ABSTRACT

The Role of Eukaryotic Initiation Factor 5A and its Cellular Localization in Developing *Zea mays* Plant Tissue

Sonya J. Saladine, M.S.

Mentor: Bryan C. Gibbon, Ph.D.

Eukaryotic initiation factor 5A is an 18 kDa protein that has been implicated in a wide variety of cellular activities, but its primary role, especially its role in translation, has been a mystery. Investigation of its localization in developing maize endosperm and root and leaf tissue have reinforced the idea that eIF5A has at least a secondary role in cellular proliferation and cell division. The interactions of eIF5A with numerous maize proteins identified through pull down analysis, have provided insight into the primary function of this elusive protein. Identified proteins that interacted with eIF5A were components of the cytoskeleton or involved in cell division (tubulin, legumin, actin), carbohydrate synthesis and modification, amino acid biosynthesis, and translation elongation factors. These interacting proteins all have a link to management and survival under low oxygen conditions in plants, suggesting a role of eIF5A as an elongation factor that is necessary when stress interferes with the cell's primary elongation factors. Three eIF5A isoforms were identified in *Zea mays*, with eIF5A-1 being the most highly expressed.

The Role Of Eukaryotic Initiation Factor 5a And Its Cellular Localization
In Developing Zea Mays Plant Tissue

by

Sonya J. Saladine, B.S.

A Thesis

Approved by the Department of Biology

_____
Robert D. Doyle, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

Approved by the Thesis Committee

_____
Bryan C. Gibbon, Ph.D., Chairperson

_____
Myeongwoo Lee, Ph.D.

_____
Bessie W. Kebaara, Ph.D.

_____
Sung-Kun Kim, Ph.D.

Accepted by the Graduate School
December 2011

_____
J. Larry Lyon, Ph.D., Dean

*Page bearing signatures is kept on file in the Graduate School.*

Copyright © 2011 by Sonya J. Saladine

All rights reserved

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

# ACKNOWLEDGMENTS

I would like to gratefully acknowledge those of you who have helped me, in any way, complete the process of completing this thesis. Most importantly, I would like to thank Dr. Bryan Gibbon, who has supported and guided me through this lengthy process. Your time, expertise, and motivation have helped me immensely. I am thankful for your help and guidance, and for putting up with my never ceasing frustrations. It's been a pleasure working with you (and sharing some maize/science jokes) over the past few years.

I would also like to thank Dr. Myeongwoo Lee, Dr. Bessie Kebaara, and Dr. Sung-Kun Kim, for your willingness to serve on my thesis committee. Much thanks and appreciation also goes out to Dr. Sang-Chul Nam, and Dr. Chris Kearney for serving on my proposal committee. Thank you again Dr. Kearney for your help with the planting of *Brachypodium* and electroporation techniques.

I am grateful to all of the people I have had the pleasure of working with in Dr. Gibbon's lab. To my labmates Mo, Hao, Kasey, Kyle, and Jason, it has been a pleasure working with you. Your technical help as well as frequent chat sessions will be greatly missed. Enormous amounts of gratitude are extended to my wonderful friend, Anica Debelica. Thank you for all of the moral support you have given me (as well as a place to crash); I wouldn't be here without you!

And finally, thank you to all of my friends and family. Your constant support and encouragement has been a blessing. I truly couldn't ask for a bigger, or better, extended family.

# DEDICATION

To my friends and family,

for putting up with all of my crazy

# CHAPTER ONE

## Introduction

### *Discovery of Hypusine and eIF5A*

The unique amino acid hypusine [$N^{\varepsilon}$-(4-amino-2-hydroxybutyl) lysine] was discovered by Japanese scientists, Shiba and others in 1971 during a search for proteinogenic, basic amino acids in bovine brain extracts. Hypusine, a modified lysine residue, has its name is derived from two of the amino acid's structural components: hydroxyputrescine and lysine (Shiba and others, 1971). In later investigations, hypusine was found existing as a free amino acid and as a protein component in various animal tissues.

Hypusine, a structurally exceptional amino acid, is known to be present in only one cellular protein, eukaryotic initiation factor 5A, or eIF5A. Initially deemed IF-M2Bα or eIF-4D, this 18 kDa protein was discovered by Benne and Hershey in 1978 during a high salt wash of rabbit reticulocytes (Benne and others, 1978). While investigating lymphocyte growth stimulation, they discovered that the rate of hypusine modification was associated with an increased rate of protein synthesis. Coupled with the fact that numerous initiation factors were found in reticulocyte extracts, eIF5A was initially thought to have a role in the initiation phase of translation. Early experiments with eIF5A showed that the protein increased methionyl-puromycin production, suggesting a role for eIF5A in the formation of the first peptide bond. When all putative initiation factors (eIF1, eIF1A, eIF3, eIF5A, eIF5B, eIF5) were tested for their impact on globin synthesis, the removal of eIF5A was the only one to not exhibit a marked decrease

in the levels of globin (Benne and others, 1978). In a later study by Kang and others, eIF5A levels were depleted and resulted in only a slight (~30%) decrease in protein synthesis (Kang and Hershey, 1994). Subsequent studies reaffirm the fact that although eIF5A is involved in protein synthesis, it is not required and its depletion results in a small decrease in synthesis levels (Zuk and Jacobson, 1998). While the essential importance of eIF5A cell proliferation has been firmly established (Chen and Liu, 1997; Frigieri and others, 2007; Nishimura and others, 2005; Park, 2006; Schnier and others, 1991), the exact cellular function for this protein is still elusive.

### *Post-translational Modification of eIF5A*

Eukaryotic initiation factor 5A is, to date, the only protein known to contain the unique amino acid hypusine. There is only one cellular pathway that results in the formation of hypusine, and there is no known biochemical pathway in its role as a free amino acid (Park and others, 1997). The immature form of the eIF5A is known to undergo two modifications after translation, the first being phosphorylation of the N-terminal acetylated serine residue (Kang and others, 1993). However, when the function of unphosphorylated eIF5A was analyzed, it was found that this protein was still able to support the growth of a yeast strain (Klier and others, 1993), suggesting that phosphorylation is not necessary for eIF5A function *in vivo*. However, it appears that the second post-translational modification of the eIF5A precursor, hypusination, is essential to its function.

The modification of the hypusine residue consists of two enzymatic steps (Figure 1.1) ( Park and others, 1982; Murphey and Gerner, 1987; Park, 2006). The process begins when deoxyhypusine synthase (DHS), an NAD-dependent tetrameric enzyme,

catalyzes the cleavage of the aminobutyl moiety of the polyamine spermidine (Joe and others, 1995; Wolff and others, 1995). This subunit is then transferred to the ε-amino group of a specific lysine residue (lys50 in humans) of the eIF5A precursor, forming an intermediate, deoxyhypusine [$N^{\varepsilon}$-(4-aminobutyl)-lysine] (Wolff and others, 1997; Wolff and others, 2000). The deoxyhypusine intermediate is then hydroxylated by a second enzyme, deoxyhypusine hydroxylase (DOHH) to complete the synthesis of the unique amino acid, hypusine, and the activation of eIF5A.

*Evolutionary Conservation of eIF5A and Hypusine Biosynthesis*

The posttranslational modification of the eIF5A precursor protein is quite unique in that the two enzymes used in the modification, DHS and DOHH, are narrowly specific to the eIF5A substrate proteins. Neither enzyme appears to be used in any other biosynthetic pathway; it seems as though they evolved exclusively for the modification of eIF5A. Spermidine, which provides the aminobutyl moiety that is cleaved and transferred by DHS (Figure 1.1), is a polyamine involved in typical cellular metabolism. Most polyamines have loose structural requirements in cellular pathways and a variety of analogs can substitute for other polyamines in cells. However, spermidine's role in the hypusination of eIF5A is quite strict and its role can only be filled by itself and very few structurally similar analogs (Byers and others, 1994; Chattopadhyay and others, 2003). Neither DHS or DOHH, the two enzymes utilized in hypusination, will modify free amino acids within the cell or short peptides that are similar to the sequence of eIF5A (Wolff and others, 2007). In a study published by Wolff et al. in 2007, a site directed mutagenesis of eIF5A identified the specific amino acid sequence and macro-molecular

structure of the substrate protein as being the factor that influenced whether DHS or DOHH modification would occur (Wolff and others, 2007).

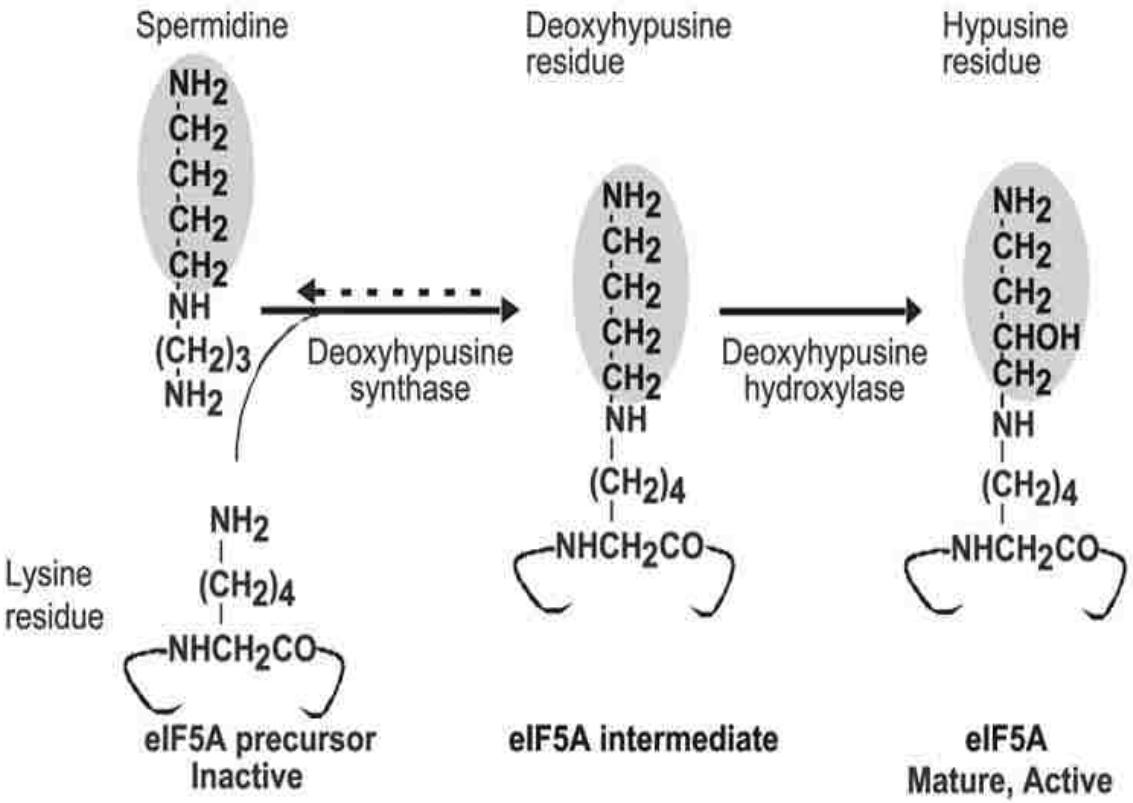


Figure 1.1. Hypusine biosynthesis pathway. Deoxyhypusine synthase (DHS) catalyzes the cleavage and subsequent transfer of the aminobutyl moiety of spermidine (shown highlighted) to the lysine residue of the eIF5A precursor protein to form the eIF5A intermediate, deoxyhypusine. The intermediate is then hydroxylated by deoxyhypusine hydroxylase (DOHH) to form hypusine. This is the mature, active form of the eIF5A protein (Park, 2006).

Gene disruption and knock down studies in *S. cerevisiae* and other eukaryotes have provided ample evidence in the importance of both modification enzymes in cellular proliferation. The post-translational modification of eIF5A is essential to the viability of yeast cells as demonstrated by Kang et al., in 1994 (Kang and Hershey, 1994), and if either modification step is inhibited it leads to arrest of the cell cycle at the $G_1$/S boundary (Park and others, 1997). This modification is also highly specific in that when

a Lys-Arg mutation is introduced, the hypusination of eIF5A by DOHH does not occur (Jin and others, 2003).

It is interesting to note that most of the major players in the hypusine biosynthetic pathway are conserved in a vast majority of organisms. The presence of initiation factor 5A occurs in all eukaryotes (eIF5A), with strong sequence conservation (Figure 1.2), and also archaebacteria (aIF5A), but not eubacteria. However, there exists in eubacteria an ortholog, an essential protein called EF-P, that is involved in translation elongation and affects the peptidyl transferase activity of the ribosome (Glick and Ganoza, 1975). EF-P, like eIF5A, also promotes methionyl-puromycin synthesis in the cell. EF-P, aIF5A, and eIF5A have a striking similarity in amino acid sequence, with eIF5A and aIF5A having the largest sequential similarity near the hypusination site (Clement and others, 2003). In addition, these three proteins differ only a small amount structurally (Hanawa-Suetsugu and others, 2004; Tong and others, 2009). DHS also has homologs in all eukaryotes and archaea, and DOHH is conserved in all eukaryotes (Park and others, 2010).

### *Isoforms of eIF5A*

There have been at least two eIF5A isoforms identified in all eukaryotic organisms studied thus far. The two isoforms in *S. cerevisiae* are expressed by the genes *TIF51A* and *TIF51B* and are regulated through the presence of oxygen, with the former expressed under aerobic conditions and the latter expressed under anaerobic conditions (Schnier and others, 1991). It seems as though the two isoforms are indistinguishable in their ability to enhance cell growth in yeast, as the two isoforms can be inactivated and substituted for each other (Magdolen and others, 1994; Clement and others, 2003). In

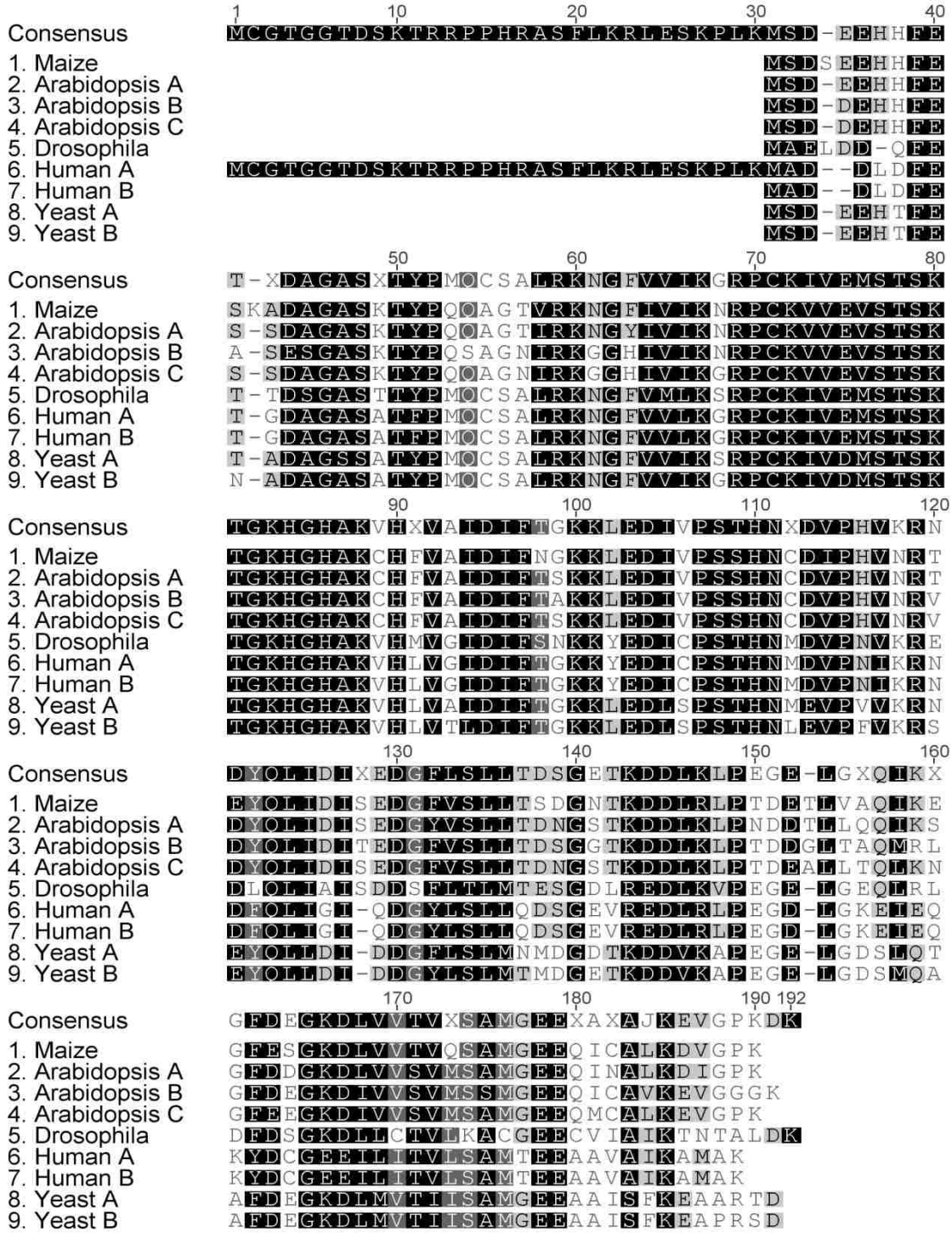


Fig. 1.2. Multiple sequence alignment of eIF5A. The eIF5A amino acid sequence from maize, three isoforms of *Arabidopsis*, *Drosophila*, two human isoforms, and two yeast isoforms were compared using the Geneious alignment tool (Biomatters Ltd., Auckland, New Zealand). The lysine at position 80 is the residue modified into hypusine in active eIF5A. The strong sequence conservation around this point suggests a role for eIF-5A in an important cellular function throughout the evolution of eukaryotes.

*C. elegans*, which has two isoforms, (IFF-1 and IFF-2) it appears that IFF-1 is needed for germ cell proliferation, whereas IFF-2 is required for growth of somatic cells (Hanazawa and others, 2004).

In humans, there are two isoforms, eIF5A-1 and eIF5A-2, which have an 84% similarity in their amino acid sequences. Amongst other vertebrates (such as fish, chicken, and amphibians) the two isoforms seem to be co-expressed (Jenkins and others, 2001; Clement and others, 2003). However, in humans, and most other mammals, there is some differentiation in expression. While eIF5A-1 is expressed in all mammalian tissues, there is tissue dependent mRNA expression with the *EIF5A-2* gene, suggesting a different function of this isoform. The *EIF5A-2* gene seems to be highly expressed in testis and moderately expressed in brain tissue, and its normally low detection in other mammalian tissues skyrockets in certain human ovarian and colorectal cancers (Guan and others, 2001; Clement and others, 2003; Guan and others, 2004). Inhibition of DHS in Chinese hamster ovary cells by Park et al. in 1994 showed that such an inhibition had anti-proliferative effects on certain cancers (Park and others, 1994), suppressed the growth of HeLa cells(Shi and others, 1996), and could lead to apoptosis (Tome and others, 1997). In addition, overexpression of eIF5A-2 in various mammalian cells has led to cellular transformation. Due to these findings, it has been postulated that the second isoform of eIF5A in mammals could be an oncogene (Guan and others, 2004). It has been suggested that the eIF5A-1 isoform is essential for embryonic growth in mammals (Park and others, 2010). These studies indicate that eIF5A has an influence on cell growth and proliferation.

*Role of eIF5A in Translation Elongation*

Although early experiments suggested that eIF5A had a role in the initiation step of translation, depletion studies with yeast from numerous laboratories have proved otherwise (Kang and Hershey, 1994; Zuk and Jacobson, 1998). While it has been known for some time that hypusination is required for protein synthesis, cellular growth, and association with translational machinery, it has been a mystery what direct role the active protein eIF5A has in this process (Wolff and others, 2007). Recently, through numerous findings, it has been proposed that eIF5A plays a role in the elongation step of translation.

Through yeast two-hybrid screens and co-purification experiments, eIF5A has been found to associate with both large and small ribosomal subunits and other elongation factors. However, it was determined by Zanelli et al. in 2006 that this binding will only occur in translating ribosomes and only if the eIF5A protein has undergone hypusination (Zanelli and others, 2006). In vitro yeast extract assays with eIF5A mutants have demonstrated that upon addition of hypusinated wild type eIF5A, but not the mutated K51R strain, a marked increase in translation of luciferase reporter mRNAs (Saini and others, 2009).

Numerous studies of polysome profiles with temperature sensitive yeast eIF5A mutant strains have further enhanced the idea of eIF5A's part in translation. At restrictive temperature, it was demonstrated that the eIF5A mutants caused an increase of the ratio between polysomes and monosomes, a detail that has also been seen in WT cells blocked at the elongation step when treated with inhibitors (Saini and others, 2009; Zanelli and others, 2006). Li et al. have suggested that while eIF5A has a modest effect

on cell growth and translation under normal conditions, it has strong implications on cell proliferation under times of great environmental stress. Under stress conditions, eIF5A knockdown cells have severely inhibited stress granule assembly, and reduced rates of ribosomal transit time (Gregio and others, 2009; Saini and others, 2009; Li and others, 2010). These results conclude that eIF5A, while not a stress granule assembly factor, could be a non-essential elongation factor that is necessary when other elongation factors are deactivated due to stress, thus suggesting eIF5A is a deciding factor of whether a cell survives during unfavorable conditions (Li and others, 2010).

### *Secondary Roles of eIF5A*

As mentioned previously, depletion of eIF5A and its precursors such as spermidine, leads to cell cycle arrest at the G1 phase. Temperature sensitive strains of yeast grown at restrictive temperatures also reveal links between eIF5A and the cell cycle. The eIF5A mutant with the temperature sensitive phenotype demonstrated altered morphology (markedly smaller cell size), weakened cell wall integrity due to a change in the actin dynamics of the cell (Park, 2006), and arrest at the G1 phase of the cell cycle (Chatterjee and others, 2006). Subsequent studies have exhibited similar results of the role of eIF-5a in cell growth. Increased levels of both eIF5A and polyamines (such as spermidine) have been found in malignant cell growth in mammals (Rosorius and others, 1999; Park, 2006). Tumor suppression has been demonstrated by treating malignant cells with D,L-α-difluoromethylornithine, a depressor of spermidine function, resulting in the arrest of cell growth at the G1/S phase of the cell cycle (Park, 1987).

Evidence that eIF5A may play a role in cellular apoptosis, seems at first glance to contradict its role in cell growth, however, an interesting link has been found between

# DEDICATION

To my friends and family,

for putting up with all of my crazy

# CHAPTER ONE

## Introduction

### *Discovery of Hypusine and eIF5A*

The unique amino acid hypusine [$N^{\varepsilon}$-(4-amino-2-hydroxybutyl) lysine] was discovered by Japanese scientists, Shiba and others in 1971 during a search for proteinogenic, basic amino acids in bovine brain extracts. Hypusine, a modified lysine residue, has its name is derived from two of the amino acid's structural components: hydroxyputrescine and lysine (Shiba and others, 1971). In later investigations, hypusine was found existing as a free amino acid and as a protein component in various animal tissues.

Hypusine, a structurally exceptional amino acid, is known to be present in only one cellular protein, eukaryotic initiation factor 5A, or eIF5A. Initially deemed IF-M2Bα or eIF-4D, this 18 kDa protein was discovered by Benne and Hershey in 1978 during a high salt wash of rabbit reticulocytes (Benne and others, 1978). While investigating lymphocyte growth stimulation, they discovered that the rate of hypusine modification was associated with an increased rate of protein synthesis. Coupled with the fact that numerous initiation factors were found in reticulocyte extracts, eIF5A was initially thought to have a role in the initiation phase of translation. Early experiments with eIF5A showed that the protein increased methionyl-puromycin production, suggesting a role for eIF5A in the formation of the first peptide bond. When all putative initiation factors (eIF1, eIF1A, eIF3, eIF5A, eIF5B, eIF5) were tested for their impact on globin synthesis, the removal of eIF5A was the only one to not exhibit a marked decrease

in the levels of globin (Benne and others, 1978). In a later study by Kang and others, eIF5A levels were depleted and resulted in only a slight (~30%) decrease in protein synthesis (Kang and Hershey, 1994). Subsequent studies reaffirm the fact that although eIF5A is involved in protein synthesis, it is not required and its depletion results in a small decrease in synthesis levels (Zuk and Jacobson, 1998). While the essential importance of eIF5A cell proliferation has been firmly established (Chen and Liu, 1997; Frigieri and others, 2007; Nishimura and others, 2005; Park, 2006; Schnier and others, 1991), the exact cellular function for this protein is still elusive.

### *Post-translational Modification of eIF5A*

Eukaryotic initiation factor 5A is, to date, the only protein known to contain the unique amino acid hypusine. There is only one cellular pathway that results in the formation of hypusine, and there is no known biochemical pathway in its role as a free amino acid (Park and others, 1997). The immature form of the eIF5A is known to undergo two modifications after translation, the first being phosphorylation of the N-terminal acetylated serine residue (Kang and others, 1993). However, when the function of unphosphorylated eIF5A was analyzed, it was found that this protein was still able to support the growth of a yeast strain (Klier and others, 1993), suggesting that phosphorylation is not necessary for eIF5A function *in vivo*. However, it appears that the second post-translational modification of the eIF5A precursor, hypusination, is essential to its function.

The modification of the hypusine residue consists of two enzymatic steps (Figure 1.1) ( Park and others, 1982; Murphey and Gerner, 1987; Park, 2006). The process begins when deoxyhypusine synthase (DHS), an NAD-dependent tetrameric enzyme,

catalyzes the cleavage of the aminobutyl moiety of the polyamine spermidine (Joe and others, 1995; Wolff and others, 1995). This subunit is then transferred to the ε-amino group of a specific lysine residue (lys50 in humans) of the eIF5A precursor, forming an intermediate, deoxyhypusine [$N^{\varepsilon}$-(4-aminobutyl)-lysine] (Wolff and others, 1997; Wolff and others, 2000). The deoxyhypusine intermediate is then hydroxylated by a second enzyme, deoxyhypusine hydroxylase (DOHH) to complete the synthesis of the unique amino acid, hypusine, and the activation of eIF5A.

*Evolutionary Conservation of eIF5A and Hypusine Biosynthesis*

The posttranslational modification of the eIF5A precursor protein is quite unique in that the two enzymes used in the modification, DHS and DOHH, are narrowly specific to the eIF5A substrate proteins. Neither enzyme appears to be used in any other biosynthetic pathway; it seems as though they evolved exclusively for the modification of eIF5A. Spermidine, which provides the aminobutyl moiety that is cleaved and transferred by DHS (Figure 1.1), is a polyamine involved in typical cellular metabolism. Most polyamines have loose structural requirements in cellular pathways and a variety of analogs can substitute for other polyamines in cells. However, spermidine's role in the hypusination of eIF5A is quite strict and its role can only be filled by itself and very few structurally similar analogs (Byers and others, 1994; Chattopadhyay and others, 2003). Neither DHS or DOHH, the two enzymes utilized in hypusination, will modify free amino acids within the cell or short peptides that are similar to the sequence of eIF5A (Wolff and others, 2007). In a study published by Wolff et al. in 2007, a site directed mutagenesis of eIF5A identified the specific amino acid sequence and macro-molecular

structure of the substrate protein as being the factor that influenced whether DHS or DOHH modification would occur (Wolff and others, 2007).

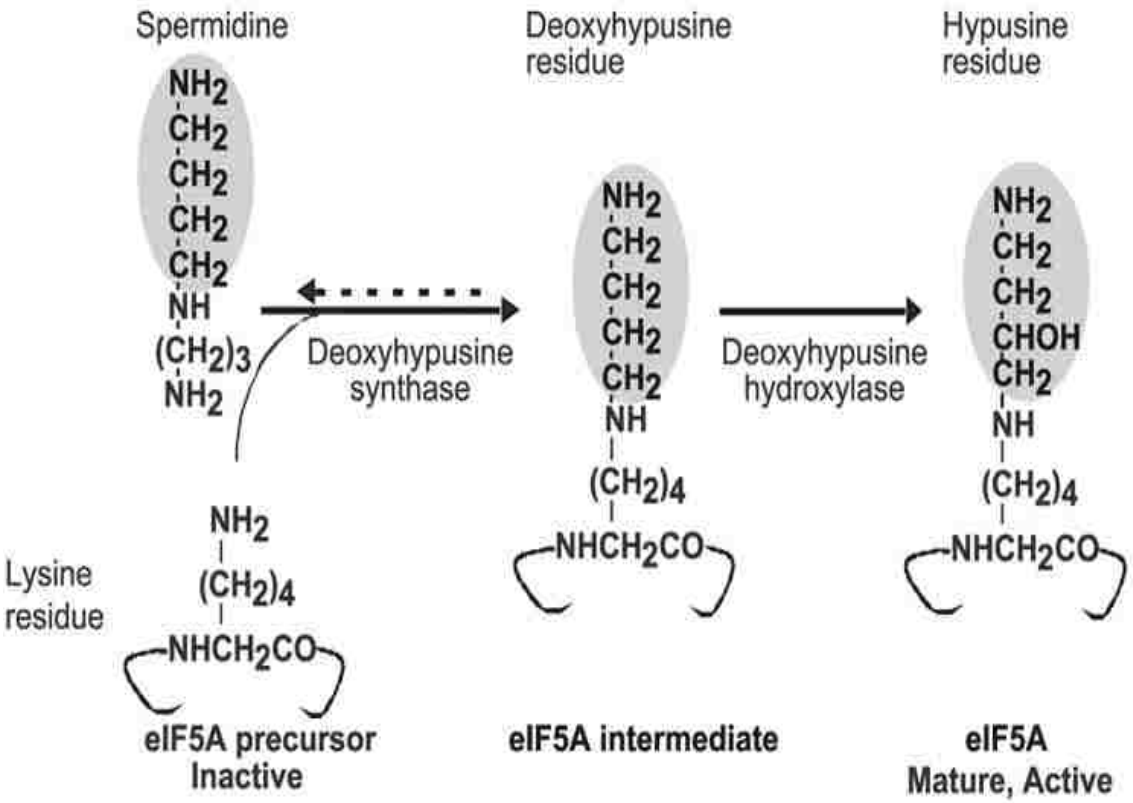


Figure 1.1. Hypusine biosynthesis pathway. Deoxyhypusine synthase (DHS) catalyzes the cleavage and subsequent transfer of the aminobutyl moiety of spermidine (shown highlighted) to the lysine residue of the eIF5A precursor protein to form the eIF5A intermediate, deoxyhypusine. The intermediate is then hydroxylated by deoxyhypusine hydroxylase (DOHH) to form hypusine. This is the mature, active form of the eIF5A protein (Park, 2006).

Gene disruption and knock down studies in *S. cerevisiae* and other eukaryotes have provided ample evidence in the importance of both modification enzymes in cellular proliferation. The post-translational modification of eIF5A is essential to the viability of yeast cells as demonstrated by Kang et al., in 1994 (Kang and Hershey, 1994), and if either modification step is inhibited it leads to arrest of the cell cycle at the $G_1$/S boundary (Park and others, 1997). This modification is also highly specific in that when

a Lys-Arg mutation is introduced, the hypusination of eIF5A by DOHH does not occur (Jin and others, 2003).

It is interesting to note that most of the major players in the hypusine biosynthetic pathway are conserved in a vast majority of organisms. The presence of initiation factor 5A occurs in all eukaryotes (eIF5A), with strong sequence conservation (Figure 1.2), and also archaebacteria (aIF5A), but not eubacteria. However, there exists in eubacteria an ortholog, an essential protein called EF-P, that is involved in translation elongation and affects the peptidyl transferase activity of the ribosome (Glick and Ganoza, 1975). EF-P, like eIF5A, also promotes methionyl-puromycin synthesis in the cell. EF-P, aIF5A, and eIF5A have a striking similarity in amino acid sequence, with eIF5A and aIF5A having the largest sequential similarity near the hypusination site (Clement and others, 2003). In addition, these three proteins differ only a small amount structurally (Hanawa-Suetsugu and others, 2004; Tong and others, 2009). DHS also has homologs in all eukaryotes and archaea, and DOHH is conserved in all eukaryotes (Park and others, 2010).

### *Isoforms of eIF5A*

There have been at least two eIF5A isoforms identified in all eukaryotic organisms studied thus far. The two isoforms in *S. cerevisiae* are expressed by the genes *TIF51A* and *TIF51B* and are regulated through the presence of oxygen, with the former expressed under aerobic conditions and the latter expressed under anaerobic conditions (Schnier and others, 1991). It seems as though the two isoforms are indistinguishable in their ability to enhance cell growth in yeast, as the two isoforms can be inactivated and substituted for each other (Magdolen and others, 1994; Clement and others, 2003). In

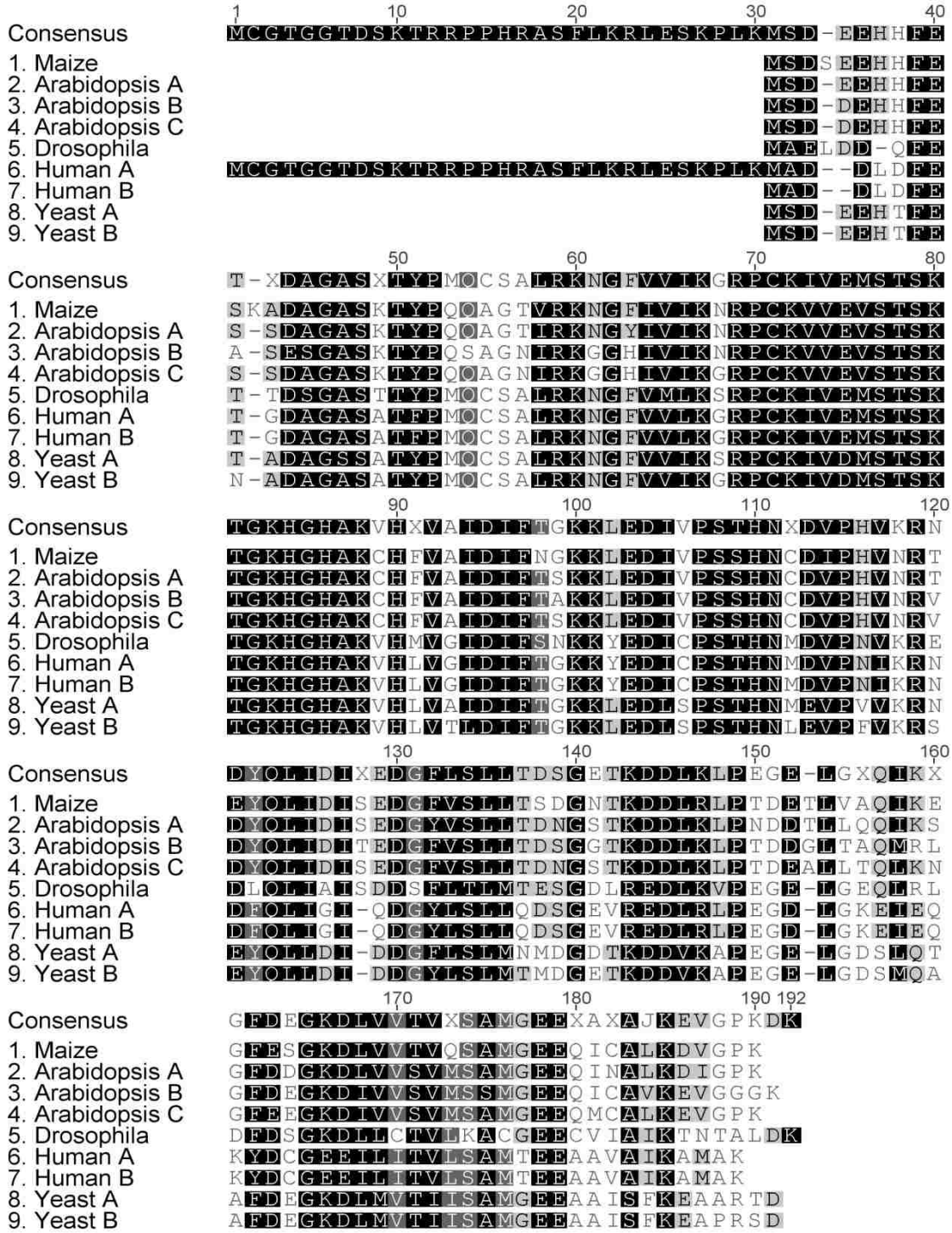


Fig. 1.2. Multiple sequence alignment of eIF5A. The eIF5A amino acid sequence from maize, three isoforms of *Arabidopsis*, *Drosophila*, two human isoforms, and two yeast isoforms were compared using the Geneious alignment tool (Biomatters Ltd., Auckland, New Zealand). The lysine at position 80 is the residue modified into hypusine in active eIF5A. The strong sequence conservation around this point suggests a role for eIF-5A in an important cellular function throughout the evolution of eukaryotes.

*C. elegans*, which has two isoforms, (IFF-1 and IFF-2) it appears that IFF-1 is needed for germ cell proliferation, whereas IFF-2 is required for growth of somatic cells (Hanazawa and others, 2004).

In humans, there are two isoforms, eIF5A-1 and eIF5A-2, which have an 84% similarity in their amino acid sequences. Amongst other vertebrates (such as fish, chicken, and amphibians) the two isoforms seem to be co-expressed (Jenkins and others, 2001; Clement and others, 2003). However, in humans, and most other mammals, there is some differentiation in expression. While eIF5A-1 is expressed in all mammalian tissues, there is tissue dependent mRNA expression with the *EIF5A-2* gene, suggesting a different function of this isoform. The *EIF5A-2* gene seems to be highly expressed in testis and moderately expressed in brain tissue, and its normally low detection in other mammalian tissues skyrockets in certain human ovarian and colorectal cancers (Guan and others, 2001; Clement and others, 2003; Guan and others, 2004). Inhibition of DHS in Chinese hamster ovary cells by Park et al. in 1994 showed that such an inhibition had anti-proliferative effects on certain cancers (Park and others, 1994), suppressed the growth of HeLa cells(Shi and others, 1996), and could lead to apoptosis (Tome and others, 1997). In addition, overexpression of eIF5A-2 in various mammalian cells has led to cellular transformation. Due to these findings, it has been postulated that the second isoform of eIF5A in mammals could be an oncogene (Guan and others, 2004). It has been suggested that the eIF5A-1 isoform is essential for embryonic growth in mammals (Park and others, 2010). These studies indicate that eIF5A has an influence on cell growth and proliferation.

*Role of eIF5A in Translation Elongation*

Although early experiments suggested that eIF5A had a role in the initiation step of translation, depletion studies with yeast from numerous laboratories have proved otherwise (Kang and Hershey, 1994; Zuk and Jacobson, 1998). While it has been known for some time that hypusination is required for protein synthesis, cellular growth, and association with translational machinery, it has been a mystery what direct role the active protein eIF5A has in this process (Wolff and others, 2007). Recently, through numerous findings, it has been proposed that eIF5A plays a role in the elongation step of translation.

Through yeast two-hybrid screens and co-purification experiments, eIF5A has been found to associate with both large and small ribosomal subunits and other elongation factors. However, it was determined by Zanelli et al. in 2006 that this binding will only occur in translating ribosomes and only if the eIF5A protein has undergone hypusination (Zanelli and others, 2006). In vitro yeast extract assays with eIF5A mutants have demonstrated that upon addition of hypusinated wild type eIF5A, but not the mutated K51R strain, a marked increase in translation of luciferase reporter mRNAs (Saini and others, 2009).

Numerous studies of polysome profiles with temperature sensitive yeast eIF5A mutant strains have further enhanced the idea of eIF5A's part in translation. At restrictive temperature, it was demonstrated that the eIF5A mutants caused an increase of the ratio between polysomes and monosomes, a detail that has also been seen in WT cells blocked at the elongation step when treated with inhibitors (Saini and others, 2009; Zanelli and others, 2006). Li et al. have suggested that while eIF5A has a modest effect

on cell growth and translation under normal conditions, it has strong implications on cell proliferation under times of great environmental stress. Under stress conditions, eIF5A knockdown cells have severely inhibited stress granule assembly, and reduced rates of ribosomal transit time (Gregio and others, 2009; Saini and others, 2009; Li and others, 2010). These results conclude that eIF5A, while not a stress granule assembly factor, could be a non-essential elongation factor that is necessary when other elongation factors are deactivated due to stress, thus suggesting eIF5A is a deciding factor of whether a cell survives during unfavorable conditions (Li and others, 2010).

### *Secondary Roles of eIF5A*

As mentioned previously, depletion of eIF5A and its precursors such as spermidine, leads to cell cycle arrest at the G1 phase. Temperature sensitive strains of yeast grown at restrictive temperatures also reveal links between eIF5A and the cell cycle. The eIF5A mutant with the temperature sensitive phenotype demonstrated altered morphology (markedly smaller cell size), weakened cell wall integrity due to a change in the actin dynamics of the cell (Park, 2006), and arrest at the G1 phase of the cell cycle (Chatterjee and others, 2006). Subsequent studies have exhibited similar results of the role of eIF-5a in cell growth. Increased levels of both eIF5A and polyamines (such as spermidine) have been found in malignant cell growth in mammals (Rosorius and others, 1999; Park, 2006). Tumor suppression has been demonstrated by treating malignant cells with D,L-α-difluoromethylornithine, a depressor of spermidine function, resulting in the arrest of cell growth at the G1/S phase of the cell cycle (Park, 1987).

Evidence that eIF5A may play a role in cellular apoptosis, seems at first glance to contradict its role in cell growth, however, an interesting link has been found between

eIF5A and p53, a transcription factor essential to apoptosis initiation in the presence of DNA damage. Li et al., demonstrated in 2004 that eIF5A interacts with a protein syntenin, to regulate the activity of p53 (Li and others, 2004). Overexpression of eIF5A resulted in the up-regulation of p53 and likewise the silencing of eIF5A did the opposite, reducing cellular p53 levels. Since spermidine and other polyamines are thought to function in the regulation of cell growth, it stands to reason that hypusinated eIF5A, having spermidine as a precursor, could function in the same manner.

The role of eIF5A in cell growth and proliferation may also be explained with the newly formed hypothesis that eIF5A may, in fact, be linked to the transport of molecules from the nucleus to the cytoplasm. Through various studies, eIF5A has been known to interact with HIV-1 Rev (Ruhl and others, 1993), CRM-1/exportin1 (Rosorius and others, 1999), exportin4 (Lipowsky and others, 2000), p53 and syntenin (Li and others, 2004), and actin (Chatterjee and others, 2006). It appears that the many proteins eIF5A can associate with are involved, in one mechanism or another, with nucleocytoplasmic transport. However, even the specific interaction eIF5A has with these proteins is contradictory from one study to another. It was discovered that a portion of cellular eIF5A was localized to the nucleus, primarily at nuclear pore-associated intranuclear filaments (Rosorius and others, 1999), and that it was necessary for the transport of Rev in *Xenopus* oocytes (Bevec and Hauber, 1997). The interaction of eIF5A with Rev and CRM1/exportin1 was directly involved with the promotion of the export of Rev-mRNA complexes from the nucleus. It has also been reported that inhibition of deoxyhypusine synthase and deoxyhypusine hydroxylase results in antiretroviral activity of HIV-1 (Andrus and others, 1998; Hauber and others, 2005). On the other hand, studies have

shown that eIF5A is localized to the cytoplasm in mammalian cells with some association with the endoplasmic reticulum (Shi and others, 1996).

Numerous reports also suggest that there is no functional interaction between eIF5A and HIV Rev. Since eIF5A cellular distribution is not altered after HIV-1 Rev expression, there is little evidence to indicate that there is a functional association between the two molecules (Shi and others, 1997). Contradicting evidence also exists in the interactions of eIF5A with CRM1/exportin1 and exportin4. While studies from Rosorius et al. indicate an interaction with the nuclear export receptor CRM1 (Rosorius and others, 1999), another study illustrates that blocking of CRM-1 dependent export does not change the localization of eIF5A (Joao and others, 1995). CRM1 was also found to bind 1,000 times more weakly to eIF5A than exportin4, and eIF5A lacking the hypusine modification bound to exportin4 weaker than its modified counterpart (Lipowsky and others, 2000). Currently there are no studies that illustrate what effects inhibition of exportin4 might have on the distribution of eIF5A.

A study published in 2009 by Lee et al. may shed some light on why it appears that eIF5A seems to have such contradictory roles depending on where it is localized. Upon activation by the posttranslational synthesis of the amino acid hypusine, eIF5A also undergoes acetylation at a specific lysine residue. These two modifications appear to have an influence on where eIF5A is localized. While the precursor eIF5A protein tends to be localized both in the cytoplasm and the nucleus, hypusinated (active) eIF5A is only found in the cytoplasm (Lee and others, 2009). Mutant eIF5A proteins with defective hypusine modifications were localized similar to the unhypusinated eIF5A precursor

protein and hypusine-modified mutant proteins were localized mainly in the cytoplasm, suggesting that hypusine plays a role in where eIF5A is localized.

The role of eIF5A in mRNA decay is likely a secondary one, due to the protein's effect on the elongation step of translation. Temperature sensitive mutants at restrictive temperature exhibited an accumulation of unstable forms of eIF5A and mRNA decay defects (Zuk and Jacobsen, 1998; Valentini and others, 2002). Another study by Schrader et al. saw severe growth phenotypes, induction of apoptosis, and accumulation of nonsense-mediated-decay targeted mRNAs (Schrader and others, 2006). Such examples would likely be attributed to the inhibition of translation elongation due to the knock down of wild type eIF5A in these cells.

*Specific Aim: Identification of eIF5A Binding Proteins and Characterization of eIF5A Expression in* Zea Mays

Identifying key proteins that bind to eIF5A, as well as quantifying the expression of isoforms of the protein may provide insight into the function of this protein. eIF5A was once proposed to have a role in transcription initiation, hence the name. However, research has found that eIF5A is not necessary for protein synthesis. Recent studies have linked eIF5A to a wide array of proteins, such as translational machinery, p53, syntenin, CRM-1, HIV-1 Rev, and actin, suggesting that above all, eIF5A may be involved in the elongation step of translation. Secondary roles may implicate the protein in the transport of molecules from the nucleus to the cytoplasm and in regulation of cell growth. To further investigate the function of eIF5A in cellular growth, the localization of the protein in developing plants was analyzed as well.

# CHAPTER TWO

## Materials and Methods

### *Developmental Time Course of eIF-5A Expression in Maize Endosperm*

Endosperm from the W64A inbred line of *Zea mays* (grown in spring 2010) was dissected from multiple kernels of varying ages: 10, 14, 18, 20 and 22 days after pollination (DAP). The endosperms were frozen in liquid nitrogen and then ground into a fine flour using a mortar and pestle. Approximately 50-100 mg of flour from each sample was added to a microcentrifuge tube and suspended in 4 volumes of extraction buffer A (20mM HEPES pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT). The tubes were incubated at room temperature with shaking for 2 hours. After incubation, the tubes were centrifuged at room temperature for 15 min; the supernatant was transferred to a new tube. 300 µl of total protein extract (supernatant) from each tube was added to 700 µl of 100% ethanol. The tubes were mixed well and incubated for 2 h at room temperature. The sample was centrifuged again to separate ethanol soluble zein proteins (supernatant) from non-zein proteins (pellet).

Zein proteins were dried in a centrifugal evaporator (Eppendorf, Hamburg, Germany) to remove all liquid. The non-zein protein pellet was washed twice with 70% ethanol and air dried until edges were transparent. The pellet was then resuspended in 200 µl of 5X Laemmli sample buffer and heated to 85°C to dissolve the pellet. Once resuspended, 10 µl of each sample was run on an SDS PAGE gel (12% acrylamide) according to the manufacturer's instructions. Proteins were then transferred to a PVDF (Pall Corporation, Port Washington, NY) membrane and a Western blot was performed

with a custom rabbit primary antibody to eIF5A at a 1:3000 dilution and a secondary goat anti-rabbit antibody at a 1:30,000 dilution. Presence of eIF5A was detected using Pierce's Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA). Integrated density of illuminated bands was then measured with ImageJ software (Rasband, 1997-2011).

### *Maize Tissue Expression of eIF-5A*

Twelve kernels from W64A inbred line of *Zea mays* were planted in Perlite as a growth matrix. Seedlings were allowed to grow for 14 days. The root of each seedling was dissected into three sections: root tip (bottom 3-4 mm), central root, and proximal root (4-5 mm from the embryo). The dissected portions were then frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. The powder was weighed, added to a microcentrifuge tube, and 12X extraction buffer A was added. Once the powder was resuspended in the extraction buffer, the tubes were incubated at 37°C with shaking for 2 h. The samples were centrifuged at 14,000 *g* and the supernatant was removed into a new tube; 300 µl of this protein extract was added to 700 µl 100% ethanol, the tube was mixed well, and incubated for 1 hr. at -20°C. The sample was centrifuged again to separate ethanol soluble zein proteins (supernatant) from non-zein proteins (pellet). The pellet was washed 2X with 70% ethanol and dried until the edges were transparent. The pellet was then dissolved in 200 µl of 8M urea, followed by the addition of 4 volumes of 5X sample buffer. The protein samples, 15 µl of each, were analyzed by SDS-PAGE and western blotting as described above.

*Expression and Purification of Recombinant Maize eIF5A Protein*

2YT broth (2% w/v Tryptone, 1% w/v Yeast Extract, 0.5% w/v NaCl, and 100 µg/l ampicillin) was inoculated with *Escherichia coli* expression cell line OX41 C07-02, which expresses 6X His-tagged maize eIF5A in the expression vector pCRT7-NT (Invitrogen, Carlsbad, CA). The culture was incubated overnight and then transferred into 10 volumes of fresh 2YT broth with ampicillin and was incubated with shaking until reaching an $OD_{600}$ of 0.6-0.8. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM and incubated again for an additional 3-4 h. The cells were collected at 10,000 x g for 5 min and resuspended in binding buffer (300 mM NaCl, 30 mM Imidazole pH 8.3). The cells were lysed using sonication three times for1 min, with 30 s on ice between each round. The lysate was centrifuged at 30,000 x *g* for 20 min at 4°C, and the supernatant containing cellular proteins was collected.

A Chelating Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden), charged with $Ni^{2+}$, was equilibrated using 30 mL binding buffer and the entire volume of bacterial cell lysate was passed over the column. The column was washed with 30 ml of wash buffer (300 mM NaCl, 60 mM Imidazole pH 8.3). The bound proteins were eluted from the column with 30 ml of elution buffer (300 mM NaCl, 300 mM Imidazole pH 8.3) and 1-1.5 ml fractions were collected. A Bradford assay was used to determine the presence of protein in the fractions and purity of eIF5A was assessed using SDS-PAGE (12% acrylamide running gel).

To remove imidazole from the protein solution, buffer exchange was performed by gel filtration using a Sephadex G-50 column. The column was first equilibrated with 30 mL of 10mM HEPES buffer, pH 7.5. Protein samples were pooled to 5mL and then

run through the column. The protein was eluted with the HEPES buffer, the concentration determined by absorbance at 280 nm using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA), flash frozen with liquid nitrogen, and then stored at -80°C until needed.

*Coupling of Recombinant eIF5A to Sepharose Beads*

Recombinant eIF5A was concentrated to 15-20 mg/ml by lyophilizing protein samples until powdered and resuspending in a reduced volume of liquid. A final concentration of 20.83 mg/ml was achieved. Each tube of lyophilized powder (5 tubes total) was resuspended in 1 mL coupling solution (0.1 M $NaHCO_3$ pH 8.3 with 0.5 M NaCl) overnight.

Approximately 1g of CNBr-activated Sepharose® powder (GE Healthcare Sciences, Pittsburgh, PA) was suspended in 1mM HCl for 30 minutes to allow swelling. A final volume of approximately 3.5 mL was achieved. The gel was then washed with 15 volumes of cold 1 mM HCl 3 times. The gel was equilibrated with coupling buffer and ligand. 5 mL of the coupling solution was added to the CNBr gel and incubated for 4 h at room temperature with end over end rotation. The gel was then transferred to a column and excess ligand was washed away with 5 volumes of 0.1 M $NaHCO_3$. The gel was resuspended in 0.1 M Tris-HCl, pH 8.0, and was incubated for 2 h to block any remaining CNBr active sites. The column was then washed with 3 cycles of alternating pH (10 mL of each): 0.1M sodium acetate, pH 4.0 with 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0 with 0.5 M NaCl. 20% ethanol was added to the column as a preservative.

*Pull-Down Analysis*

Beads coupled to eIF5A were added to microcentrifuge tubes for a final bed volume of 25-50µl. Sepharose beads not bound to eIF5A were used as a control for trapping and non-specific binding. Each of the samples was washed with 1ml ice cold HNTG buffer [20mM HEPES pH 7.5, with 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% (w/v) glycerol] and centrifuged at 3,000 x g at 4°C. The beads were washed a total of three times.

Whole protein samples were extracted from corn endosperm (inbred line W64A, 18 days after pollination) using extraction buffer (20 mM HEPES pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT). One ml of the endosperm lysate was added to each tube and incubated for either 90 min or overnight. The bound proteins were collected by centrifugation at 3,000 x *g* for 2 min at 4°C. The pellet was washed four times with HNTG and then resuspended in 25-100 µl of Laemmli sample buffer. The samples were heated at 95°C for 5 minutes and centrifuged at room temperature at 12,000 x g. The supernatant was collected and analyzed using SDS-PAGE (12% acrylamide). The gel was stained for 60 min with Sypro tangerine (Molecular Probes, Inc., Eugene, OR) at a 1:5000 dilution in 7% (v/v) acetic acid, and visualized on a Typhoon FLA 9000 (GE Healthcare, Piscataway, NJ) at a PMT voltage of 600 V.

*In-Gel Digestion and Purification of Protein Spots*

Visible bands of interest were excised from the gel using a razor blade and cut into cubes of less than 1 mm. The cubes were placed in Fisherbrand siliconized low retention microcentrifuge tubes (Thermo Fisher Scientific Inc., Pittsburgh, PA). The pieces were covered with 40 µl of 100mM $NH_4HCO_3$ and 40 µl of acetonitrile and

incubated 15 min at room temperature. Supernatant was removed and the pieces were covered with another 1:1 application of acetonitrile/ $NH_4HCO_3$. The second wash was removed and the gel pieces were then dried in a speedvac concentrator (Concentrator plus, Eppendorf, Hamburg, Germany).

The dried gel pieces were rehydrated in 40 µl of trypsin digestion buffer (50 mM $NH_4HCO_3$, 5 mM $CaCl_2$, 15 µg/µl trypsin) and samples were incubated for 45 min on ice. The excess solution was removed, 40 µl of digestion buffer was added, and samples were incubated for 16 h at 37°C. Following incubation, the supernatant was removed and saved in a low retention microcentrifuge tube. 20 µl of 25mM $NH_4HCO_3$was then added to the samples and incubated for 15 min, followed by addition of 20 µl of acetonitrile (15 min. incubation). The supernatant was removed and added to the previously saved extraction in a low retention tube. Three additional washed were performed using 20 µl of 5% formic acid was then added to each sample and incubated for 15 min, followed by 15 min incubation with 20 µl acetonitrile. The supernatant from these washes was added to those removed previously. Sufficient 10 mM DTT was added to each sample to give a final concentration of 1 mM. The extracted peptides were dried an Eppendorf Concentrator plus, and then resuspended in 20 µl of 5% formic acid. Purification of extracted peptides was performed using Zip-Tip pipette tips (Millipore, Billerica, MA) according to the manufacturer directions. LC-MS/MS was performed on LTQ-Orbitrap Velos with a one hour elution gradient and database search at the Donald Danforth Plant Science Center Proteomics and Mass Spectrometry Facility (St Louis, MO).

*Immunoprecipitation*

*Zea mays* endosperm from strain W64A was dissected and the protein extracted with 1 ml of extraction buffer (containing 20 mM HEPES pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT). The extracted material was centrifuged for 15 min and the supernatant was removed and transferred to a new tube. A small portion of the whole protein extract was saved for further analysis.

Protein G Mag Sepharose beads (GE Healthcare, Indianapolis, IN) were gently resuspended in their tubes to create a slurry. 25 µl of the slurry (~ 5 µl bead volume) was transferred to a microcentrifuge tube and placed into a magnetic rack to allow for the removal of the storage solution. The tube was removed from the rack and 500 µl of binding buffer (50 mM tris, 150 mM NaCl pH 7.5) was added for equilibration of the beads. The tube was manually inverted to allow resuspension of the beads and replaced in the magnetic rack to allow removal of the equilibration buffer. Immediately after equilibration, a 2% solution of crude rabbit serum, raised against eIF5A, diluted in binding buffer was added to the beads and incubated with end-over-end mixing for one hour. The liquid was then removed and saved for further analysis. The beads were then washed with 500 µl of binding buffer. A buffer change was then performed and the beads were resuspended in 500 µl of 200 mM triethanolamine, pH 8.9 (crosslink solution A). The solution was removed and bound antibodies were crosslinked to the magnetic beads using 500 µl of crosslink solution A with 50 mM dimethyl pimelimidate dihydrochloride. The magnetic beads were incubated for 1 h with slow end-over-end mixing and the crosslinking solution was subsequently removed.

After crosslinking, the beads were washed with 500 µl of crosslink solution A. To block any active crosslink sites on the beads, 500 µl of 100 mM ethanolamine, pH 8.9 was added to the tubes and incubated for 15 min with end-over-end mixing. The liquid was removed, and the beads were washed with 500 µl of elution buffer (0.1 M glycine-HCl, 2M urea, pH 2.9) to remove any non-bound antibody. The beads were washed twice with binding buffer and total protein extract from *Zea mays* was added, followed by a room temperature incubation of 1 h with end-over-end mixing. After incubation, the extract was removed and saved, then the beads were washed 3X with 500 µl of wash buffer (50 mM tris, 150 mM NaCl, pH 7.5). Samples of each of the washes were saved. Bound target proteins were then eluted by adding 150 µl of elution buffer to the beads, incubating 2 min at room temperature, and removing the liquid. The elution step was repeated and the liquids from each extraction were combined. 5X Laemmli buffer was added to each of the samples and analyzed by SDS PAGE gel (12% acrylamide) and Western blotting with antibody to eIF5A (1:3000 dilution) to ensure presence of target protein. The gel was stained for 60 min with Sypro tangerine at a 1:5000 dilution in 7% (v/v) acetic acid. The gel was then visualized on a GE Typhoon FLA 9000 at a PMT voltage of 700 V.

### *RNA Extraction and cDNA Synthesis*

Endosperm from ≥ 5 kernels each of 10, 14, 18, and 22 DAP were dissected and placed in liquid nitrogen. Endosperm were dissected from kernels from 3 different ears at each DAP (12 samples total). The frozen endosperms were then ground into flour using a mortar and pestle, and approximately 0.1 g was weighed into microcentrifuge tubes and kept at -80°C until use.

Twelve kernels from W64A inbred line of *Zea mays* were planted in Perlite as a growth medium and allowed to grow for 14 days. Roots and leaves were dissected and pooled as described for protein extraction. Root and leaf frozen samples were ground into a fine powder using a mortar and pestle with liquid nitrogen. The leaf powder was weighed to approximately 0.1 g and the root powder was weighed to 0.05-0.07g. All samples were kept frozen at -80°C until use.

Into each frozen sample (for endosperm, leaf, and root tissue), 0.5 mL of Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA) was added, and the samples were incubated for 5 min. with end-over-end rotation at room temperature. The samples were then clarified by centrifuging 2 min at 12,000 *g* at room temperature and the supernatant was transferred to a new tube. To each clarified extract, 0.1 ml of 5 M NaCl was added and mixed gently, followed by an addition of 0.3 ml of chloroform with mixing by inversion of the tubes. The samples were then centrifuged at 4°C and 12,000 *g* for 10 min, and the top, aqueous phase was then transferred to another tube. To the aqueous phase, an equal volume of isopropyl alcohol was added, mixed thoroughly, and incubated at room temperature for 10 min. After the incubation, the sample was centrifuged at 4°C and 12,000 *g* for 10 min.

The supernatant was decanted, and the pellet was washed with 1 ml of 75% ethanol. The samples were centrifuged for 1 min at 12,000 *g* and the supernatant removed again. The pellets were dissolved in 30 µl of RNase-free water and the concentration was determined using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The samples were diluted with distilled water to a concentration of ~100 ng/µl. The cDNA was created from 2 µg of RNA using qScript™

cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and was diluted 10-fold prior to use in qPCR.

### *Quantitative PCR Analysis of eIf5A Isoforms*

Three isoforms of eIF5A were identified in the *Zea mays* genome using a BLAST search. A set of primers were then created for each individual isoform and one set of primers was created to act as a universal primer for all three isoforms to analyze the total expression level of the eIF5A protein. Primers were designed for a 62°C annealing temperature and to span exon junctions (Table 2.1).

Table 2.1. Primers created for qPCR analysis.

| Primer Name | Sequence |
|---|---|
| eIF5A universal forward | ACAGTGGCAACACTAAGGATGACCT |
| eIF5A universal reverse | GCGCAGATCTGTTCCTCGCCCAT |
| eIF5A 2974 forward | ACAGTGGCAACACTAAGGATGACCTC |
| eIF5A 2974 reverse | GCTGCTGCCGATCCTACAAGGCA |
| eIF5A 3696 reverse | AGGGTTCCGTCTCCAAAGCCAAGG |
| eIF5A 3696 and 4030 forward | TCAGATGGCAACACTAAGGATGATCTT |
| eIF5A 4030 reverse | AGCGCGGGTAAACTGCCCACC |

qPCR was performed in a 72-well rotor in the Corbett Quantitative PCR Rotor-Gene$^{TM}$ 3000 (Qiagen, Velancia, CA). The volume of each reaction was 20 µl; 10 µl of PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, MD), 1 µM of

the forward and reverse primers, 2 µl cDNA template or 1 µl of the plasmid control standard (copy numbers from $10^5$ to $10^8$). Expression levels of the three eIF5A isoforms were measured against the expression of the control protein, retinoblastoma-related protein 1 (RRb1). The PCR program was as follows: 2 min 50°C hold, 95°C for 10 min, 50 cycles of 95°C 15 sec, and 60°C for 1 min. The melting curves were obtained through a 55°C to 95°C ramp with 1°C ramp rate. The resulting data was analyzed using the Q-Gene Core Module file.

# CHAPTER THREE

## Results

### *Abundance of eIF5A in Developing* Zea Mays *Endosperm*

eIF5A has been implicated in cell proliferation in numerous studies (Schnier and others, 1991; Chen and Liu, 1997; Nishimura and others, 2005; Park, 2006; Frigieri and others, 2007). Because levels of cell division are increased in developing plant tissues, it could be postulated that there would be a high expression of eIF5A in developing endosperm, root, and leaf tissues of *Zea mays.*

Endosperm dissected from five different developmental stages of *Zea mays* were separated by SDS-PAGE, analyzed by a Western blot with a primary antibody to eIF5A, and stained with Coomassie blue (Figure 3.1). Using the Coomassie stained gel as a control for the amount of protein loaded from the maize endosperm extracts, the total protein abundance was low in the 10 DAP stage, but fairly constant in the remaining stages (14-22 DAP). In the Western blot analysis of the developmental time course, when incubated with antibody to eIF5A, the level of fluorescence is highest at the earliest developmental stage, 10 DAP. In the 14 and 18 DAP endosperm, the levels of eIF5A decrease approximately 50% as determined by densitometry. Interestingly, in the 20 and 22 DAP endosperm, the levels rise somewhat nearing the levels they were at 10 DAP.

### *Abundance of eIF5A in Developing* Zea Mays *Root and Leaf Tissue*

Root tissues from three sets of biological replicates of 14 day old seedling were dissected. The first areas examined were the root tips (bottom 3-4 mm, below the root hairs), which were removed from the dissected portion and frozen in liquid nitrogen. The

second area examined was a central area where the root hairs were prevalent. The third area examined was the proximal root (4-5 mm from the embryo), which was a pink color. From the same plants, leaf tissue was dissected and split into three categories: outer edge (outer 3 mm), midrib, and central tissue (tissue located between midrib and outer 3-4 mm of leaf).

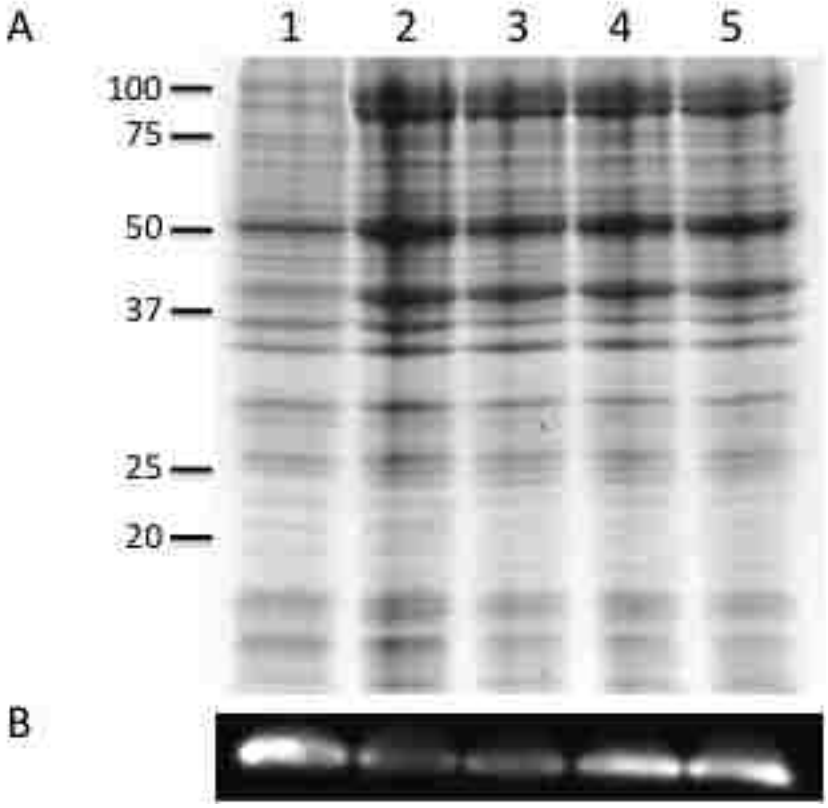


Figure 3.1. Western blot analysis of eIF5A abundance in developing *Zea mays* endosperm.
10 µl of non-zein proteins of dissected endosperm from 10, 14, 18, 20, and 22 DAP from the W64-A inbred line of *Zea mays* were analyzed by Western blot analysis. Lane 1 = 10 DAP, lane 2 = 14 DAP, lane 3 = 18 DAP, lane 4 = 20 DAP, and lane 5 = 22 DAP. (A) Coomasie Blue stained gel. Molecular mass standards are kDa. (B) Western blot with antibody against eIF5A. Band sizes are approximately 18 kDa.

The protein from the samples was extracted, separated by SDS-PAGE, analyzed by Western blot with a primary antibody to eIF5A, and Coomassie stained (Figure 3.2). In the Coomassie stained gel used as a control, there was a large amount of total protein

in the root tip extract, but very little in the central or proximal root. In the leaf tissue, the midrib protein extract exhibited the lowest levels of protein abundance, while the central and outer leaf were similar, with the central leaf having slightly darker bands. In the Western blot analysis, the only signal observed in the root tissues was from the root tip tissue (Figure 3.2, lane1). The central root and proximal root exhibited no visible signal (Figure 3.2, lanes 2 and 3). It is possible that the low fluorescence seen in the central and proximal root is due to low levels of protein loading, as observed in the Coomassie stained control. In leaf tissue, the highest signal was observed from the outer edge of the leaf (Figure 3.2, lane 6). The central leaf tissue had a slightly lower intensity than the outer edge (Figure 3.2, lane 5), while the lowest expression of eIF5A was observed in the leaf midrib samples (Figure 3.2, lane 4).

*Protein Interactions with eIF5A by Pull Down Analysis and Immunoprecipitation*

The exact function of eIF5A is not known, despite it being implicated in numerous cellular activities. Investigating interactions between eIF5A and other cellular proteins could aid in the delineation of the function of this elusive protein since it is likely that proteins involved in the same or similar roles as eIF5A should also associate and bind with it *in vivo*. A pull-down experiment was performed using eIF5A-coupled beads and maize endosperm extract (Figure 3.3). The flow-through of the maize endosperm that did not bind to the control beads or Sepharose beads was analyzed to ensure adequate binding. Elutions were performed for the control, and for the 90 minute and overnight incubation periods. Elution for the control beads showed very little non-specific binding, while both incubations had multiple bands indicative of protein bound to eIF5A. Bands were excised and digested from the overnight incubation as they were much more

pronounced and distinct. Band sizes that were excised, from largest to smallest, were approximately 100 kDa (PD 1), 90 kDa (PD 2), 50 kDa (PD 3), 45 kDa (PD 4), 42 kDa (PD 5), 40 kDa (PD 6), 23 kDa (PD 7).

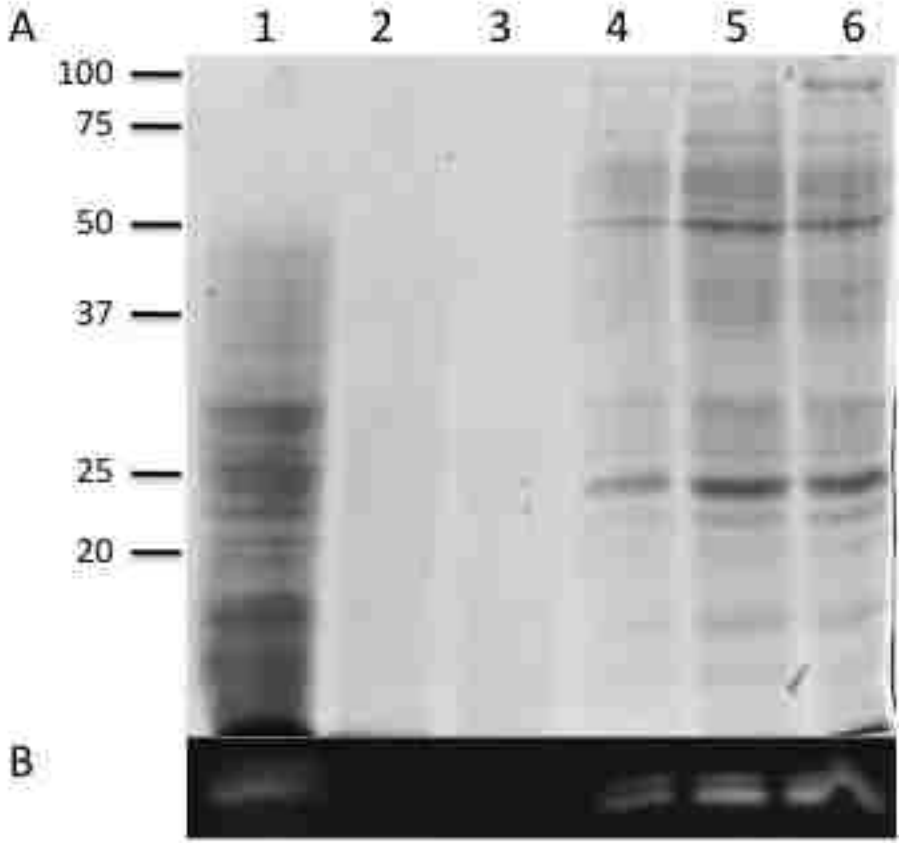


Figure 3.2. Western blot analysis of eIF5A abundance in root and leaf tissue of *Zea mays*. 15µl of protein extracted from root tip (RT; lane 1), central root (CR; lane 2), proximal root (PR; lane 3), leaf midrib (ML; lane 4), central leaf tissue (CL; lane 5), and tissue from the outer edge of leaves (OL; lane 6) from 14 day old *Zea mays* seedlings was run on SDS-PAGE. A) Coomasie Blue stained gel. Molecular mass standards are kDa. B Western blot with antibody against eIF5A. Band sizes are approximately 18 kDa.

These bands were excised, digested with trypsin, and analyzed by LC-MS/MS and the resulting mass spectra searched against the Genbank non-redundant protein database. Proteins were identified with a minimum protein identification probability of 99.0% and a minimum of two unique peptides. Among the proteins that had interaction

with recombinant eIF5A were numerous proteins related to the cytoskeleton: actin, cell division protein FtsZ, and three types of tubulin. Legumin, a storage protein, was also pulled down. Multiple translation factors (elongation factor 1-α, elongation factor 1-gamma 3, translational elongation factor EF-TuM) and metabolic enzymes (1,4-α-glucan-branching enzyme 2, sorbitol dehydrogenase homolog1, UDP-glycosyltransferase, alanine transaminase, alanine aminotransferase, acetolactate synthase, pyruvate phosphate dikinase 1) as well as an ATP-dependent 26S proteasome regulatory subunit were pulled down in the experiment (Figure 3.3; Table 3.1).

Protein identification matches were found for bands labeled PD 2, PD 3, PD 4, PD 5, and PD 6. Possible interactions were identified for the bands labeled PD 1 and PD 7, but they are not considered relevant since they proteins identified were Chain L from the apical membrane antigen of *Plasmodium vivax*, and immunoglobulin heavy chain (Igh) from mouse, *Mus musculus.* It is likely that the Chain L protein is a highly conserved protein since it has numerous homologs. Both Chain L and the Igh were likely contaminants from sample preparation, trays used for antibody incubation for Western blotting, or on the columns used in the pulldown experiment. Proteins identified in bands PD 1 or PD 7, are not included in Table 3.1 since they are not maize proteins and are irrelevant to the pulldown study and investigations into interactions with eIF5A.

Protein interactions with eIF5A were also investigated by immunoprecipitation using protein extract from *Zea mays* endosperm. Interacting proteins were separated by SDS-PAGE and stained with Sypro tangerine (Figure 3.4). Proteins were not analyzed by LC-MS/MS because signals were not as strong as observed in the pull-down analysis. Instead, to confirm that similar proteins interact with native, hypusine modified eIF5A

Western blots were performed with antibodies to interacting proteins that were seen in the pull-down image (Figure 3.4). Antibodies to initiation factor 2A (IF2A), initiation factor 4G, tubulin, elongation factor 1-α (EF1-α), actin, and ribosomal protein S6 were used in the Western blot analysis of both the immunoprecipitation and the pull-down experiments.

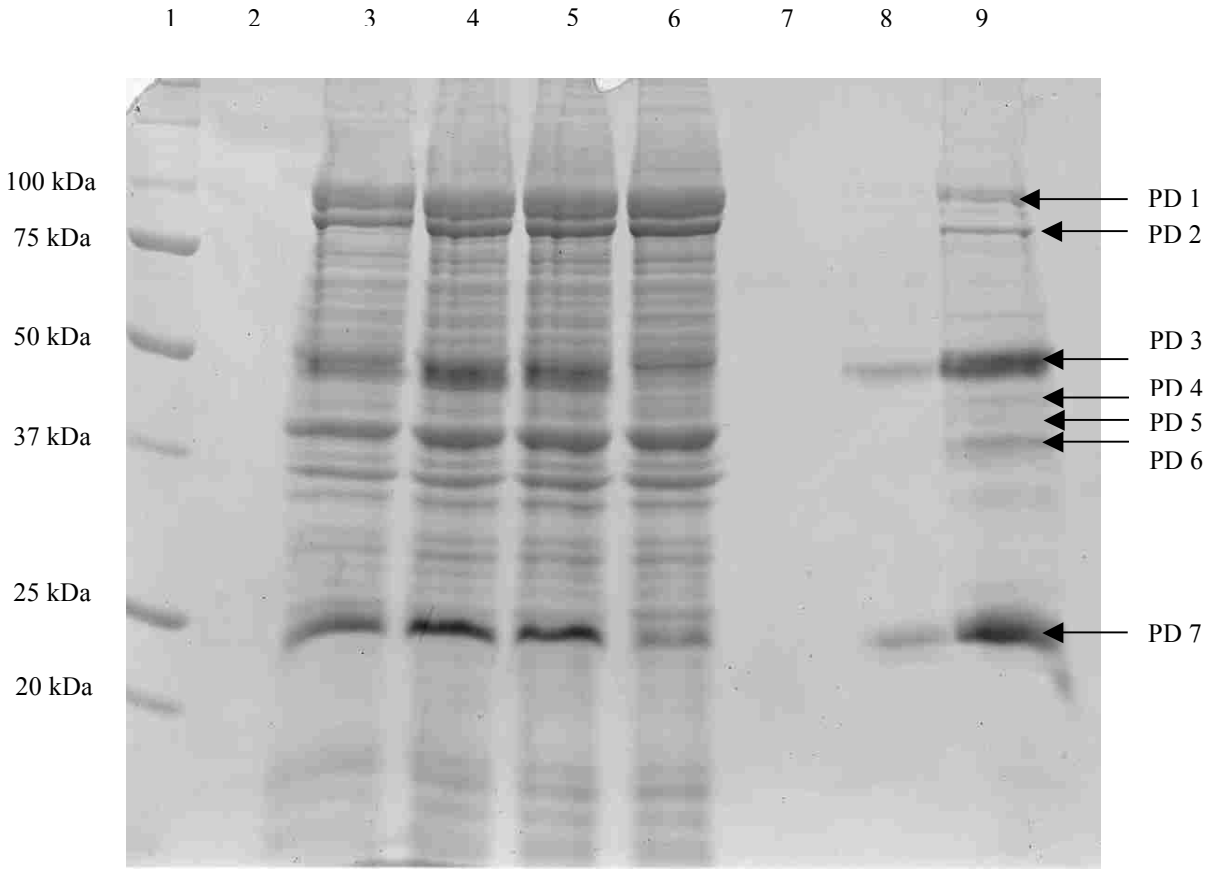


Figure 3.3. Sypro tangerine stained SDS-PAGE gel of protein interactions with eIF5A from pull down analysis. Lane 1= marker (band sizes to the left of figure), lane 2 = blank, lane 3= whole endosperm protein extract from *Zea mays*, lane 4 = G-50 sepharose (control) flow through, lane 5 = CNBr beads (90 min. incubation) flowthrough, lane 6 = CNBr beads (overnight) flow through, lane 7 = elution of G-50 beads, lane 8 = elution of 90 min. incubation, lane 9 = elution of overnight incubation. Bands highlighted with arrows were digested with trypsin and analyzed by LC-MS/MS and correlate to the numbered bands appearing in Table 3.1.

Table 3.1. Identified protein interactions with eIF5A by LC-MS/MS. Protein interactions from the pull-down experiment were digested and analyzed by LC-MS/MS. Proteins with at least 99% identification probability and 2 unique peptides were compared to the NCBI Protein bank using their accession numbers. Table includes the name of identified proteins, accession numbers, relative molecular weight, numbered band on the initial SDS-PAGE image (Figure 3.3), and function.

| Protein Name (from *Zea mays* unless otherwise stated) | Accession Number | Molecular Weight | Band on Pull-Down Image | Function |
|---|---|---|---|---|
| legumin 1 | gi 162460908 | 53 kDa | PD 3 | storage in seed vacuoles; inductor of chromatin condensation production |
| actin | gi 212274479 | 42 kDa | PD 4 | component of cytoskeleton; roles in cell division and cytokinesis |
| tubulin beta-7 chain | gi 162460038 | 50 kDa | PD 3 | major constituent of microtubules; microtubule-based movement and cell division; protein polymerization; GTP binding |
| tubulin alpha-1 chain | gi 135398 | 50 kDa | PD 3 | same as above |
| beta-tubulin (*Oryza sativa*) | gi 1076737 | 50 kDa | PD 3 | same as above |
| cell division protein FtsZ (*Oryza sativa*) | gi 115454331 | 47 kDa | PD 4 | initiation of cell division in bacteria (also found in plant chloroplasts); GTP-binding protein; similar to tubulin |
| elongation factor 1-α | gi 195620072 | 49 kDa | PD 3 | elongation phase of translation; role in nuclear export of proteins |
| elongation factor 1-gamma 3 | gi 195628630 | 47 kDa | PD 3 | elongation factor; component of eukaryotic translation elongation factor 1 complex |

Table 3.1. Continued.

| Protein Name (from *Zea mays* unless otherwise stated) | Accession Number | Molecular Weight | Band on Pull-Down Image | Function |
|---|---|---|---|---|
| translational elongation factor EF-TuM | gi 11181616 | 49 kDa | PD 4 | elongation factor in mitochondria; GTPase that delivers amino-acylated tRNAs to the ribosome |
| 1,4-α-glucan-branching enzyme 2 | gi 162459706 | 91 kDa | PD 2 | glycogen biosynthesis; required for sufficient glycogen accumulation |
| sorbitol dehydrogenase homolog1 | gi 226504732 | 39 kDa | PD 3, 4, 6 | interconversion of glucose and fructose |
| UDP-glycosyltransferase | gi 293335525 | 51 kDa | PD 3 | catalyzes transfer of glycosyl moiety |
| alanine transaminase | gi 212722888 | 19 kDa | PD 3 | catalyzes alanine production specifically in response of plants to anaerobic stress |
| alanine aminotransferase | gi 3694807 | 53 kDa | PD 3 | same as "alanine transaminase" |
| acetolactate synthase (small subunit) | gi 293333562 | 39 kDa | PD 3 | feedback inhibition of biosynthesis of branched chain amino acids |
| pyruvate phosphate dikinase 1 | gi 162460730 | 103 kDa | PD 2 | starch/protein balance in endosperm; influence on alanine-aromatic amino acid synthesis |
| ATP-dependent 26S proteasome regulatory subunit (*Oryza sativa*) | gi 115444877 | 45 kDa | PD 3, 4 | non-lyosomal protease; component of ubiquitin-mediated substrate degradation pathway |

The presence of tubulin, actin, and EF1-α in the LC-MS/MS data was confirmed in the Western blot of the pull-down experiment. The Western blot of the immunoprecipitation resulted in bands of similar size. The bands were more pronounced in the pull-down (with the exception of actin) when compared to the immunoprecipitation, due to the abundance of protein that was eluted in the pull-down experiment. Antibodies to initiation factor 2A were used to illustrate whether any other initiation factors may be associated with eIF5A that might have not been identified during the pull-down and subsequent LC-MS/MS. IF2A was present in samples from both experiments, but exhibited a more intense illumination in the pull-down. Using antibodies to RPS6 and initiation factor 4G resulted in very little signal. RPS6 was only present in the immunoprecipitation, while IF4G was not present in the elution of the immunoprecipitation or the pull-down (not shown in Fig. 3.4).

### *Gene Expression Analysis by qPCR*

Three different isoforms of eIF5A in *Zea mays* were identified through a BLAST search of the maize reference genome release 5b.60. Primers were designed for each individual isoform and a primer was designed to amplify all isoforms of eIF5A, and these were subsequently used in qPCR. The raw data were analyzed and normalized using the Q-gene Core Module file and statistical analysis using a student's t-test ($\alpha = 0.05$).

To observe if eIF5A gene expression varied amongst different *Zea mays* tissues (leaf and root that were studied previously) qPCR was performed with all four primer sets using extracted protein from plant tissue. Leaf tissue was not dissected into separate regions as it was with previous analyses, but instead removed from the plant and frozen in liquid nitrogen whole. Root tissue was divided into two categories, root tip and

proximal root (areas closer to the embryo, with root hairs removed) because there was such a large difference in eIF5A protein expression between the root tip and proximal root. As shown in Table 3.2, relative expression of eIF5A isoforms were the highest in the leaf tissue and lowest in the proximal root. Ribosomal protein S6 (RPS6) was compared against the eIF5A data since it is believed to have a similar role to eIF5A in controlling cell growth and proliferation through regulation of translation.

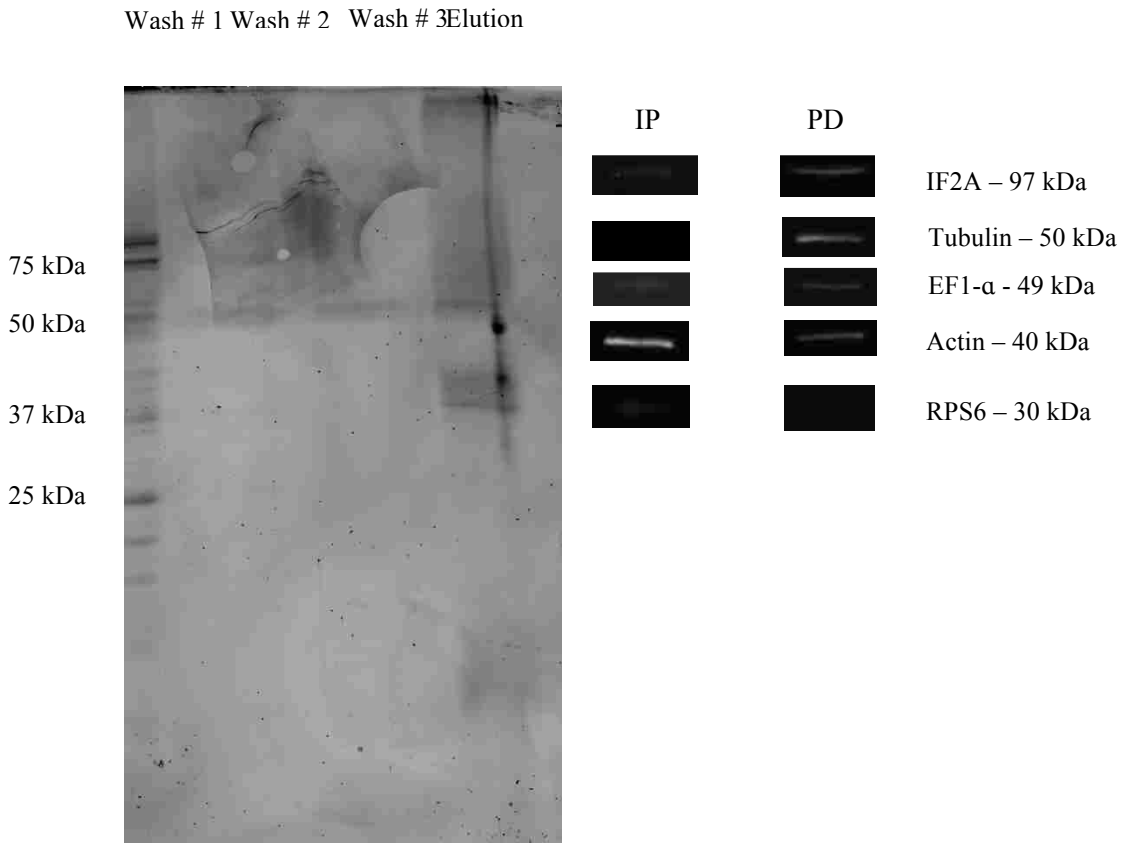


Figure 3.4. Protein interactions with eIF5A from immunoprecipitation and pull-down. Image to the left is the Sypro tangerine stained SDS-PAGE gel of the cross-linked immunoprecipitation. Lanes that included the protein marker, eIF5A antibody, antibody flow through, whole protein extract, and extract flow through were not included in figure because of the high voltage used for visualization. The first lane on the left, appearing in the image is the first wash, followed by wash #2, wash #3, and the elution in the final lane. Approximate protein sizes are given to the left of the figure. To the right of the image are the illuminated bands from Western blots of the immunoprecipitation (IP) and pull-down (PD) experiments. Approximate band sizes are given to the right of the image (tubulin and EF1-α are have been separated slightly due to their similar molecular weights).

Table 3.2. Average expression and SEM for eIF5A isoforms in various *Zea mays* tissues.

| Primer | Leaf | Root Tip | Proximal Root | Leaf SEM | Tip SEM | Proximal SEM |
|---|---|---|---|---|---|---|
| eIF5A all | 20.0333 | 13.55 | 8.243 | 0.0333 | 3.6459 | 3.7562 |
| eIF5A-1 | 32.7333 | 19.9667 | 14.6863 | 3.209 | 4.7259 | 6.9015 |
| eIF5A-2 | 0.013 | 0.0155 | 0.0138 | 0.0016 | 0.00045 | 0.0017 |
| eIF5A-3 | 0.0477 | 0.0906 | 0.0433 | 0.0058 | 0.0391 | 0.0058 |
| RPS6 | 10.6167 | 18.58 | 12.9307 | 0.4868 | 5.8039 | 6.8087 |

Similar isoform gene expression analysis was performed with the four primers sets using endosperm extract from various developmental stages in maize. Endosperm was harvested from three sets of biological replicates for 10, 14, 18, and 22 DAP developmental stages. The qPCR and subsequent analysis was performed as described in previous sections. From the resulting data shown in Figure 3.5, the relative expression of all isoforms of eIF5A is highest around 10 DAP, when cell proliferation and mitotic activity is at its peak. The expression levels begin to decrease until 22 DAP, where they become slightly elevated.

Isoform eIF5A-1 had the highest expression levels out of the three isoforms. The levels of expression for the other two isoforms, eIF5A-2 and eIF5A-3 were quite low in comparison. In addition, the eIF5A-1 isoform has a more pronounced increase in expression at the 22 DAP stage than the universal eIF5A does (Figure 3.5). Levels of expression of ribosomal protein S6, although higher than that of eIF5A-1, follow a similar expression pattern. Both proteins have an initial expression that is high at 10 DAP, followed by a decrease in expression levels until 22 DAP where a slight elevation is observed.

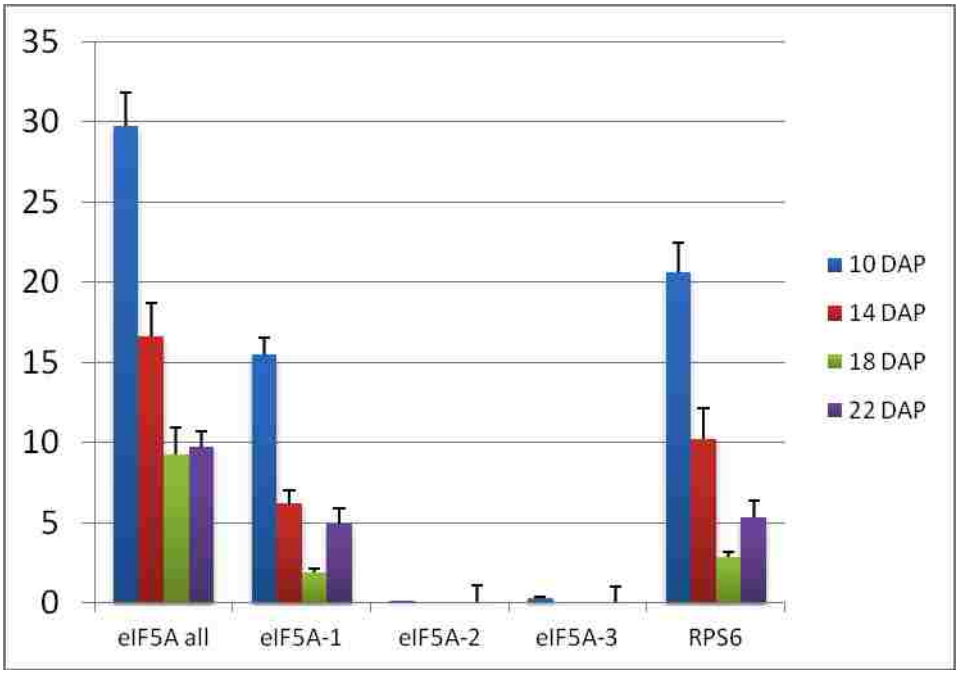


Figure 3.5. Graphical representation of expression levels of eIF5A isoforms in developing *Zea mays* endosperm. Three isoforms of eIF5A in maize protein extract had expression levels analyzed by qPCR. Y-axis is the relative abundance of each isoform and X-axis is the protein isoform. Also compared is the total expression of all eIF5A isoforms (eIF5A all) and RPS6, a protein thought to have a similar influence on eukaryotic translation rates.

# CHAPTER FOUR

## Discussion and Conclusions

In this study, the role of eIF5A and its abundance in various tissues of *Zea mays* were investigated. The importance of eIF5A in cellular proliferation has been established using a variety of eukaryotic organisms, such as *S. cerevisiae* and mice, but little work has been done with plants (Schnier and others, 1991; Chen and Liu, 1997; Nishimura and others, 2005; Park, 2006; Frigieri and others, 2007).

The endosperm of cereal plants, such as *Zea Mays*, function in the storage of materials like proteins and starch for the developing maize seed. This important tissue comprises 85-90% of the mature dry kernel weight and serves a crucial role in the maize plant development. Mitotic activity in the developing endosperm reaches its peak around 8-10 days after pollination (DAP), and then sharply drops after that time (Kowles and Phillips, 1985). Programmed cell death then begins near 16 DAP in the starchy endosperm, and continues until nearly 28 DAP (Young and Gallie, 2000).

In plant roots, the apical meristem is an area located near the tip of the root. The meristematic cells will divide continuously, with little to no differentiation due to an abundance of the plant hormone auxin. This high rate of cell division will cause the root to grow and cellular differentiation will begin when the hormone cytokinin balances auxin levels proximal to the meristem (Moubayidin and others, 2010). In areas outside of the root tip, there is little cell division occurring and the cells will eventually elongate, differentiate, and mature into specialized cells of the root tissue. In leaf tissue, the tip of the blade is formed first and new cells tend to form near the leaf base. Growth is often

more rapid near the outer edge of the blade than the center (midrib), and a characteristic V-shaped auricle is formed (Kiesselbach, 1949).

Using *Zea mays*, tissues that were known to have high levels of proliferation or translational activity, such as seed endosperm, root tip, and leaf blades, were examined both at the genetic and protein level. Maize endosperm, root, and leaf extracts were examined for expression of eIF5A protein and three distinct isoforms of the eIF5A gene.

Because the root tip contains the apical meristem, an area of very high mitotic activity, eIF5A expression is expected to be high if it is correlated with cell proliferation in plants as it is in animals and fungi. Consistent with this hypothesis, expression of eIF5A is highest at the root tip as shown in Figure 3.2. The central area and proximal area of the root have little to no cell proliferation and did not exhibit significant accumulation of eIF5A. As previously discussed, the absence of eIF5A protein may also be due to low levels of protein loading. Likewise, the outer edge of the plant leaf is an area with high levels of cell division, and the rate of cell division tapers closer to the midrib of the leaf. Similar to the root, proteins that are involved in cell proliferation should also be highest in leaf tissues closest to the edge of the leaf blade because this is the region with the highest rates of cell division. Consistent with this expectation, eIF5A protein levels are highest at the leaf edge and decrease closer to the midrib as shown in Figure 3.2. The leaf midrib exhibits a mid-range expression of eIF5A since there would still be some cell proliferation in this tissue, but not at as high of a level as the outer edge.

In the maize endosperm, eIF5A levels were highest in early developmental stages, and then decreased over time with an elevation again around 20 DAP. Tissues that were previously known to have high levels of cellular proliferation and rates of

mitosis were also the tissues that had elevated levels of eIF5A, thus confirming at least a secondary role of eIF5A in cell proliferation in plants. Similar results were seen in the genetic analysis of eIF5A isoforms.

The only conflicting data in protein expression was seen in the 20 DAP and 22 DAP endosperm. During these developmental stages, cell proliferation should be low since the endosperm is undergoing programmed cell death (Young and Gallie, 2000). A possible explanation may lay in another secondary role of eIF5A. This protein has been found to interact and bind to syntenin, a regulator of p53 dependent apoptosis. In a yeast two-hybrid screen, an up-regulation of eIF5A was found to increase levels of p53 and its silencing reduced p53 levels. Both interactions were dependent on the hypusination of eIF5A; if the protein was not modified to include hypusine, the previous effects were not seen. The up-regulation of eIF5A and increase in p53, lead to increased rates of p53-dependent apoptosis, but when eIF5A interacted with syntenin, the effects were diminished (Li and others, 2004).

Three isoforms of eIF5A were identified in *Zea mays* by BLAST analysis. These isoforms were named eIF5A-1, -2, and -3, with the first isoform being a previously cloned gene in the database for maize eIF5A. Isoforms of eIF5A have been identified in various other eukaryotic organisms, such as yeast, amphibians, chickens, mammals, and plants. Until this point, isoforms of eIF5A in *Zea mays* had not been identified. Most eukaryotic organisms have two isoforms of eIF5A, while *Arabidopsis* and *Zea mays* have three. These distinct isoforms can be co-expressed, as in chickens and amphibians (Clement and others, 2003), or expressed at different levels in specific tissues and one form is typically predominant (Guan and others, 2001; Jenkins and others, 2001). In *Zea*

*mays*, these data have shown that while all three isoforms are present in maize root, leaf, and endosperm tissue, eIF5A-1 is expressed at a significantly higher level than the other two in these tissues.

The relative expression of the eIF5A isoforms and their increased levels in leaf tissue (Table 3.2) may be due to the stage of development of the maize plant that was harvested. It is possible that, due to an increased need for photosynthetic tissue and subsequent leaf growth and development, that the relative expression of eIF5A in the leaf would be higher than the mitotically active root tip. The expression levels are still quite low in tissues experiencing little cell proliferation, like the proximal roots.

In developing maize endosperm, expression levels of eIF5A and RPS6 were similar in pattern (see Table 3.3), with the highest levels in 10 DAP, an initial decrease, and then an increase seen in 22 DAP, consistent with the pattern of eIF5A protein accumulation. RPS6, located in the mRNA-binding site of the 40S subunit of ribosomes, is thought to play a role in regulating translation in eukaryotes. Previous studies have shown that the phosphatases and kinases that modify RPS6 have differential activities when subjected to various stressors. Heat shock and oxygen deprivation led to an reduction in hyper-phosphorylated forms of RPS6, where cold stress led to an increase in these forms (Williams and others, 2003). Similar stress conditions have also had effects on eIF5A modifications and functions. Under low oxygen conditions, eIF5A knockdown cells have inhibited stress granule assembly and ribosomal transit time (Gregio and others, 2009; Saini and others, 2009; Li and others, 2010). An increase in deoxyhypusine synthase mRNA and eIF5A mRNA has been observed in tomato plant leaves

experiencing osmotic stress and undergoing programmed cell death (Wang and others, 2001).

Similar gene expression patterns between a known ribosomal subunit component and eIF5A, coupled with studies of the two proteins during stressful conditions, suggests a potential role for eIF5A in translational elongation, particularly during times of high stress. As mentioned previously, eIF5A could be a non-essential elongation factor that is necessary when other elongation factors are inactivated due to unfavorable conditions, thus, being the deciding factor in an organism's survival.

Proteins that have associations with eIF5A were investigated through pull-down analysis and immunoprecipitation. As seen in Table 3.1, numerous proteins associated with the cell cytoskeleton and cell division, such as actin, tubulins, legumin1, and FtsZ, were pulled down along with eIF5A. A secondary role of eIF5A in cell proliferation might explain the prevalence of these proteins. Areas in the plant that would be high in mitotic activity, such as maize endosperm, would need to have an increased rate of cellular machinery involved in cell replication. If eIF5A does indeed function as an elongation factor, eukaryotic elongation factor 1A (eEF1A) may provide aid in determining why eIF5A is associating with such proteins. The presence of legumin and FtsZ, as well as elongation factor TuM (EF-TuM), may also be artifacts from disruption of subcellular compartments due to grinding of endosperm in liquid nitrogen.

eEF1A, as a cytoskeleton-associated protein, is known to interact with numerous proteins, including actin microfilaments and microtubules (Moore and Cyr, 2000; Lopez-Valenzuela and others, 2003). In a pH-dependent manner, eEF1A is involved in the bundling of actin through its association with microtubules and microfilaments. It is

thought that eEF1A is involved in the cross-linking of the cytoskeleton, allowing for the translation of mRNAs coding for microfilaments and microtubules (Lopez-Valenzuela and others, 2003). It is possible that eIF5A functions in a similar manner, thus explaining its association with a vast array of cytoskeletal components. The presence of these proteins may also be due to the association of eEF1A with these cellular elements. If eIF5A functions as an elongation factor or co-factor, it is possible that it may associate with eEF1A, resulting in a pull-down of cytoskeletal proteins.

The remainder of the associated proteins, such as metabolic enzymes and elongation factors, initially seem somewhat unrelated. However, when the role of eIF5A as an elongation factor, particularly in times of anoxic stress, are taken into consideration, these seemingly discordant pieces seem to fit together. Carbohydrate modification and storage abilities of a plant are drastically changed when subjected to environments low in oxygen due to the decreased ability of the plant to produce energy and maintain membrane intactness. In order to survive under such conditions, anoxia-resistant plants must have steady access to fermentable sugars (such as glucose and fructose) and a means to rapidly mobilize storage carbohydrates (Rawyler and others, 2002). Due to an increase in fermentation under stressful conditions, and increase in fermentation by-products (ethanol, lactate, and particularly alanine) will be seen in anoxic plants. Maize endosperm, due to the low oxygen tension levels present, could provide the conditions necessary for this type of cellular metabolism. Another explanation for the presence of these metabolic enzymes may be due to the possibility that they are part of a multicomponent system of enzymes. This aggregate of proteins has been implemented in

an association with microtubules in order to channel cell wall modification substrates throughout the cell (Chuong and others, 2004).

The first key players, identified through pull-down analysis, in the role of eIF5A in translation elongation, are three different elongation factors: elongation factor 1-α, elongation factor 1-gamma 3, and translational elongation factor EF-TuM. eEF1A is a known actin-binding protein, and may provide explanation of the presence of cytoskeletal components in the pull-down. Also of significant importance is elongation factor EF-TuM, a protein found in abundance in bacteria as well as plant cell mitochondria. Mitochondria, whose functions are dependent on the presence of oxygen, do not fare well under anoxic stress. An association with eIF5A could indicate that the latter protein may be recruited by some signal from the mitochondria under anoxic, or other high stress, conditions. eIF5A may then function as either an elongation factor, when all other elongation factors have degraded due to high stress, or possibly a co-factor that stabilizes elongation under such conditions.

Also indentified with the pull-down analysis are various proteins functioning as enzymes, particularly enzymes related to modification of carbohydrates. Sorbitol dehydrogenase homolog1, 1,4-α-glucan-branching enzyme 2, UDP-glycosyltransferase, and acetolactate synthase are all enzymatic proteins involved in the biosynthesis of carbohydrates, particularly those involved in glycolysis and cellular respiration (Chipman and others, 1998; Pauly and others, 2003). As stated previously, plants undergoing high stress must be efficient in the requisition of fermentable sugars in order to survive. eIF5A may aid an anoxic plant in increased translation of proteins that may enable the plant to synthesize such sugars.

The remaining interacting proteins, alanine aminotransferase (also called alanine transaminase) and pyruvate phosphate dikinase 1, both play a role in either the production or modification of alanine containing compounds. Alanine transaminase has been shown to be involved in the breakdown of alanine, particularly alanine produced under anoxic conditions in *Arabidopsis thaliana*. In *Arabidopsis*, it was concluded that alanine transaminase is important in low-oxygen stress tolerance and nitrogen metabolism (Miyashita and others, 2007). Pyruvate phosphate dikinase 1 (PPDK1) is a protein involved in alanine-aromatic amino acid synthesis (Chastain and others, 2006) and has recently been implicated in the balance between starch and protein production in developing endosperm (Mechin and others, 2007). Mechin et al. also demonstrated that an increase in PPDK expression is a result of anoxic stress under low oxygen conditions. There are areas in maize endosperm, near the interior of the endosperm, that lay in an "anoxic region", where the availability of oxygen is low (Rolletschek and others, 2005). PPDK may play a role in storage product composition in plant endosperm (Mechin and others, 2007) and the role may be intensified when under stress. Thus, eIF5A may regulate an increase in translation of PPDK1 in unsuitable environmental conditions.

While the developmental time course of eIF5A expression in maize endosperm and leaf and root tissue may have corroborated previous studied that suggested that eIF5A has a role in cell proliferation, it is likely that this role is secondary. The similar expression patterns of isoforms of eIF5A and RPS6, as well as the interaction of eIF5A with numerous proteins involved in oxygen-induced stress coping mechanisms, it is likely that the primary role of eIF5A in plants involves translation elongation, particularly under times of high stress. eIF5A may possibly be recruited by mitochondrial regulatory

factors when oxygen levels are low, and then proceed to function as an elongation factor in the translation of proteins involved in carbohydrate metabolism and modification of fermentation by-products.

### *Future Directions*

Interactions of maize endosperm proteins with eIF5A during pull-down analysis resulting in interesting insight into the function of eIF5A. It would be advantageous to use various plant tissues, such as root tip, to investigate if protein interactions may be different in tissues that are more susceptible to anoxic conditions. During the pull-down, recombinant eIF5A from an *E.coli* vector was used. Despite numerous attempts, using a eukaryotic vector for production of eIF5A was unsuccessful. Because hypusine modification plays a large role in the functionality of eIF5A, and prokaryotes are unable to hypusinate IF5A, it would be of use to create recombinant protein in a eukaryotic vector for use in another pull-down analysis.

# BIBLIOGRAPHY


Andrus L, Szabo P, Grady RW, Hanauske AR, Huima-Byron T, Slowinska B, Zagulska S, Hanauske-Abel HM. 1998. Antiretroviral effects of deoxyhypusyl hydroxylase inhibitors: a hypusine-dependent host cell mechanism for replication of human immunodeficiency virus type 1 (HIV-1). Biochemical pharmacology 55(11):1807-1818.

Benne R, Brown-Luedi ML, Hershey JW. 1978. Purification and characterization of protein synthesis initiation factors eIF-1, eIF-4C, eIF-4D, and eIF-5 from rabbit reticulocytes. The Journal of biological chemistry 253(9):3070-3077.

Bevec D, Hauber J. 1997. Eukaryotic initiation factor 5A activity and HIV-1 Rev function. Biological signals 6(3):124-133.

Byers TL, Lakanen JR, Coward JK, Pegg AE. 1994. The role of hypusine depletion in cytostasis induced by S-adenosyl-L-methionine decarboxylase inhibition: new evidence provided by 1-methylspermidine and 1,12-dimethylspermine. The Biochemical journal 303 ( Pt 2):363-368.

Chastain CJ, Heck JW, Colquhoun TA, Voge DG, Gu XY. 2006. Posttranslational regulation of pyruvate, orthophosphate dikinase in developing rice (Oryza sativa) seeds. Planta 224(4):924-934.

Chatterjee I, Gross SR, Kinzy TG, Chen KY. 2006. Rapid depletion of mutant eukaryotic initiation factor 5A at restrictive temperature reveals connections to actin cytoskeleton and cell cycle progression. Molecular genetics and genomics : MGG 275(3):264-276.

Chattopadhyay MK, Tabor CW, Tabor H. 2003. Polyamines protect Escherichia coli cells from the toxic effect of oxygen. Proceedings of the National Academy of Sciences of the United States of America 100(5):2261-2265.

Chen KY, Liu AY. 1997. Biochemistry and function of hypusine formation on eukaryotic initiation factor 5A. Biological signals 6(3):105-109.

Chipman D, Barak Z, Schloss JV. 1998. Biosynthesis of 2-aceto-2-hydroxy acids: acetolactate synthases and acetohydroxyacid synthases. Biochimica et biophysica acta 1385(2):401-419.

Chuong SD, Good AG, Taylor GJ, Freeman MC, Moorhead GB, Muench DG. 2004. Large-scale identification of tubulin-binding proteins provides insight on subcellular trafficking, metabolic channeling, and signaling in plant cells. Molecular & cellular proteomics : MCP 3(10):970-983.

Clement PM, Henderson CA, Jenkins ZA, Smit-McBride Z, Wolff EC, Hershey JW, Park MH, Johansson HE. 2003. Identification and characterization of eukaryotic initiation factor 5A-2. European journal of biochemistry / FEBS 270(21):4254-4263.

Frigieri MC, Thompson GM, Pandolfi JR, Zanelli CF, Valentini SR. 2007. Use of a synthetic lethal screen to identify genes related to TIF51A in Saccharomyces cerevisiae. Genetics and molecular research : GMR 6(1):152-165.

Glick BR, Ganoza MC. 1975. Identification of a soluble protein that stimulates peptide bond synthesis. Proceedings of the National Academy of Sciences of the United States of America 72(11):4257-4260.

Gregio AP, Cano VP, Avaca JS, Valentini SR, Zanelli CF. 2009. eIF5A has a function in the elongation step of translation in yeast. Biochemical and biophysical research communications 380(4):785-790.

Guan XY, Fung JM, Ma NF, Lau SH, Tai LS, Xie D, Zhang Y, Hu L, Wu QL, Fang Y and others. 2004. Oncogenic role of eIF-5A2 in the development of ovarian cancer. Cancer research 64(12):4197-4200.

Guan XY, Sham JS, Tang TC, Fang Y, Huo KK, Yang JM. 2001. Isolation of a novel candidate oncogene within a frequently amplified region at 3q26 in ovarian cancer. Cancer research 61(9):3806-3809.

Hanawa-Suetsugu K, Sekine S, Sakai H, Hori-Takemoto C, Terada T, Unzai S, Tame JR, Kuramitsu S, Shirouzu M, Yokoyama S. 2004. Crystal structure of elongation factor P from Thermus thermophilus HB8. Proceedings of the National Academy of Sciences of the United States of America 101(26):9595-9600.

Hanazawa M, Kawasaki I, Kunitomo H, Gengyo-Ando K, Bennett KL, Mitani S, Iino Y. 2004. The Caenorhabditis elegans eukaryotic initiation factor 5A homologue, IFF-1, is required for germ cell proliferation, gametogenesis and localization of the P-granule component PGL-1. Mechanisms of development 121(3):213-224.

Hauber I, Bevec D, Heukeshoven J, Kratzer F, Horn F, Choidas A, Harrer T, Hauber J. 2005. Identification of cellular deoxyhypusine synthase as a novel target for antiretroviral therapy. The Journal of clinical investigation 115(1):76-85.

Jenkins ZA, Haag PG, Johansson HE. 2001. Human eIF5A2 on chromosome 3q25-q27 is a phylogenetically conserved vertebrate variant of eukaryotic translation initiation factor 5A with tissue-specific expression. Genomics 71(1):101-109.

Jin BF, He K, Wang HX, Wang J, Zhou T, Lan Y, Hu MR, Wei KH, Yang SC, Shen BF and others. 2003. Proteomic analysis of ubiquitin-proteasome effects: insight into the function of eukaryotic initiation factor 5A. Oncogene 22(31):4819-4830.

Joao HC, Csonga R, Klier H, Koettnitz K, Auer M, Eder J. 1995. The polypeptide chain of eukaryotic initiation factor 5A occurs in two distinct conformations in the absence of the hypusine modification. Biochemistry 34(45):14703-14711.

Joe YA, Wolff EC, Park MH. 1995. Cloning and expression of human deoxyhypusine synthase cDNA. Structure-function studies with the recombinant enzyme and mutant proteins. The Journal of biological chemistry 270(38):22386-22392.

Kang HA, Hershey JW. 1994. Effect of initiation factor eIF-5A depletion on protein synthesis and proliferation of Saccharomyces cerevisiae. The Journal of biological chemistry 269(6):3934-3940.

Kang HA, Schwelberger HG, Hershey JW. 1993. Translation initiation factor eIF-5A, the hypusine-containing protein, is phosphorylated on serine in Saccharomyces cerevisiae. The Journal of biological chemistry 268(20):14750-14756.

Kiesselbach TA. 1949. The structure and reproduction of corn. Lincoln, NB: Agricultural Experiment Station, University of Nebraska.

Klier H, Wohl T, Eckerskorn C, Magdolen V, Lottspeich F. 1993. Determination and mutational analysis of the phosphorylation site in the hypusine-containing protein Hyp2p. FEBS letters 334(3):360-364.

Kowles RV, Phillips RL. 1985. DNA amplification patterns in maize endosperm nuclei during kernel development. Proceedings of the National Academy of Sciences of the United States of America 82(20):7010-7014.

Lee SB, Park JH, Kaevel J, Sramkova M, Weigert R, Park MH. 2009. The effect of hypusine modification on the intracellular localization of eIF5A. Biochemical and biophysical research communications 383(4):497-502.

Li AL, Li HY, Jin BF, Ye QN, Zhou T, Yu XD, Pan X, Man JH, He K, Yu M and others. 2004. A novel eIF5A complex functions as a regulator of p53 and p53-dependent apoptosis. The Journal of biological chemistry 279(47):49251-49258.

Li CH, Ohn T, Ivanov P, Tisdale S, Anderson P. 2010. eIF5A promotes translation elongation, polysome disassembly and stress granule assembly. PloS one 5(4):e9942.

Lipowsky G, Bischoff FR, Schwarzmaier P, Kraft R, Kostka S, Hartmann E, Kutay U, Gorlich D. 2000. Exportin 4: a mediator of a novel nuclear export pathway in higher eukaryotes. The EMBO journal 19(16):4362-4371.

Lopez-Valenzuela JA, Gibbon BC, Hughes PA, Dreher TW, Larkins BA. 2003. eEF1A isoforms change in abundance and actin-binding activity during maize endosperm development. Plant physiology 133(3):1285-1295.

Magdolen V, Klier H, Wohl T, Klink F, Hirt H, Hauber J, Lottspeich F. 1994. The function of the hypusine-containing proteins of yeast and other eukaryotes is well conserved. Molecular & general genetics : MGG 244(6):646-652.

Mechin V, Thevenot C, Le Guilloux M, Prioul JL, Damerval C. 2007. Developmental analysis of maize endosperm proteome suggests a pivotal role for pyruvate orthophosphate dikinase. Plant physiology 143(3):1203-1219.

Miyashita Y, Dolferus R, Ismond KP, Good AG. 2007. Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in Arabidopsis thaliana. The Plant journal : for cell and molecular biology 49(6):1108-1121.

Moore RC, Cyr RJ. 2000. Association between elongation factor-1alpha and microtubules in vivo is domain dependent and conditional. Cell motility and the cytoskeleton 45(4):279-292.

Moubayidin L, Perilli S, Dello Ioio R, Di Mambro R, Costantino P, Sabatini S. 2010. The rate of cell differentiation controls the Arabidopsis root meristem growth phase. Current biology : CB 20(12):1138-1143.

Murphey RJ, Gerner EW. 1987. Hypusine formation in protein by a two-step process in cell lysates. The Journal of biological chemistry 262(31):15033-15036.

Nishimura K, Murozumi K, Shirahata A, Park MH, Kashiwagi K, Igarashi K. 2005. Independent roles of eIF5A and polyamines in cell proliferation. The Biochemical journal 385(Pt 3):779-785.

Park MH. 1987. Regulation of biosynthesis of hypusine in Chinese hamster ovary cells. Evidence for eIF-4D precursor polypeptides. The Journal of biological chemistry 262(26):12730-12734.

Park MH. 2006. The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). Journal of biochemistry 139(2):161-169.

Park MH, Cooper HL, Folk JE. 1982. The biosynthesis of protein-bound hypusine (N epsilon -(4-amino-2-hydroxybutyl)lysine). Lysine as the amino acid precursor and the intermediate role of deoxyhypusine (N epsilon -(4-aminobutyl)lysine). The Journal of biological chemistry 257(12):7217-7222.

Park MH, Lee YB, Joe YA. 1997. Hypusine is essential for eukaryotic cell proliferation. Biological signals 6(3):115-123.

Park MH, Nishimura K, Zanelli CF, Valentini SR. 2010. Functional significance of eIF5A and its hypusine modification in eukaryotes. Amino acids 38(2):491-500.

Park MH, Wolff EC, Lee YB, Folk JE. 1994. Antiproliferative effects of inhibitors of deoxyhypusine synthase. Inhibition of growth of Chinese hamster ovary cells by guanyl diamines. The Journal of biological chemistry 269(45):27827-27832.

Pauly TA, Ekstrom JL, Beebe DA, Chrunyk B, Cunningham D, Griffor M, Kamath A, Lee SE, Madura R, McGuire D and others. 2003. X-ray crystallographic and kinetic studies of human sorbitol dehydrogenase. Structure 11(9):1071-1085.

Rasband WS. 1997-2011. ImageJ. U.S. National Institutes of Health, Bethesda, Maryland, USA.

Rawyler A, Arpagaus S, Braendle R. 2002. Impact of oxygen stress and energy availability on membrane stability of plant cells. Annals of botany 90(4):499-507.

Rolletschek H, Koch K, Wobus U, Borisjuk L. 2005. Positional cues for the starch/lipid balance in maize kernels and resource partitioning to the embryo. The Plant journal : for cell and molecular biology 42(1):69-83.

Rosorius O, Reichart B, Kratzer F, Heger P, Dabauvalle MC, Hauber J. 1999. Nuclear pore localization and nucleocytoplasmic transport of eIF-5A: evidence for direct interaction with the export receptor CRM1. Journal of cell science 112 ( Pt 14):2369-2380.

Ruhl M, Himmelspach M, Bahr GM, Hammerschmid F, Jaksche H, Wolff B, Aschauer H, Farrington GK, Probst H, Bevec D and others. 1993. Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. The Journal of cell biology 123(6 Pt 1):1309-1320.

Saini P, Eyler DE, Green R, Dever TE. 2009. Hypusine-containing protein eIF5A promotes translation elongation. Nature 459(7243):118-121.

Schnier J, Schwelberger HG, Smit-McBride Z, Kang HA, Hershey JW. 1991. Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast Saccharomyces cerevisiae. Molecular and cellular biology 11(6):3105-3114.

Schrader R, Young C, Kozian D, Hoffmann R, Lottspeich F. 2006. Temperature-sensitive eIF5A mutant accumulates transcripts targeted to the nonsense-mediated decay pathway. The Journal of biological chemistry 281(46):35336-35346.

Shi XP, Yin KC, Ahern J, Davis LJ, Stern AM, Waxman L. 1996. Effects of N1-guanyl-1,7-diaminoheptane, an inhibitor of deoxyhypusine synthase, on the growth of tumorigenic cell lines in culture. Biochimica et biophysica acta 1310(1):119-126.

Shi XP, Yin KC, Waxman L. 1997. Effects of inhibitors of RNA and protein synthesis on the subcellular distribution of the eukaryotic translation initiation factor, eIF-5A, and the HIV-1 Rev protein. Biological signals 6(3):143-149.

Shiba T, Mizote H, Kaneko T, Nakajima T, Kakimoto Y. 1971. Hypusine, a new amino acid occurring in bovine brain. Isolation and structural determination. Biochimica et biophysica acta 244(3):523-531.

Tome ME, Fiser SM, Payne CM, Gerner EW. 1997. Excess putrescine accumulation inhibits the formation of modified eukaryotic initiation factor 5A (eIF-5A) and induces apoptosis. The Biochemical journal 328 ( Pt 3):847-854.

Tong Y, Park I, Hong BS, Nedyalkova L, Tempel W, Park HW. 2009. Crystal structure of human eIF5A1: insight into functional similarity of human eIF5A1 and eIF5A2. Proteins 75(4):1040-1045.

Valentini SR, Casolari JM, Oliveira CC, Silver PA, McBride AE. 2002. Genetic interactions of yeast eukaryotic translation initiation factor 5A (eIF5A) reveal connections to poly(A)-binding protein and protein kinase C signaling. Genetics 160(2):393-405.

Wang TW, Lu L, Wang D, Thompson JE. 2001. Isolation and characterization of senescence-induced cDNAs encoding deoxyhypusine synthase and eucaryotic translation initiation factor 5A from tomato. The Journal of biological chemistry 276(20):17541-17549.

Williams AJ, Werner-Fraczek J, Chang IF, Bailey-Serres J. 2003. Regulated phosphorylation of 40S ribosomal protein S6 in root tips of maize. Plant physiology 132(4):2086-2097.

Wolff EC, Folk JE, Park MH. 1997. Enzyme-substrate intermediate formation at lysine 329 of human deoxyhypusine synthase. The Journal of biological chemistry 272(25):15865-15871.

Wolff EC, Kang KR, Kim YS, Park MH. 2007. Posttranslational synthesis of hypusine: evolutionary progression and specificity of the hypusine modification. Amino acids 33(2):341-350.

Wolff EC, Lee YB, Chung SI, Folk JE, Park MH. 1995. Deoxyhypusine synthase from rat testis: purification and characterization. The Journal of biological chemistry 270(15):8660-8666.

Wolff EC, Wolff J, Park MH. 2000. Deoxyhypusine synthase generates and uses bound NADH in a transient hydride transfer mechanism. The Journal of biological chemistry 275(13):9170-9177.

Young TE, Gallie DR. 2000. Programmed cell death during endosperm development. Plant molecular biology 44(3):283-301.

Zanelli CF, Maragno AL, Gregio AP, Komili S, Pandolfi JR, Mestriner CA, Lustri WR, Valentini SR. 2006. eIF5A binds to translational machinery components and affects translation in yeast. Biochemical and biophysical research communications 348(4):1358-1366.

Zuk D, Jacobson A. 1998. A single amino acid substitution in yeast eIF-5A results in mRNA stabilization. The EMBO journal 17(10):2914-2925.