “Spike-In Control” graph. The graph contains three different lines representing the 1P, 1C, and 3C time point. Once this is obtained the average (Mean532-B532) values that constitute the most linear portion that overlaps between all three lines, is averaged (Figure 8). For example, if (+) E1A_r60_a135, (+) E1A_r60_a20, (+) E1A_r60_a22, (+) E1A_r60_a97 constitute the most linear portion found in all three lines, then the corresponding average (Mean532-B532) values used to plot the graph, and again averaged. This produces an average fluorescent intensity for the linear most portion of each line. These values are then used to calculate the fold-change between each microarray. The array with the highest average is set as the reference. Once the fold-change value is obtained, this value can then be used to normalize the arrays by multiplying the fold-change value at each time point to every Mean532-B532 value in the corresponding array. (Figure 7)
Once normalization is complete fluorescent intensity spots that fall below the given cut-off value are removed. The cut-off value is based on the average fluorescent intensities across all time points for known maternal genes. Since TU-Tagging was performed after MTZ all tagged transcripts should be strictly zygotic and synthesis of any remaining maternal genes will be minimal, and should therefore exhibit low fluorescent intensity on each microarray. The cut-off value is also based on the average fluorescent intensity of the lowest dilution Spike In control, (+) E1A_r60_3. For example, if the average value of all 22 (+) E1A_r60_3 spots is 72, then any genes with a 1-hour pulse fluorescent intensity less than 72 are removed. This ensures that spots that are comprised of all background fluorescence and no real signal are removed from the analysis.

In order to check that normalization is accurate, taking the average of all now normalized (MeanF532-B532) fluorescent values should produce a value close to 1.0.

**CALCULATION OF mRNA DECAY KINETICS AND TRANSCRIPT HALF-LIFE**

Decay coefficient, transcript half-life, and $R^2$ values were obtained utilizing R program software. Overall methods used for calculations were similar to those used by Munchel et al. The equation for a single exponential decay curve is $A = A_0 e^{-kt}$ where $A$ is the number of mRNA transcripts present at some time, $t$, given the number of mRNA transcripts at 0 minutes and the decay constant ($k$). A single exponential curve was fitted to the mRNA abundance for all time points between 0 and 3 hours by calculating the decay constant that gave the greatest nonlinear regression ($R^2$) value. The half-life for each gene was then calculated using the equation $t_{1/2} = \ln(2) / k$. 

![Spike In Controls Processed Signal vs. Concentration](image)
CHAPTER 3: RESULTS

4-THIOURIDINE PULSE-CHASE ALLOWS GLOBAL mRNA DECAY MEASUREMENTS IN INTACT DROSOPHILA EMBRYOS

In order to obtain mRNA decay rates for all zygotically-transcribed genes, a variation of the TU-Tagging approach was used to label nascent mRNAs transcribed in all tissues of stage 12-15 embryos, corresponding to approximately 9.3-12.3 hours after egg laying (AEL). This modified TU-Tagging method utilized 4sUd to tag mRNAs independent of UPRT activity, therefore tagging all nascent mRNAs with no cell-type specificity. A 1-hour 4sUd pulse was followed by a chase period in which embryos were transferred to D22 media containing a molar excess of unmodified uridine. 4sUd-RNA was purified at three separate time points: following the 1 hour pulse, a 1 hour “chase” in uridine, and a three hour “chase” in uridine (Figure 8A).

One potential limitation of this approach is continued incorporation of 4sUd into nascent mRNAs during the chase period, either through recycling of 4sUd from degraded transcripts, or incomplete replacement of 4sUd with unmodified uridine in nucleotide pools. This could result in over estimating mRNA half-lives, as transcripts would seem more stable over time due to inappropriate incorporation of 4sUd. In order to test if such an effect does exist, two experiments were performed in parallel: 4sUd tagging both with and without the transcriptional inhibitor actD in the chase media. Inclusion of actD would ensure that no nascent mRNAs, that could incorporate 4sUd, are made during the chase (Figure 8B).

Analysis revealed that the distribution of mRNA half-lives was similar for pulse-chase experiments performed with or without actD included in the chase media (Figure 8C). The median mRNA half-life (HL) was slightly higher in uridine only experiments, reflecting a minor increase in mRNA HL for the majority of genes. These results suggest that there may be a low level of 4sUd, or 4TU, tagging during the chase; however, alternatively, this shift in median HL could be due to the effects of actD on mRNA transcripts. ActD has been shown to either stabilize specific mRNAs, or accelerate the degradation of mRNAs, like those encoding ribosomal proteins in S. cerevisiae. Therefore it is a possibility that treatment with actD alters cellular processes in a way that accelerates mRNA decay.

Overall, differences in individual HL values were minimal between the two data sets: 92.2% of mRNAs exhibited no significant differences HL measurements done with or without actD, 1.1% of mRNAs had a two-fold or greater HL value based on the actD experiments, and 6.7% of mRNAs had a two-fold or greater HL value based on the uridine only experiments (Figure 8D). Importantly, mRNAs encoding proteins in distinct functional classes had similar decay kinetics in both datasets; such as slow decay of ribosomal protein transcripts and rapid decay of mRNAs encoding transcription factors and cell cycle regulators (Figure 8E). This trend, of mRNAs in distinct functional class having similar decay rates across both data sets, was further confirmed by gene ontology (GO) analysis.
mRNAs EXHIBIT NEURAL-SPECIFIC DECAY KINETICS

Modification of the TU-Tagging protocol allowed pulse-chase analysis of mRNA decay in neural tissues. 1-hour pulse labeling with 4TU in intact pros>TgUPRT embryos was followed by either a 1-hour or 3-hour chase in media containing a molar excess of unmodified uridine. 4TU-RNA was purified at the same time points as the 4sUd pulse-chase experiments, as described above. pros>TgUPRT experiments allowed for the comparison of neural-specific HL calculations to whole embryo HL calculations (which provide the average decay kinetics of a transcript across multiple tissues). The overall distribution of HL values from neural-specific and whole embryo decay analyses were very similar: median HLs for whole embryo and nervous system was 74.5 and 75.7 minutes, respectively (Figure 9A). This data suggests that global decay kinetics and mRNA turnover is not significantly different in the nervous system compared to other embryonic tissues.

Although the majority of mRNA transcripts show similar decay kinetics in nervous system-specific tissues and whole embryo tissues, there is however, a subset of mRNA transcripts that show differential decay kinetics between the two datasets (Figure 9B). Genes with a FlyBase annotated neural development functional and a 2-fold or greater change in HL between the two datasets are labeled. Interestingly, mRNAs that show increased stability in the nervous system are important for nervous system development and maintenance, and mRNAs with decreased stability in the nervous system are important for inducing changes in gene expression (Table 3 and Table 4).

Gene ontology categories based on high and low stability mRNAs were compared for whole embryo and nervous system specific datasets (Figure 9C, Figure 9D). As previously displayed, by Munchel et al. and Sharova et al., GO categories of mRNAs from whole embryo identify that high stability mRNAs have functional roles in
translation and cytoskeleton organization, while low stability mRNAs have functional roles in cell fate determination and transcription (50,53). Nervous system specific GO categories also identify cytoskeleton organization and translation as major functions of high stability mRNAs, as well as mRNAs that function in cell fate determination in the low stability group. However, also enriched in this low stability group are mRNAs that have specific roles in nervous system function. This indicates the importance of cell-type specific mRNA decay studies that are able to identify mRNAs exhibiting differential decay kinetics in a specific cell-type compared to the whole organism. These nervous system specific mRNAs, which may play critical roles in nervous system development, may otherwise have been overlooked or decay rates would have been mis-calculated if only whole embryo decay measurements had been made.

### Table 3

<table>
<thead>
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<th>Gene</th>
<th>Annotated Function</th>
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<tr>
<td>Shet1</td>
<td>A-type transient outward potassium channel activity, synaptic transmission</td>
</tr>
<tr>
<td>taf1</td>
<td>Border follicle cell migration and motility, 6-24hr AEL</td>
</tr>
<tr>
<td>Foss2</td>
<td>Synaptic organization, learning and memory, expressed in PNS 6-24hr AEL</td>
</tr>
<tr>
<td>Kbp54D</td>
<td>Microtubule motor activity, axon guidance, anterograde transport, sensory perception of sound and smell</td>
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### Table 4

<table>
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<tr>
<th>Gene</th>
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<td>Rpl23</td>
<td>Protein binding function, involved in neurogenesis, expressed in CNS throughout embryogenesis</td>
</tr>
<tr>
<td>btf</td>
<td>Photoreceptor morphogenesis, associates with differentiating cells, negative regulator of axon guidance and extension, 0-6hr AEL</td>
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<tr>
<td>Art72F</td>
<td>GTPase activity, triggers actin polymerization, lamellipodium formation</td>
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<tr>
<td>tsfB</td>
<td>Actin-binding, regulates actin dynamics and remodeling</td>
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<tr>
<td>Snap24</td>
<td>Neurotransmitter secretion, vesicle-mediated transport</td>
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<td>tuff</td>
<td>Synaptic target recognition, regulation of dendrite morphogenesis and axon guidance, lateral inhibition, 6-24hr AEL</td>
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<tr>
<td>ey</td>
<td>Termination of cell fate in the eye, neuron differentiation, expressed in PNS 8-12 AEL</td>
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NEURAL mRNA STABILITY CORRELATES WITH LOCALIZATION AND FUNCTION

Multiple studies in various model organisms measuring global and cell-type specific mRNA decay kinetics has revealed an important principle of posttranscriptional gene regulation: mRNA decay rates correlate with gene localization and/or function. This principle was confirmed through analysis of the nervous system specific dataset. Exponential decay curves of neural specific transcripts demonstrate that mRNAs segregate to distinct functional categories based on their stability (Figure 10A). High stability mRNAs encode synaptic proteins, including several whose mammalian orthologs are known to be locally translated at synaptic terminals. Low stability mRNAs encode proteins that regulate cell fate specification and are known to be differentially expressed between neural progenitors and progeny or between different subsets of neurons (Table 5 and Table 6).
Odds ratio analysis, which is the measure of the strength of association between two sets of values, is defined as the odds of an event occurring in one group compared to the odds of that same event occurring in another group. An odds ratio greater than 1.0 indicates that the condition is more likely to occur in set 1 compared to set 2, while an odds ratio less than 1.0 indicates that the condition is less likely to occur in set 1 compared to set 2.

Odds ratio analysis compared the likelihood of high versus low stability mRNAs to be present or absent in a group of transcripts known to be localized to dendrites. Of the 154 rat hippocampal CA 1 region dendritic-localized mRNAs identified by Zhong et al., 20 mRNAs had *Drosophila* orthologs. The odds ratio test compared the odds of finding these 20 dendrite-localized mRNAs in the 1000 highest stability mRNAs versus the odds of finding the 20 mRNAs in the 1000 lowest stability genes. The odds ratio test found a significant enrichment of the 20 dendritic mRNAs in the high stability group, while they were completely absent from the low stability group. Odds ratio analysis also compared the likelihood of high versus low stability mRNAs to be present or absent in a group of known neural fate transcription factors. The 34 transcription factors were obtained from Flybase.org, and compared against the 1000 most and least stable mRNAs. As expected, significant enrichment of the 34 transcription factors were found in the low stability mRNAs, and were absent from high stability genes (Figure 10B).

Odds ratio tests indicate that dendrite-localized mRNAs distinctly appear among highly stable mRNAs, whereas mRNAs encoding neural transcription factors solely appear among low stability transcripts. This may suggest that increased mRNA stability is a common feature of genes that are trafficked and locally translated with the nervous system; while decreased stability is a potential requirement of neural fate transcription factors.
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![Diagram](image)

**Figure 10. Neural mRNA Stability Correlates with Localization and Function.**

**A.** mRNAs highlighted in red are high stability and have mammalian orthologues that are known to be locally translated in synaptic terminals. mRNAs highlighted in blue are low stability and have mammalian orthologues that are known to be cell fate determinants. **B.** Odds ratio analysis of high stability mRNAs (highlighted in red) and low stability mRNAs (highlighted in blue). High stability mRNAs are enriched in a group of 20 known dendrite-localized mRNA (Zhang et al.). Low stability mRNAs are enriched in a group of 34 known Drosophila neural fate transcription factor mRNAs (Interactive Flybase.org). *** = p < 0.001

*Table 5 and Table 6. Neural mRNA Stability Correlates with Localization and Function.*

*Genes from Figure 10A. Annotated function (Interactive Flybase.org). Peak expression listed if annotated.

*Table 5. High stability mRNAs, locally translated. Table 6. Low stability mRNAs, cell fate determinants.*
IDENTIFICATION OF AU-RICH ELEMENTS ENRICHED IN DISTINCT NEURAL mRNA DECAY CLASSES

Odds ratio analysis of neural-specific mRNAs revealed significant enrichment of both high and low stability mRNAs in a group of 1,004 predicted ARE-containing mRNAs (Figure 11A, Supplemental Figure 1A). This indicates that in Drosophila, similar to mammalian cells, regulation by AUBPs does not solely promote mRNA decay, but rather a portion of AUBPs act to stabilize mRNAs. Odds ratio analysis also indicates that regulation of gene expression through ARE-mediated decay is a widely used mechanism in the nervous system.

Comparison of predicted ARE-containing mRNAs to the dataset of mRNAs known to be dendrite-localized (Zhong et al.) revealed three mRNAs: pum, mub, and Spn to be present in both groups. Each of the three mRNAs is predicted to contain a functional class I ARE. All three mRNAs have important functional roles in development of the Drosophila nervous system, and therefore may be preferentially stabilized by AUBPs during embryogenesis. However, comparison of these mRNAs to the pros>TgUPRT dataset of neural-specific mRNAs reveals no significant enrichment. None of the three transcripts is present in the 1000 most stable or the 1000 least stable group of mRNAs (Supplemental Figure 1B).

Comparison of predicted ARE-containing mRNAs to the dataset of known Drosophila neural-fate transcription factors (Interactive Flybase.org) revealed that 17 mRNAs are present in both groups. 13 mRNAs contain a functional class I ARE, while the remaining 4 mRNAs contain a functional class II ARE. Each of the mRNAs has an important functional role in fate determination, differentiation, or morphogenesis during nervous system development. These mRNAs may therefore be targeted for rapid degradation by AUBPs during embryogenesis. Comparison of these mRNAs to the pros>TgUPRT dataset of neural-specific mRNAs reveals no significant enrichment in neither the high or low stability groups (Supplemental Figure 1C). However, two mRNAs: longitudinal lacking (lola) and seven up (svp) are present in the distinct decay classes. lola, which functions through protein binding and is involved in neuron differentiation, immune response, and apoptosis; is found in the 1000 least stable neural-specific mRNAs. Interestingly, svp, which is a transcription factor involved in protein binding, synaptic transmission, cell fate determination, differentiation, and proliferation; is present in both the 1000 most stable mRNAs, as well as the 1000 least stable mRNAs.

The 1000 most and 1000 least stable neural-specific mRNAs from the pros>TgUPRT dataset were compared against the five different ARE subgroups identified by ARE database (Cairrao et al.). No significant enrichment was found in ARE Groups 1-4 (Supplemental Figure 1D-G). However, ARE Group 5, which contains transcripts with a WWW[UAUUUAU]WWW motif (W = A or U), was significantly enriched in both the 1000 most stable mRNAs and the 1000 least stable mRNAs, with p-values of .002 and .02 respectively (Figure 11B). This indicates that this motif may be recognized by both stabilizing and destabilizing AUBPs; and may induce cooperative and/or competitive binding of AUBPs to regulate mRNA stability.

miR-315 database, which consists of 415 mRNAs, was compared against the ARE database (ARED) to identify mRNAs that are both putative targets of miR-315 and
contain a functional ARE. This produced 114 “ARE-miR-315” mRNAs. These mRNAs were then compared against the 1000 most and the 1000 least stable neural-specific mRNAs from the pros>TgUPRT dataset. ARE-miR-315 mRNAs were compared against both high and low stability mRNA groups. 11 ARE-miR-315 mRNAs were found in the high stability group, and 8 ARE-miR-315 mRNAs were found in the low stability mRNA group, however no significant enrichment was found in either group (Supplemental Figure 1H).

Neural-specific mRNAs from the pros>TgUPRT dataset were also compared against the database of 400 neural mRNAs that undergo 3'UTR extension during embryogenesis (Smibert et al.) Significant enrichment was found for 3'UTR extended mRNAs within the high stability mRNA group, with a p-value of 0.0007 (Figure 11C). Although no significant enrichment for ARE-miR-315 mRNAs was found within the low stability group (Supplemental Figure 1E), both high and low stability 3'UTR extended transcripts were further analyzed. Transcripts previously annotated by ARED were excluded from the analysis, since the 3'UTRs of these transcripts were already predicted to contain a functional ARE. Within the high stability mRNA group, 23 neural-specific mRNAs with extended 3'UTRs were found, while 18 were found within the low stability mRNA group; none of which had previously been annotated to contain AREs. 3'UTR extensions are thought to harbor regulatory motifs that subject transcripts to unique neural-specific posttranscriptional regulation. Therefore it was hypothesized that these mRNAs may contain regulatory motifs within the extended 3'UTRs sequences. 3'UTR sequences of each mRNA was obtained from Interactive FlyBase and searched for AU-rich motifs.

Each 3'UTR was searched for the following five elements: 1) number of AUUUA motifs 2) number of repeats of AUUUA motif (repeats of the AUUUA motif within an AU rich context is a hallmark of AREs) 3) Group 5 ARE: WWW[UAUUUAU]WWW 4) UUAUUUAUUU (predicted to be the most fundamental AU-rich motif capable of conferring instability) and 5) UUUUAAA and UUUGUUU (predicted targets of stabilizing AUBP ELAV) (Table 7 and Table 8). Both high stability and low stability extended 3'UTR mRNAs contain multiple AUUUA motifs. 61% of high stability transcripts and 55% of low stability transcripts contain AUUUA repeats within their extended 3'UTRs. Although the percentage of mRNAs with AUUUA repeats is higher for the high stability group, mRNA transcripts from the low stability group contain more AUUUA repeats per 3'UTR, with a maximum of 8 repeats found in fs(1)h mRNA. This supports the observation that mRNAs containing multiple AUUUA motifs, especially motifs that occur in tandem, often exert a destabilizing effect on mRNA.

A search for the Group 5 ARE motif within the extended 3'UTRs revealed that this ARE motif is present in both high and low stability mRNAs. 48% of high stability transcripts and 61% of low stability transcripts contain the WWW[UAUUUAU]WWW motif. Similar to the previous results, this finding also supports the observation that the Group 5 ARE motif may preferentially confer transcript instability. Since the UUAUUUAUUU motif is thought to cause transcript instability it is expected that there would be a significant enrichment of this motif in the low stability mRNA group; however no significant difference was found between the two stability groups for presence of the UUAUUUAUUU motif. Also, no significant difference was found between both groups for the presence of either the UUUUAAA or UUUGUUU motif. 89% of high stability mRNAs
contained either motif, and 87% of low stability transcripts contained either motif. However, once again, the low stability mRNAs had a much higher occurrence of these motifs per 3’UTR.

A

Most stable mRNAs: ave. + 1s.d. (n=739)
Least stable mRNAs: ave. - 1s.d. (n=713)

Predicted ARE-containing mRNAs (n=1,004)

(Cairraco et al., MCB, 2009)

B

All Data
ARE-Group 5

10236
895
506

1000 Least Stable
1000 Most Stable

ARE-Group 5

893
100
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Figure 11: Identification of ARE-Rich Elements Enriched in Distinct Neural mRNA Decay Classes.
A. Odds ratio analysis of high stability mRNAs (highlighted in red) and low stability mRNAs (highlighted in blue). Both high and low stability mRNAs are enriched in a dataset of 1,004 mRNAs predicted to contain functional ARE (Carrap et al.) *** p < 0.001 B. Venn Diagram showing enrichment of both high stability mRNAs (p = 0.022) and low stability mRNAs (p = 0.002) in Group 5 AREs compared to whole data set C. Venn Diagram showing enrichment of high stability mRNAs (p = 0.0007) within group of 3'UTR extended mRNAs compared to the whole dataset.

Table 5 and Table 6. Neural mRNA Stability Correlates with Localization and Function.
Genes from Figure 11C. 3'UTR coordinates were obtained from Smibart et al. 3'UTR sequences were obtained from Interactive Flybase.org. Each sequence was searched for AUUUA pentamer, and AUUUA repeats. Group 5 ARE. UUAUUAUUU, UUUUAAA, and UUUUUGUUU motifs. Table 7. High stability mRNAs with extended 3'UTRs, not annotated in ARE5. Table 6. Low stability mRNAs with extended 3'UTRs, not annotated in ARE5.
CHAPTER 4: DISCUSSION

TU-TAGGING AS A METHOD TO MEASURE mRNA DECAY

This work further demonstrates that metabolic labeling using 4sUd or 4TU in combination with pulse-chase experiments is an effective and accurate method for measuring mRNA decay kinetics. TU-Tagging not only provides an approach for in vivo cell-type specific decay measurements, it also does not require the use of a transcriptional inhibitor. Comparison of median, as well as individual transcript, mRNA HLs for pulse-chase experiments performed with and without an actD chase revealed similar decay kinetics overall. Gene ontology categories further confirmed this similarity by producing similar functional groups for the high and low stability mRNA decay classes. GO analysis also revealed that global mRNA decay kinetics produces distinct functional classes that share similar decay rates, a reoccurring principal in mRNA decay studies. Analysis of both data sets also importantly shows that mRNA decay typically follows simple first order kinetics, and that the majority of transcripts can be fit to a single exponential decay curve with $R^2 \geq 0.8$. This suggests that the chase kinetics are efficient, and that most Drosophila genes are degraded with a first order decay rate (4,54).

The actD chase and uridine only chase datasets were also used to test for potential experimental biases in TU-Tagging decay measurements. Since the thio-containing nucleoside is incorporated approximately one in every 26 uridine, there is the possibility that longer transcripts, that contain higher uridine frequencies, will falsely appear more stable due to the increased number of 4sUd, or 4TU, that can be incorporated. However, there was no detected correlation between uridine number and HL for each transcript (data not shown). Experimental bias also has potential to arise from the abundance of each transcript at the beginning of the chase period. If a bias exists transcripts with higher abundance at the onset of the chase period may appear to have longer HLs, even though they are decayed relatively rapidly; while transcripts with low abundance at the onset of the chase period will appear to have extremely short HLs although they are stable transcripts. Test of this bias revealed no correlation between transcript abundance and HL (data not shown) (61,62).

The multiple analyses described validate that 4-thiouridine pulse-chase effectively and accurately measures mRNA decay rates in intact embryos, without the use of actD or other transcriptional inhibitors. Overall, measurements of mRNA decay by TU-Tagging, with uridine only chase, is not stifled by recycling of 4sUd, and significantly mirrors decay measurements made with actD. Although it is impossible to definitively conclude which dataset displays “true” mRNA HL values, the effects of 4sUd or 4TU recycling on mRNA decay measurements are nominal in comparison to the effects of transcriptional inhibition using actD. Treatment with actD does not produce uniform transcriptional shutoff, causes developmental arrest, and often interferes with transcription and decay machinery leading to alterations in decay rates (Figure 12).
Contrastingly, 4sUd recycling appears to occur at a minimal rate uniformly across the whole embryo, therefore mRNA decay measurements remain reliable. Even though, the true value for mRNA HL may not be obtained, the stability of mRNA transcripts relative to one another remains accurate. mRNA transcripts can still be correctly identified as having high, medium, or low stability. mRNA decay studies in mammalian cells indicate that although data obtained by transcriptional shutoff with actin versus in vivo pulse labeling differ, overall classifications of mRNA as having short, medium, or long HLs coincide between studies (4). Also, if the rate at which 4sUd is recycled during the chase period is known, then bias caused by recycling can be corrected during array normalization. Studying relative mRNA stability also addresses one potential caveat of TU-Tagging: the effect of cell doubling and dilution of tagged mRNAs. If cells are growing through either increase in average cell volume or cell number, mRNA concentration is reduced by dilution and degradation (4). In the case of TU-Tagging experiments, since transcription is not inhibited during the chase period cell volume and/or cell number may increase, diluting the concentration of pulse-labeled mRNAs, making transcripts appear to be unstable. However, because all mRNAs are diluted uniformly, relative mRNA stability does not change; and accurate HL calculations can still be made. It is for these reasons that TU-Tagging utilizing a uridine only chase is the most accurate method for measuring mRNA decay in vivo.

NEURAL-SPECIFIC DECAY KINETICS

TU-Tagging using 4TU and pros>TgUPRT highlighted one of the key features of the TU-Tagging method: cell-type specific decay measurement. This experiment demonstrated three important findings. The first finding was that global decay kinetics
between the nervous system and whole embryo are very similar. Since the majority of factors influencing mRNA decay are present across all tissue, as expected, the median HL for whole embryo and nervous system specific showed 98% similarity. This suggests that decay regulation in the nervous system mirrors mechanisms of decay for the whole embryo.

Secondly, this experiment identified transcripts that are differentially expressed between the whole embryo and nervous system. Although the majority of transcripts had similar HLs between the datasets, two groups of differentially expressed mRNAs, approximately 14% of transcripts, were identified. Transcripts that are known to play a role in neural development had nervous-system specific differences in mRNA stability. Interestingly, each of the highlighted transcripts exhibit peak expression during early embryonic development, suggesting that neural-specific trans-acting factors such as RBPs or miRNAs, may play an active role in either stabilizing or destabilizing these mRNAs. High stability mRNAs tend to be involved in learning and memory, synaptic activity, cell motility, axon guidance and cell motility, while low stability transcripts seem more to be involved in cell fate determination and dynamic actin polymerization (Table 3).

This experiment also demonstrated the ability for the TU-Tagging method to isolate cell-type specific mRNAs. Gene ontology categories for high stability mRNAs produced functional groups involved in translation and cytoskeleton organization. This is expected since decay studies have shown that transcripts with long HL function in translation, cytoskeleton organization, and metabolism regardless of the cell-type. This finding is supported by decay studies in *E.coli*, *S. cerevisiae*, *Drosophila*, and mice that report stable mRNA transcripts, with long HLs, encode proteins involved biosynthesis, posttranscriptional modifications, translation, energy metabolism, and cell-envelope maintenance (50,53,55,73). In contrast, unstable mRNAs, particularly short HLs, encode transcription factors and regulatory genes, as well as proteins involved in cell cycle, growth and development, differentiation, histone modification and chromatin remodeling, and cellular signaling. In *Drosophila*, it is proposed that unstable mRNAs encoding cell cycle and DNA replication factors may be linked to the well-timed elimination of cell cycle regulators known to slow down mitotic cycles at the onset of gastrulation, and readjustment of their expression once cell division is restricted to specific domains in the embryo (54,57,58,63).

Interestingly, most GO analysis for global decay kinetics, in these and previous experiments do not show enrichment for nervous system specific functional groups in the low stability mRNAs. However, gene ontology of pros>TgUPRT identifies an enrichment for groups with specific nervous system function in the low stability mRNAs, such as neuroblast proliferation, asymmetric neuroblast division, and neuroblast fate determination (Table 3).

Overall this experiment demonstrates the need for cell-type specific decay experiments, as well as TU-Tagging as an effective method for accomplishing this. Comparison of neural-specific decay to whole embryo decay identified neural-specific mRNAs that are differentially expressed, which were found to play critical roles in neural development. Lack of cell-type specificity would have overlooked these mRNAs. The ability to study nascent versus total RNA pools, in a cell-type specific manner, provides a
higher level of resolution for identifying important temporal and spatial mRNAs, not revealed at the total RNA level. For example, while studying serum-starvation of primary mouse embryonic fibroblasts, Kenzelmann et al. identified only 50 differentially expressed mRNAs from analysis of total RNA; however analysis of nascent mRNA identified 247 differentially expressed genes (63). Thus, cell-type specific decay analysis of nascent mRNA pools is crucial for elucidating fine-tuned decay regulatory networks.

**NEURAL-SPECIFIC mRNAs: SYNAPTIC-LOCALIZATION VERSUS CELL FATE DETERMINANTION**

mRNA decay studies across several organisms demonstrate that a common principle of decay kinetics is that transcript HL correlates with function (57,63). In yeast and mammalian cells there is a relationship between decay rate and gene function. mRNAs encoding proteins that function in the same pathways, or process; generally have similar decay rates. Grouping of genes by their decay patterns also reveals significant enrichment of GO terms (35,56,59,72).

One hypothesis of nervous system development is that mRNAs that are locally translated must exhibit low, or slow, $k_d$; whereas mRNAs that determine cell fate must exhibit high rapid $k_d$. Analysis of neural-specific mRNAs HL was used to test this hypothesis. Within the 1000 most stable neural mRNAs, many transcripts encode proteins whose mammalian orthologs are known to be locally translated at synaptic terminals. Within the 1000 least stable neural mRNAs, many transcripts encode regulators of cell fate determination, several of which are differentially expressed between neural progenitors and progeny or between different subsets of neurons. This hypothesis was further confirmed by analyzing known *Drosophila* neural mRNAs that are known to be either locally translated or cell-fate determinants (Figure 13). Results from this analysis confirm that mRNAs, such as *pum, Lim1*, and GluRA (which are translated at synaptic terminals) are all highly stable; while mRNAs, such as *hb, cas, pdm*, and *mir* (which are differentially expressed in dividing neuroblasts) all highly unstable.
The odds ratio analyses applied to distinct populations of stable or unstable mRNAs support a model in which mRNAs synthesized in the cell body and trafficked the long distance to synaptic terminals for localized translation, must exhibit high stability in order not to be degraded in route. *Drosophila* mRNAs *pum*, *Lim1*, and *GluRA* each play important roles in nervous system development specifically at synaptic terminals. *pum* mRNA, which encodes PUM1 RBP, may play a role in regulating local translation that underlies synapse-specific modifications during memory formation (78). *Lim1* mRNA encoding LIM1 kinase is involved synaptic target formation. *GluRA*, an AMPA receptor subunit, is important for activity-dependent insertion of AMPA receptors in synaptic junctions during long-term potentiation.

The odds ratio analyses also support a model of neural development in which mRNAs that specify cellular fate must be tightly regulated at the level of mRNA decay allowing expression of those genes to be activated and shut off in a precise and rapid manner, ensuring proper cellular diversity (2). *Drosophila* neural mRNAs *mira*, *hb*, *cas*, and *pdm* each play an important role in determining neuroblast or GMC fate (Figure 14). Miranda (*mira*), a key component cell fate determination during *Drosophila* neurogenesis, mediates asymmetric segregation of transcription factors into only one daughter cell during neural stem cell division. Miranda, an adaptor protein that colocalizes with Prospero in mitotic neuroblasts, tethers Prospero to the basal cortex of the dividing neuroblast directing Prospero into the newly made GMC (80). *mira* mRNA is readily detectable in mitotic neuroblasts, however never found in GMCs; suggesting that *mira* specifies neuroblast fate and is rapidly degraded in daughter GMCs in order to maintain proper cell fate specification. *hb*, *kru*, *pdm*, and *cas* mRNAs encode transcription factors that are sequentially expressed in dividing neuroblast 2-4, 7-1, and 7-3; and produce differentiated neural progeny that maintain the transcription factor
profile present at their birth, thus specifying a distinct motor neuron identity (79). During this stage of neurogenesis mitotic neuroblasts divide approximately once every hour, indicating that the expression level of each transcription factor must be dramatically altered within a short time window. For example, *hb* mRNA is highly expressed in the first born neuroblast and must then be rapidly degraded, within 60 minutes, at the onset of second neuroblast division in which *kr* is highly expressed and then rapidly degraded upon the birth of the third neuroblast. Thus, each mRNA experiences tight temporal regulation at the level of mRNA stability, as evidenced by their rapid decay and short HLs.

![Diagram of neural cell fate determination](image)

**Figure 14. Low Stability mRNAs are Known Cell Fate Determinants**

Genes from Figure 13 known to play a role in NB temporal identity and differentiation. During neural development NBs both differentiate into GMCs and self renew, *mira*, which specifies NB fate, must be rapidly degraded each time the NB differentiates. Also each time the NB self renews it will sequentially express *hb*, *kru*, *pdm*, and *cas* mRNAs. At each division the cell fate determinant activated in the previous division is rapidly degraded. *hb*, *kru*, *pdm*, and *cas* are then able to specify the fate of their corresponding GMC.

**AU-RICH ELEMENTS IN DISTINCT NEURAL mRNA DECAY CLASSES**

ARE-mediated decay activity has been shown to play an important role in posttranscriptional regulation of gene expression. ARE-bearing mRNAs, as well as AUBPs play critical roles in nervous system development and function. For example, in *Drosophila* ARE-mediated decay of *gcm* mRNA ensures proper glia development and balance between the number of glia and neuron produced. (11). Dysfunction of ARE-mRNAs or AUBPs contributes to neurological disorders such as Down Syndrome, Alzheimer’s, and Parkinson’s disease; and has been implicated in many types of cancers (15,16,18).

Odds ratio tests of neural-specific mRNAs revealed significant enrichment of ARE-bearing mRNAs in both the 1000 most stable, termed high stability and the 1000 least stable, termed low stability, mRNAs. This suggests that regulation of gene expression by AUBPs occurs through targeting both high stability and low stability neural-specific mRNAs. This also suggests that ARE-mediated decay is a widely used
mechanism throughout the *Drosophila* nervous system and that AUBPs within the nervous system are used both to stabilize and destabilize ARE-bearing mRNAs.

For example, *Drosophila* mRNA *unc-5* is predicted to contain a functional class I ARE and is present in the high stability mRNA class. *unc-5* is a protein-coding gene that encodes a Netrin receptor involved in chemorepulsion. *unc-5* has been shown to play a role in motor neuron guidance, axon guidance, salivary gland boundary specification, and glial cell migration (Interactive Flybase.org). *unc-5* promotes axon pathfinding, connections between growing motor neurons and muscle targets, and migration of glia derived from the ventricular zone of the neural tube or from the neural crest. The molecular and biological function of *unc-5* suggests that this transcript may be preferentially stabilized by AUBPs during embryogenesis in order to help establish the developing nervous system. *Drosophila* mRNA *CycE* is also predicted to contain a functional class I ARE, but differing from *unc-5*, is present in the low stability mRNA class. *CycE* is a cyclin-dependent protein serine/threonine kinase that functions in many biological processes, but is most well known for its function as a cell cycle regulator. Through regulation of the cell cycle, *CycE* has been shown to be involved in stem cell differentiation, cell fate commitment, neuroblast fate commitment, and neuron differentiation (Interactive Flybase.org). The molecular and biological function of *CycE* suggests that this transcript may be preferentially destabilized by AUBPs during specific stages of embryogenesis in order to tightly regulate cell-fate specification in the developing nervous system. *CycE* mRNAs are likely induced during differentiation and self-renewal of neural progenitors, and then rapidly degraded by AUBPs in order to ensure proper temporal and spatial expression of this potent cell-fate determinant.

In order to determine whether regulation via AREs affects mRNA localization and function, predicted ARE-containing mRNAs were compared against the dataset of dendrite-localized mRNAs and the dataset of neural fate transcription factors. mRNA transcripts that were present in either the “ARE-Local” group or the “ARE-Cell Fate” group were then compared against high stability mRNAs and low stability. If ARE-mediated mRNA does play a role in mRNA localization and function expect results should find an enrichment of “ARE-Local” mRNAs in the high stability group, and an enrichment of “ARE-Cell-Fate” mRNAs in the low stability group. However no significant enrichment was found for any comparison.

Although, no significant enrichment was found when compared to the whole embryo, comparisons of dendrite-localized and neural fate transcription factors to the ARED provided insight into regulation of neural-specific mRNAs through ARE-mediated decay. For example, comparison of ARED to the dataset of 20 mRNAs known to be dendrite-localized (Zhong et al.) revealed three mRNAs: *pum*, *mub*, and *Spn* to be present in both groups. Each of the three mRNAs contains a functional class I ARE, and has an important role in *Drosophila* nervous system development. *pum*, which encodes the Pumilio RBP, is involved in regulation of synaptic growth and transmission, dendrite morphogenesis, formation of long-term memory, behavior response to ethanol, and negative regulation of epidermal growth factor receptor signaling pathway which controls cell adhesion, migration, and proliferation (Interactive Flybase.org). MUB protein, encoded by *mub* mRNA, is thought to be an RNA binding protein that binds and stabilizes mRNAs specifically in the mushroom bodies, and may therefore be involved in learning and memory in *Drosophila*. *Spinophilin* (*Spn*), which is far less studied than *pum*
and mub, has protein phosphatase 1 binding activity, and is involved in male-aggressive behavior and olfactory behavior; suggesting that similar to mub, Spn has a role in learning and memory. Each of these three mRNAs has an important role in Drosophila nervous system development, and therefore may be preferentially stabilized by AUBPs during embryogenesis. One explanation for why these mRNAs were not found in the high stability or low stability pros>TgUPRT dataset could be attributed to the stringent R² values. For these analyses, and R² value of 0.8 was utilized as a cut off point. It would be interesting to see whether these three mRNAs are present when R² values are lower, or when high stability versus low stability ranking is solely based on transcript half-life. Nevertheless, pum, mub, and Spn are likely regulated by ARE-mediated mechanisms; and due to their functional roles are good candidate mRNAs to be stabilized by AUBPs during nervous system development.

Comparison of “ARE-Cell Fate” mRNAs against the high and low stability groups produced two mRNAs present in the distinct decay classes. longitudinal lacking (lola) and seven up (svp) mRNAs were found within the low stability mRNA group. Interestingly, svp was also found within the high stability mRNA group. lola has been shown to play a role in neuron differentiation, immune response, and apoptosis. Similar to other neural-fate transcription factors like hb, kru, pdm, and cas, which are sequentially expressed and then rapidly degraded during each round of neuroblast division; due to its role in the nervous system, lola is likely to be rapidly degraded at specific stages of neural development. The dominant mediator of this degradation may occur through recognition of AREs by destabilizing AUBPs, promoting lola degradation. svp, which is a transcription factor involved in protein binding, synaptic transmission, cell fate determination, differentiation, and proliferation; was found in both the high and low stability mRNA groups. svp mRNA is unique in that its annotated functional roles can be segregated into two categories: high stability mRNA functions and low stability mRNA functions. Function associated with protein binding and synaptic transmission are often attributed to high stability mRNAs, while function associated with cell fate determination, differentiation, and proliferation are often attributed to low stability mRNAs. Therefore it is proposed that svp may belong to either stability class, explaining its appearance in both the high and low stability groups. This difference may be due differentially expressed and thus, differentially regulated transcript variants of svp. There is some evidence that neural-specific variant of svp is highly stable, potentially due to interactions with stabilizing AUBPs, while the non-neural variants are rapidly degraded, which also may be due to association with destabilizing AUBPs. Since the Agilent microarray does contain probes for transcript variants, in future analyses it will be worthwhile to study the changes in mRNA HL based on differentially expressed transcript variants within the nervous system. However, this analysis provides a better understanding of how synaptic-localized mRNAs, and neural-fate determinant mRNAs may be regulated through ARE association with AUBPs. Elucidating these mechanisms will provide further insight into understanding how mRNAs are trafficked to synaptic terminals, which is important in neurodegenerative diseases like Alzheimer’s, as well as how cell fate is regulated, which is integral for reprogramming differentiated cells into iPS cells.

High and low stability mRNA groups were compared against each of the five ARE subgroups identified in ARED. Both high and low stability mRNAs were found to be significantly enriched in Group 5 only. Enrichment in both mRNA groups suggests that this ARE motif may be recognized by both stabilizing and destabilizing AUBPs, and may
allow for cooperative or competitive binding to regulate ARE-mRNA stability. Of the 5 different ARE subgroups, Group 5 contains the “weakest” ARE, lacking any repeats of the AUUUA pentamer which is a hallmark of potent AREs. The weakness of this motif could explain its enrichment in both stability groups. Alternatively, this motif may play an important role in multidimensional posttranscriptional regulation of ARE-mRNAs. For example, the robustness, as opposed to weakness, of this motif may promote recognition by a larger cohort of RBPs, compared to Group 1-4 AREs. This motif may facilitate cooperative and competitive binding of AUBPs, as well as other RBPs such as Pumilio, Smaug, and Staufen, and even neural-specific miRNAs. Thus creating a more dynamic network of posttranscriptional regulators that associate with Group 5 ARE-mRNAs. It will be interesting, in future analyses, to identify whether Group 5 ARE-mRNAs interact with more RBPs compared to Group 1-4 ARE-mRNAs.

miR-315 is a stem loop miRNA that possess a large set of mRNA targets. Although the function of miR-315 is not known, it is most notably known to be involved in activation of Wg signaling in Drosophila through inhibition of two negative regulators of the Wg pathway, Axin and Notum. miR-315 is of interest to this project since miR-315 has neural-specific mRNA targets and the seed sequence of miR-315 recognizes the ARE motif. ARE-miR-315 mRNAs were compared against both high and low stability mRNA groups. Both the high and low stability groups contain ARE-miR-315 mRNAs; suggesting that miR-315 may function to both stabilize and destabilize neural-specific ARE-containing mRNAs. One explanation is that miR-315 acts in a similar fashion to miR-369-3. miR-369-3 also recognizes AREs in 3′UTRs, and in TNFα mRNA binding by miR-369-3 activates translation in quiescent cells during serum starvation, and inhibits translation in proliferating cells (89,19). Similarly, miR-315 may stabilize or destabilize ARE-mRNAs under different cellular conditions. For low stability mRNAs, miR-315 solely binds to ARE-mRNAs, or cooperatively binds with other AUBPs, to promote mRNA degradation; while regulation of high stability mRNAs may occur through competitive binding of miR-315 and stabilizing AUBPs. In future experiments it will be interesting to identify AUBPs that share ARE-mRNA targets with miR-315; and whether these ARE-mRNAs are upregulated or down regulated in the absence of stabilizing or destabilizing AUBPs. This would help further elucidate the interaction between ARE-mRNAs and miR-315 during nervous system development. An alternative explanation is that high stability ARE-mRNAs are predicted targets of miR-315 based on seed sequence complementarity, however, in vivo these ARE-mRNAs may be protected from degradation by associating with stabilizing AUBPs during nervous system development.

High and low stability mRNA groups were compared against a database of neural-specific mRNAs that have been shown to undergo 3′UTR extension during nervous system development. This analysis identified 41 neural-specific mRNAs that undergo 3′UTR extension, but are not annotated in ARED. Since the 3′UTR is thought to harbor regulatory transcripts, it was hypothesized that a portion of these mRNAs may contain AREs in the extended 3′UTR, which had previously been overlooked by ARED. High and low stability transcripts were searched for the following: 1) number of AUUUA motifs 2) number of repeats of AUUUA motif 3) Group 5 ARE: WWW[UAUUUAU]WWW 4) UUAUUAUU and 5) UUUUAAA and UUUGUUU.

High and low stability extended 3′UTR mRNAs contain multiple AUUUA motifs, as well as AUUUA repeats. However, low stability mRNAs contain a higher number of
AUUUA repeats within 3′UTRs compared to high stability mRNAs; suggesting that the presence of AUUUA repeats are an important property of a destabilizing ARE. Similarly, there is enrichment for the Group 5 ARE motif in low stability transcripts, also suggesting that allow this ARE motif may be recognized by both stabilizing and destabilizing AUBPs; the presence of the motif may preferentially contribute to transcript instability. Extended 3′UTR mRNAs were also searched for UUAUUUAU, UUUGAA, and UUUGUUU motifs. Since UUAUUUAU is proposed to be the most basic motif with the ability to cause destabilizing activity, it would be expected that UUAUUUAU motif be preferentially enriched in low stability 3′UTR extensions. However, no difference was found for this motif between the two stability groups. This may suggest that the UUAUUUAU motif may not be specific to neural transcripts, and may be a more potent destabilizing element in other tissues, like muscle. Alternatively, UUAUUUAU may be preferentially present in the 3′UTR of short mRNA isoforms, and since it may not have a role in neural-specific ARE-mediated regulation, it may not be present in extended neural-specific 3′UTRs. It will be interesting to investigate whether UUAUUUAU motif is significantly enriched in low stability transcripts in other tissues. No significant difference was found between the percentages of high and low stability transcripts that contain either a UUUUAAA or UUUGUUU motif in the extended 3′UTR. However, similar to previous results, the low stability mRNAs exhibit a much higher occurrence of both UUUUAAA and UUUGUUU motifs within the 3′UTR. This result conflicts with the previous finding that these U-rich motifs are putative binding sites for ELAV AUBP, since ELAV is proposed to be a stabilizing AUBP. However, this conflict may be attributable to many causes. For example, although ELAV is proposed as a stabilizing AUBP, ELAV activity may not solely be stabilizing. Similar to HuR, an ortholog of ELAV, which has been shown to have both stabilizing and destabilizing activity; binding of ELAV to U-rich motifs may decrease mRNA stability. Alternatively, the U-rich motifs may also be recognized by destabilizing AUBPs, such as AUF1, which has been shown to associate with HuR to induce destabilization of ARE-mRNAs. Overall this analysis suggests that the extended 3′UTR sequence of both high and low stability mRNAs may contain both stabilizing and destabilizing cis-elements. A potential explanation of this phenomena is that high stability transcripts may in fact contain destabilizing elements, however destabilizing activity may be offset by binding of stabilizing RBPs or through inhibition of destabilizing RBPs through competitive binding, thus minimizing the degradation of these transcripts. In a similar manner, low stability mRNAs may contain stabilizing elements; however destabilizing activity may outcompete stabilizing activity through competitive binding of RBPs or cooperation with miRNAs; increasing the rate of degradation. Interestingly, there may be a need for the majority of neural-specific transcripts to harbor both stabilizing and destabilizing cis-elements in order to rapidly adjust mRNA abundance to fit the critical needs of the developing nervous system.

Overall analysis of 3′UTR extensions of neural-specific mRNAs supports the hypothesis that these extended mRNA isoforms harbor multiple regulatory motifs within their 3′UTRs that subject the mRNA to a unique process of neural-specific posttranscriptional regulation during development. From this analysis two mRNAs, Cirl and fs(1)h, are particularly good examples of the posttranscriptional regulation mediated by 3′UTR extensions.

Cirl is a protein-coding gene with latrotoxin receptor activity, and is predicted to be involved in G-protein coupled receptor pathway signaling during nervous system
development. *Cirl*, which is part of the high stability mRNA group, having a HL of approximately 154 minutes, has also been shown to undergo 3'UTR extension during neurogenesis, resulting in five transcript variants with 3'UTRs spanning 1.7 kilobases, and three transcript variants spanning 0.5 kilobases. Analysis of both the long and short isoforms of *Cirl* mRNA reveals that the shorter mRNA isoforms contains both an AUUUAA repeat and Group 5 ARE motif, whereas the longer isoforms lacks these two destabilizing motifs and instead contains both a UUUUUAA and UUGUUUU motif. This suggests that the shorter *Cirl* mRNA isoform may be rapidly degraded through binding of destabilizing AUBPs. However, during early nervous system development extended *Cirl* isoforms, which may result in the stabilization of *Cirl* mRNA through either association with stabilizing AUBPs, such as ELAV, which may recognize the U-rich motifs; or through inability for destabilizing AUBPs to bind since AUUUAA motifs are absent in the extended isoform.

Conversely, Female sterile (1) homeotic (*fs(1)h*) mRNA has protein kinase activity and is involved in terminal region determination, imaginal disc wing morphogenesis, and neuroblast proliferation. *fs(1)h*, which is part of the low stability mRNA group and has a HL of 41.6 minutes, has also been shown to undergo 3'UTR extension, resulting in two extended transcript variants with 3'UTRs spanning 5.2 kilobases, and seven shorter *fs(1)h* isoforms spanning 2.1 kilobases. Analysis of both the long and short isoforms of *fs(1)h* mRNA reveals that the shorter mRNA isoforms lack AUUUAA repeats, Group 5 ARE motif, and the proposed destabilizing UUAUUUAUU motif; whereas the longer isoforms contains eight AUUUAA repeats, four Group 5 ARE motifs, and the UUAUUUAUU motif. This result suggests that the shorter *fs(1)h* mRNA isoform may be stabilized, or very slowly degraded, due to lack of destabilizing elements in the 3'UTR. However, synthesis of *fs(1)h* mRNA with extended 3'UTRs results in the addition of destabilizing elements causing *fs(1)h* to be rapidly degraded by AUBPs during neural development.

This analysis reveals the unique mechanism of regulation of mRNAs through ARE-mediated decay and/or stabilization in extended 3'UTRs in the *Drosophila* nervous system. In addition to ARE motif searches, future analysis will identify binding sites of other neural-specific RBPs, such as PUM, as well as neural-specific miRNAs, like miR-315 and miR-124, within the extended 3'UTRs. These results will provide further evidence that extended 3'UTRs harbor binding sites for multiple regulatory elements, in addition to AUBP binding sites, as well as further establish the synergistic regulation of mRNAs by both RBPs and miRNAs; thus building a dynamic regulatory decay network.
CHAPTER 5: FUTURE DIRECTIONS AND CONCLUSION

IMMEDIATE GOALS OF PROJECT

Immediate future directions for this project include three primary experiments: neural specific decay measurements during 1) overexpression of FNE 2) knockdown of FNE and 3) knockdown of TIS11. UAS-FNE, UAS-FNE{RNAi} and TIS11{RNAi} flies have been obtained from the Bloomington Drosophila Stock Center. By genetically engineering each of these fly lines to also express HA-UPRT, then subsequently crossing these lines to Pros-GAL4; neural specific decay measurements can be obtained during gain of function and loss of function experiments. Calculation of mRNA HLs can then be compared to nervous system specific HL measurements. Data from these three experiments will help in the identification of neural mRNA targets of FNE and TIS11. These experiments will also help elucidate the function of FNE as either a stabilizing or destabilizing AUBP, which has yet to be identified.

LONG-TERM GOALS OF PROJECT

Long-term goals of this project include three specific aims: 1) Measure mRNA decay kinetics in neuroblasts and post-mitotic motoneurons. TU-Tagging techniques in combination with RNA-sequencing will provide more accurate genome wide neural-specific decay measurements. Comparison of decay kinetics in neuroblasts versus motoneurons will provide mRNAs with altered stability in proliferating cells versus differentiated cells. To identify candidate targets of RBPs expressed in the nervous system, neural-specific mRNA decay analyses will be performed in embryos with RNAi-mediated knockdown and overexpression of RBPs, such as IMP, BRU3, FMRP, STAU, SMG, PUM, and MUB. Each of these RBPs is known to affect mRNA stability, however their neural-specific targets are largely unidentified. 2) Data from aim 1 will be used to identify cis-elements and miRNAs that determine mRNA stability. Linear sequences and structural elements that are significantly enriched among transcripts with similar decay kinetics, including mRNAs with altered stability in RBP mutant embryos will be identified. Candidate cis-elements will be tested in wildtype and RBP mutant embryos using neural-specific decay analysis of reporter transcripts with insertions, deletions, and mutations of the candidate element. mRNAs predicted to be targets of miRNA-mediated decay will be tested in embryos lacking expression of the corresponding miRNA and embryos over expressing decoy RNAs. 3) Identify physical and spatial mRNA-RBP interactions. Binding of specific RBPs to cis-elements identified in aim 2 will be tested with in vitro electrophoretic mobility shift assays using recombinant RBPs, and RNA pull-down assays using cell and embryo lysates. Binding of RBPs to target mRNAs will be tested in vivo using RNA Immunoprecipitation followed by qRT-PCR detection of target mRNAs. Confocal microscopy will be used to visualize the spatial dynamics of mRNA decay, using fluorescent mRNAs, RBPs, and components of the general RNA decay machinery in cultured neurons.

CONCLUDING REMARKS

Utilizing various components of the analysis, thus far, a simple model of a neural ARE-mediated decay network can be constructed. As future experiments identify
candidate cis- and trans-acting elements of mRNA decay a larger network can be constructed, combing mRNA regulation via RBPs, miRNAs, ARE-mediated decay, and NMD. Overall this work hopes to generate a comprehensive network map of neural mRNA decay dynamics, thus filling a significant gap in current model of gene expression and posttranscriptional regulation during neural development.

Regulation of gene expression through mRNA decay is a powerful mechanism used across multiple organisms and various cell types. Although much has been elucidated about mRNA decay, many components of this regulatory mechanism have yet to be identified. For example, many mRNA targets of destabilizing and stabilizing RBPs are still unknown. Common sequence and secondary structure features shared between mRNA decay regulators, even those with shared targets, also remain unidentified. Principally there is a lack of decay regulatory networks connecting RBPs and miRNAs, to their mRNA targets as well as to one another.

Difficulty identifying and predicting novel cis and trans regulatory motifs may be due to the lack of a simple molecular code relating mRNA decay regulatory with sequence elements in their target mRNAs. mRNA decay regulation may require a highly specific combination of sequence and structural elements to be present in mRNA. On the other hand, a complex combination of multiple RBPs and/or miRNAs may be needed to determine the decay of a specific transcript (50). This indicates the need for future cell-type specific mRNA decay studies in order to compose temporally and spatially accurate neural decay regulatory networks. TU-Tagging provides an efficient and accurate method to accomplish such a task.
SUPPLEMENTAL INFORMATION AND FIGURES

A

1000 Most Stable  ARE Database

854  137  1616

1000 Least Stable  ARE Database

866  126  1627

B

All Data  ARE-Local

11641  3  0

1000 Least Stable  ARE-Local

1000  0  3

1000 Most Stable  ARE-Local

1000  0  3
Supplemental Figures 1. Analysis of AU-Rich Elements in High and Low Stability mRNA Groups
High and low stability mRNA groups compared against the following: A. ARE Database B. ARE-Local mRNAs C. ARE-Cell Fate mRNAs D. Group 1 ARE E. Group 2 ARE F. Group 3 ARE G. Group 4 ARE H. ARE-miR-315 mRNAs. For all high and low stability mRNAs $R^2 > 0.8$. No significant enrichment was found in any comparisons.
REFERENCES


24. Uckun, F.M., Goodman, P., Ma, H., Dibirdik, I., Qazi, S. CD22 EXON 12 deletion as


49. Dolken, L., Ruzsics, Z., Radle, B., Friedel, C.C., Zimmer, R., Mages, J., Hoffmann,


