CHARACTERIZATION OF THE EXPRESSION AND REGULATION OF VEGF AND 4E-BP1 IN THE RETINA DURING DIABETES AND UNDER HYPERGLYCEMIC CONDITIONS

A Dissertation in
Physiology
by
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ABSTRACT

Diabetic retinopathy is the leading cause of blindness among working-age adults, occurring in the majority of individuals who have had diabetes for twenty or more years. Among the causes implicated in the development of diabetic retinopathy, increased expression of vascular endothelial growth factor (VEGF) has been implicated in the development of both abnormal neovascularization increased vascular permeability of nascent retinal vessels. Currently, the only treatment for diabetic retinopathy is laser-mediated ablation of the peripheral retina. Because this treatment results in permanent laser scars and is only effective in completely relieving the neovascularization in half of the patients undergoing the procedure, subsequent treatments are often required. This leads to a reduction in peripheral vision, night vision, and color detection. Additionally, this procedure only relieves the symptoms, but does not approach the molecular basis behind the disease. Therefore alternate molecular approaches to treatment are required.

The first aim of this project was to define the time course of VEGF protein upregulation in the retina of diabetic rats to better understand the early progression of the disease. Retinas were isolated from rats 1, 2, 4, 6, and 12 weeks after streptozotocin (STZ) treatment and assessed for VEGF mRNA and protein expression. VEGF protein, but not mRNA expression was upregulated after 2 weeks of diabetes, and was returned to control levels after 12 weeks. The absence of a change in the expression of VEGF mRNA suggested a post-transcriptional mechanism for upregulated protein expression. The VEGF mRNA is complex and
contains multiple elements that allow for translational control, including a uORF, 5 initiation codons, and 2 internal ribosome entry sites (IRESs). VEGF IRES utilization in response to cell stress has been well documented, but the factors involved in its utilization have not been identified. Therefore, the expression of proteins involved in translational control was evaluated. Eukaryotic initiation factor-4E-binding protein 1 (4E-BP1), a key repressor of translation initiation, was upregulated following the same time course as VEGF protein expression. Altered expression of 4E-BP1 correlated with increased sequestration of eIF4E, the mRNA cap-binding protein, an event that would be expected to repress global rates of mRNA translation. Ablation of 4E-BP1/2 in mice prevented the diabetes-induced increase in VEGF, suggesting that 4E-BP1 is required in this process.

The second aim was designed to delineate the mechanism by which 4E-BP1 expression is increased in Müller cells exposed to hyperglycemic conditions. 4E-BP1 expression was maximally increased in the absence of a change in its mRNA after exposure to hyperglycemic conditions for 10 hours. Phosphorylation of 4E-BP1 by mammalian target of rapamycin complex 1 (mTORC1) results in release from eIF4E and targets the protein for ubiquitination and subsequent degradation. Analysis of the retinal lysates revealed a decrease in the relative phosphorylation of 4E-BP1 on T37/46. O-GlcNAcylation of 4E-BP1 was increased, which, unlike phosphorylation, does not interfere with the ability of 4E-BP1 to bind to eIF4E. O-GlcNAcylation of 4E-BP1 did, however, interfere with its ubiquitination, which would be expected to contribute to the stabilization and increased expression of the protein under hyperglycemic conditions.
Overall the data are consistent with a model in which hyperglycemia-induced O-GlcNAcylation decreases the phosphorylation and ubiquitination of 4E-BP1. This leads to increased expression of 4E-BP1 and sequestration of eIF4E, which would be expected to repress cap-dependent translation, making complex mRNAs and those that can be translated cap-independently more competitive for translation, including the mRNA encoding VEGF.
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<th>Description</th>
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<tbody>
<tr>
<td>4E-BP</td>
<td>eukaryotic initiation factor 4E binding protein</td>
</tr>
<tr>
<td>4E-HB</td>
<td>eukaryotic initiation factor 4E homogenization buffer</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation endproduct</td>
</tr>
<tr>
<td>Akt</td>
<td>v-akt murine thymoma viral oncogene homolog 1</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>Ang2</td>
<td>angiotensin 2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>activator protein</td>
</tr>
<tr>
<td>AS-160</td>
<td>Akt substrate 160</td>
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<tr>
<td>ATF4</td>
<td>activating transcription factor 4</td>
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<tr>
<td>BiP</td>
<td>immunoglobulin heavy chain binding protein</td>
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<tr>
<td>Bmx</td>
<td>nonreceptor Tec family tyrosine kinase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>c-myc</td>
<td>chicken-myelocytic leukemia</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>cat-1</td>
<td>cool-associated, tyrosine-phosphorylated-1</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic adenosine monophosphate responsive element binding protein</td>
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<tr>
<td>DKO</td>
<td>double knockout</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DPI</td>
<td>diphenyl iodonium chloride</td>
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<td>DRBP</td>
<td>double-stranded ribonucleic acid binding protein</td>
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<tr>
<td>E1</td>
<td>ubiquitin-activating enzyme</td>
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<tr>
<td>E2</td>
<td>ubiquitin conjugating enzyme</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine-N, N', N'-tetraacetic acid</td>
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<tr>
<td>eIF</td>
<td>eukaryotic initiation factor</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FGF2</td>
<td>fibroblast growth factor 2</td>
</tr>
<tr>
<td>Flt</td>
<td>Fms-related tyrosine kinase</td>
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<tr>
<td>FoxO</td>
<td>Forkhead box-containing protein</td>
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<tr>
<td>G-C</td>
<td>Guanine-Cytosine</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>GFAT</td>
<td>glutamine-fructose-6-phosphate-amidotransferase</td>
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GLAST | L-glutamate/L-aspartate transporter
GLUT | glucose transporter
GS | glutamine synthetase
GTP | guanosine triphosphate
HBP | hexosamine biosynthetic pathway
HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF | hypoxia inducible factor
hnRNP | heterogeneous nuclear ribonucleoprotein
HRE | hypoxia response element
HSB | high salt buffer
HSV | herpes simplex virus
ip | intraperitoneal
IFN | interferon
Ig | immunoglobulin
IGF | insulin-like growth factor
IL | interleukin
IP | immunoprecipitation
IRES | internal ribosome entry site
IRS | insulin receptor substrate
ITAF | internal ribosome entry site trans-activating factor
iv | intravenous
kd | dissociation constant
KDR | kinase insert domain receptor
LPS | lipopolysaccharide
LSB | low salt buffer
MAPK | mitogen activated protein kinase
miRNA | micro ribonucleic acid
Mnk | mitogen activated protein kinase interacting serine/threonine kinase
mRNA | messenger ribonucleic acid
MS | mass spectrometry
mTOR | mammalian target of rapamycin
mTORC1 | mammalian target of rapamycin complex 1
NADPH | nicotinamide adenine dinucleotide phosphate
NF-κB | nuclear factor κB
NRP | neuropilin
O-GlcNAc | mono O-linked β-N-acetylglucosamine
ODC | ornithine decarboxylase
OGA | O-GlcNAcase
OGT | O-GlcNAc transferase
ORF | open reading frame
p38 | protein 38
p70/S6K1 | S6 kinase 1
PAGE | polyacrylamide gel electrophoresis
PBS | phosphate buffered saline
PDGF | platelet derived growth factor
PDX | pancreatic and duodenal homeobox
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<tr>
<td>PEDF</td>
<td>pigment epithelial growth factor</td>
</tr>
<tr>
<td>PGC</td>
<td>peroxisome proliferator-activated receptor γ coactivator</td>
</tr>
<tr>
<td>PHD</td>
<td>pleckstrin chain homology domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3'-OH kinase</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<td>PKB/Akt</td>
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<td>PIGF</td>
<td>placental growth factor</td>
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<td>PP2A/1A</td>
<td>protein phosphatase 2A/1A</td>
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<td>PR</td>
<td>photoreceptor</td>
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<td>PTB</td>
<td>polypyrmidine tract binding</td>
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<td>PUGNAc</td>
<td>O-(2-acetamido-2deoxy-D-gluco-pyranosylidene)amino-N-phenylcarbamate</td>
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<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
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<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<td>ribonucleic acid-induced silencing complex</td>
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<td>ribonucleic acid</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RPE</td>
<td>retinal pigment epithelium</td>
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<td>sample buffer</td>
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<td>sodium dodecyl sulfate</td>
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<td>standard error of the mean</td>
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<td>small interfering ribonucleic acid</td>
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<td>signal transduction and transcription</td>
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<td>streptozotocin</td>
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<td>tris-buffered saline tween-20</td>
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<td>tumor necrosis factor</td>
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<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis-inducing ligand</td>
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<td>upstream open reading frame</td>
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<td>untranslated region</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
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<td>VEGF-R</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VPF</td>
<td>vascular permeability factor</td>
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<td>wildtype</td>
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ACKNOWLEDGMENTS

“Many times a day I realize how much my own life is built on the labors of my fellowmen, and how earnestly I must exert myself in order to give in return as much as I have received.” – Albert Einstein

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goals have been or how far away they have taken me. My mom has gotten me
through many hard days, and will undoubtedly get me through hundreds more. My
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For my grandfather, whom I miss dearly, Mom, Dad, and Matthew, who have been there through the good and the bad, and whose support and encouragement have made everything possible, and for everyone who has helped me along the way.
“Und sehe dass wir nichts wissen können”

-Johann Wolfgang von Goethe, Faust
CHAPTER I

INTRODUCTION
1.1 Overview of diabetic retinopathy

Diabetic retinopathy is the leading cause of blindness in working age adults in the United States and the most frequent diabetic complication. After 20 years of diabetes there is some evidence of retinopathy in all Type 1 and approximately 60% of Type 2 diabetic patients. Diabetic retinopathy is characterized by abnormal neovascularization, followed by increased vascular permeability. Molecular alterations in the endothelial cells that maintain the blood-retinal-barrier lead to its breakdown (1), increased vascular permeability and subsequent accumulation of extracellular fluid in the macula. In the proliferative phase capillary occlusion induces localized retinal ischemia, which stimulates abnormal neovascularization mediated by increased expression of VEGF; 20% of the individuals with diabetic retinopathy progress to the proliferative stage (2). Currently the only treatment for neovascularization involves laser-mediated ablation of the peripheral retinal vasculature. This procedure, while relieving local neovascularization and permeability, results in permanent scarring of the retina, and reduced night vision, peripheral vision, and color perception. Additionally, this procedure has no effect on the molecular pathology leading to the disease, and as such, nearly half of the patients that undergo this treatment will require subsequent procedures. Thus it is imperative to identify targets for intervention at the molecular level that will lead to the treatment of the cause, rather than the effect.
1.2 Molecular mechanisms of diabetic retinopathy

A majority of the molecular pathways implicated in the development of diabetic retinopathy are dependent upon intracellular accumulation of glucose. These pathways include induction of protein kinase C (PKC), activation of the polyol pathway, and the generation of advanced glycation endproducts (AGEs) (3). In fact, large clinical trials have shown that aggressive glycemic control alone can reduce the risk and severity of diabetes-induced macro- and microvascular complications, which highlights the important of the presence of hyperglycemia in mediating diabetic complications (4-7). Although a recent evaluation of men enrolled in the Epidemiology of Diabetes Interventions and Complications follow-up study of the Diabetes Control and Complications Trial, suggests that even proper glycemic control for up to 10 years may not suffice to eliminate all diabetes-related complications, including the development and severity of lower urinary tract symptoms (8). These results indicate that while hyperglycemia plays an important role in the development of complications, there is a strong genetic component to the disease. Two important pathways activated by hyperglycemic conditions are the production of reactive oxygen species (ROS) and increased flux through the hexosamine biosynthetic pathway (HBP), both of which have been linked to the development and progression of diabetic retinopathy.
1.2.1 Production of ROS through NADPH oxidase

The production of ROS plays a major role in hyperglycemia-induced retinal pathology (9-11). One of the major enzymes involved in the production of ROS in response to diabetes is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase is a multi-subunit complex composed of 2 membranous subunits, gp91phox and p22phox, and 3 cytosolic subunits, p40phox, p47phox, and p67phox. Present primarily within phagocytes, this enzyme is responsible for the respiratory bursts in active leukocytes. The homologues Nox1 and Nox4 have been identified in endothelium and are thought to mediate low-level basal and induced ROS production (12). Interestingly, these homologues are not present within leukocytes, and are found associated with intracellular organelles, rather than located at the membrane (13).

Diabetes-induced vascular dysfunction and VEGF expression can be prevented by inhibition of ROS production. In fact oxidative stress has been strongly implicated in VEGF upregulation as VEGF requires superoxide production for its angiogenic properties (14). Additionally, VEGF-induced activation of its receptor, KDR, requires superoxide production. Activation of NADPH oxidase in the retinal vasculature stimulates angiogenesis and activates VEGF receptors; NADPH oxidase production of ROS induces HIF-1α expression, and in turn, VEGF mRNA expression. The use of apocynin, a drug that inhibits oxidase assembly (15), inhibits the ischemia-induced increase in ROS production and prevents neovascularization both in vivo and in vitro. The same outcome is achieved with the expression of a
dominant negative gp91phox-tat transgene (16). Finally, NADPH oxidase subunit expression is increased during diabetes, an effect that can be prevented with apocynin (17). Because the production of ROS represents an overlap for many pathways activated by hyperglycemia (9), NADPH oxidase serves as an attractive target in studying hyperglycemia-induced retinal changes.

1.2.2 Increased flux through the HBP

The hexosamine biosynthetic pathway, a relatively minor branch of glycolysis, converts glucose, through a series of reactions, to uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a precursor molecule utilized in the synthesis of amino sugars, and the post-transcriptional protein modifier, mono O-linked β-N-acetylglucosamine (O-GlcNAc) (Figure 1). O-GlcNAc is transferred to serine or threonine residues of substrate proteins by the enzyme O-GlcNAc transferase (OGT), and removed in a reversible reaction by O-GlcNAcase (OGA) (18-20). This modification usually occurs on or near phosphorylation sites in a mutually exclusive manner with phosphorylation. O-GlcNAcylation, like phosphorylation, represents a rapid modification that plays significant roles in modulating cell signaling, transcription, and protein degradation (21-25).

Increased flux through the HBP has been extensively linked to impaired glucose uptake and insulin resistance (10; 26-28). While the HBP accounts for only 2-3% of total glucose metabolism under physiological conditions (29), during diabetes, glucose concentrations are significantly elevated, presenting additional substrate
for flux through this pathway (30). Further, diabetes-induced dysregulation of glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), inhibits flux of glucose through the glycolytic pathway, which acts to shunt glucose to alternate paths, including the HBP (9; 31; 32). Diabetes-induced O-GlcNAcylation of proteins such as Akt-1 and -2 and IRS-1 and -2 leads to an impairment in the insulin signaling pathway (18; 33; 34). Additionally, altered expression of transcription factors involved in β-cell function, glucose uptake, and insulin signaling, including PDX-1, FoxO1 and NFκB contribute to diabetic pathology (35-38).

1.3 Overview of retinal organization and development

The retina is a highly organized and heterogeneous tissue, made of up several neural layers of 10-20 distinct neuronal cell subtypes with distinct morphology and functions. The inner limiting membrane forms the innermost boundary of the retina and is comprised primarily of Müller cell endfeet, which extend through the depth of the retina forming both of the retinal boundaries. The inner limiting membrane is followed by the nerve fiber layer, made up of ganglion cell axons which join to form the optic nerve, and then the ganglion cell layer, in which the bodies of the ganglion cells lie. The inner plexiform layer, inner nuclear layer, outer plexiform layer, and outer nuclear layer, which contain axons and cell bodies for other neuronal cell subtypes, including amacrine, horizontal, and bipolar cells, follow the ganglion cell layer. The final layer, the photoreceptor layer, contains the light and color sensitive
rods and cones. Photoreceptors undergo constant renewal and are thus positioned in proximity to the retinal pigment epithelium (RPE) and choroid, which aid in the renewal process and in the delivery of trophic agents. Light passes through the layers of the retina before reaching the light sensing photoreceptors with little deflection, as the layers are unpigmented, whereas the RPE and following choroid are opaque, preventing the reflection of light that would distort image formation.

Histologically and embryologically a part of the central nervous system, the retina forms concurrently with brain tissue during development. A majority of the cells in the retina derive from a single progenitor cell, and are subdivided into two developmental phases. During the early phase, neurons of the neuroepithelium differentiate into cones, ganglion cells, and astrocytes, whereas the late phase gives rise to rods, amacrine cells, bipolar cells, and Müller cells. The developing cells migrate toward their final positions guided along the Müller cell processes (39).

1.3.1 Müller cell function

The absence of oligodendrites, and the presence of astrocytes only in mammalian species, makes Müller cells the principal glial cell of the retina. Müller cells span the entire depth of the retina, their processes forming the limits at the inner and outer limiting membranes; their cell bodies lie within the inner nuclear layer. In addition to maintaining structural integrity, Müller cells maintain the functional integrity of the retina. Their processes completely envelop neuronal cell bodies and processes; in fact, retinal neurons are only in direct contact at their synapses. This orientation
puts them in an ideal position for providing nutritional support for the surrounding cells. Glycogen storage is entirely restricted to Müller cells (40). Upon stimulation by local neuronal activity, Müller cell glycogenolysis is activated, allowing the cells to release the lactate required for the metabolic support of the inner retinal neurons. Müller cells also have comparatively high levels of the GLUT1 glucose transporter and elevated rates of glycolysis, which serves to fuel the highly oxidative photoreceptors (41; 42). High expression of the L-glutamate/L-aspartate transporter (GLAST), present only in retinal glial cells, allows for the fast and efficient uptake of glutamate, the excitatory neurotransmitter utilized and synthesized by retinal neurons (43; 44). The glutamate taken up by Müller cells is converted to glutamine by glutamate synthetase (GS), the only source of GS within the retina, and delivered back to the retinal neurons for resynthesis of glutamate (45; 46). Alternatively, glutamate taken up by the Müller cells may be converted to glutathione, which acts as an intraretinal antioxidant. In addition to interacting with other neuronal cells, Müller cell endfeet are in direct contact with the retinal vasculature. Müller cells release factors that induce the formation of tight junctions in the endothelial cells that maintain the blood-retinal-barrier, conferring barrier properties to the retinal vessels (42).

Due to their location and extensive regulatory roles within the retina, Müller cells are particularly susceptible the metabolic environment of the retina. The envelopment of retinal neurons by Müller cell endfeet, and provision of trophic factors to neurons and photoreceptors also makes Müller cells a prime candidate for the release of factors, such as mitogens and cytokines, which affect neighboring
cells. In fact, many studies have implicated Müller cell activation in the pathogenesis of diabetic retinopathy, suggesting that the Müller cell may be the primary cell type in the retina producing VEGF (39; 40; 47-51).

1.4 VEGF

VEGF was first identified as vascular permeability factor (VPF), a vascular permeabilizing agent (52). Since its discovery, VEGF has been classified as a highly potent and fairly specific endothelial cell mitogen, as most of the VEGF receptors are present on the endothelium (53-55). Expression of VEGF is especially important for angiogenesis and vasculogenesis during embryonic development and wound healing and is essential for tumorigenesis (54; 55). Loss of even a single VEGF allele is inconsistent with survival past the first few weeks of life (54; 56-58). Furthermore, VEGF expression is required after embryonic development, as reduced expression of VEGF beyond development results in growth arrest, formation of defective organs, and increased mortality (59).

1.4.1 Gene and protein structure

VEGF belongs to the placental growth factor (PIGF) family of dimeric cysteine knot growth factors (60), each monomer containing an intrachain disulfide bonded knot motif at the end of a 4-stranded β-sheet. The family is comprised of VEGF-A (identified first and referred to simply as, "VEGF"), -B, -C, -D, and PIGF. Whereas
VEGF-A is mostly involved in permeabilization and stress-mediated angiogenesis, VEGF-B is involved in embryonic angiogenesis, -C and -D, primarily in lymphangiogenesis, and PlGF, vasculogenesis.

The *vegfa* gene is approximately 14kb in length and contains 8 exons (61; 62). Alternative splicing of a single VEGF pre-mRNA yields 3 main isoforms, VEGF121, VEGF165, and VEGF189, and other minor isoforms VEGF145, VEGF148, VEGF183, and VEGF206, the significance of which have yet to be determined (61; 63; 64). Also identified are “b isoforms,” VEGF121b, VEGF145b, VEGF165b, VEGF183b, and VEGF189b, generated by the utilization of a proximal splice site within exon 8, resulting in a differential C-terminus amino acid sequence (63). While these isoforms bind to VEGF receptors, they lack the ability to elicit signal transduction, thereby acting as competitive inhibitors of their similar length counterparts (65). All of the isoforms contain exons 1-5 and 8, but they differ by combinations of additional exons. VEGF121 lacks any additional exons, while VEGF165 contains exon 7, and VEGF189 contains both exons 7 and 8 (61; 66-68). These isoforms are distinct in their subcellular localization, action, and clinical significance. Whereas VEGF121 is entirely secreted and released from the cell, for lack of the basic heparin-binding region within exon 7, VEGF165 (30-50% is released from the cell), VEGF189 and VEGF206 are mostly or entirely retained upon the cell surface (69; 70).
1.4.2 Retinal localization

In the retina VEGF immunoreactivity is detected in Müller cell intermediate and thick processes and endfeet. Co-localization with vimentin, a Müller cell marker, also demonstrates immunoreactivity within cell bodies and nuclei. VEGF mRNA and protein can be found in all retinal neurons, co-localizing with the neuronal marker enolase (71-75) and within the RPE and retinal vasculature (74; 76). Studies have demonstrated that while immunoreactivity is relatively low, cells within the ganglion cell layer may be induced to secrete VEGF as well (77). VEGF expression in the retina under physiological conditions is nearly undetectable (78). However, immunoreactivity significantly increases during diabetic retinopathy. Further, in non-proliferative diabetic retinopathy VEGF co-localizes with vimentin, suggesting Müller cell production (79).

1.4.3 Receptors

VEGF signal transduction is achieved through activation of a family of high affinity class III receptor tyrosine kinases, VEGF-R1, R2, and R3. This family features 7 extracellular IgG domains, one membrane spanning domain, and an intracellular tyrosine kinase domain with a kinase insert region (80-83). Upon ligand binding receptors dimerize, undergo autotransphosphorylation, and initiate a signaling cascade involving PI3K, Akt, and the MAPK signaling pathways. While the majority
of receptors are present on endothelial cells, conferring relative endothelial cell specificity, in the retina, Müller cells and RPE also contain receptors.

VEGF-R1 (Flt-1) was the first receptor identified and has the highest binding affinity for VEGF with a $k_d=10-30\text{pM}$ (81; 84; 85). This receptor also accommodates PI GF and VEGF-B. Expressed primarily within the endothelium in adults, Flt-1 participates in wound healing and endothelial cell maintenance. However, there is some debate on the functionality of this receptor in adult tissues. Tyrosine phosphorylation after VEGF induction is difficult to detect and VEGF-R2 (KDR) is thought to mediate most observed endothelial cell responses, implicating Flt-1 perhaps as a decoy receptor that mediates negative VEGF regulation (86). In fact, signaling through Flt-1 leads to repression of P13K activation (87). Regardless of this implication, Flt-1 expression is required for normal development as in its absence there is a defect in the formation of organized blood vessels, despite the presence of mature endothelial cells (88). A soluble Flt-1 receptor (sFlt-1), an alternate splice form of Flt-1 containing 6 IgG domains, was discovered in placental tissue (89). This receptor has the ability to form non-signaling heterodimers with both Flt-1 and KDR, sequestering VEGF from signaling, perhaps facilitating in the differential activation of the VEGF receptors (90; 91).

VEGF-R2 (Flk-1/KDR) has a lower affinity for VEGF and appears to be downregulated in adults (85; 92; 93). However, KDR is more efficiently tyrosine phosphorylated upon VEGF stimulation. KDR knockout is lethal and results in a defect in the formation of endothelial cell precursors (94; 95). Co-activation of KDR is thought to be mediated through neuropilin-1 (NRP1) (96).
NRP1 and NRP2, neuronal receptors of the collapsin/semaphorin family, are capable of recognizing VEGF_{165} through an interaction with an encoded domain in exon 7. These receptors are expressed in a wide variety of tissues and mediate axonal growth as well as vasculogenesis (97-99). Genetic studies have indicated that tight regulation of NRP1 is essential for proper development, while absence of this receptor leads to early mortality due to vascular abnormalities (98). Conversely, overexpression results in mortality due to vascular overgrowth (99). However, NRP1 does not appear to mediate angiogenic effects unassisted, failing to elicit signaling in the absence of KDR. These results would suggest that perhaps NRP1 acts as a co-receptor for KDR, enhancing the binding of VEGF to KDR (96). This suggestion is supported by the extensive co-expression of NRP1 and KDR in the retina (100).

VEGF-R3 (Flt-4) has restricted localization to the adult lymphatic endothelium, binding to VEGF-C and -D, but not VEGF-A. Signal transduction from this receptor facilitates lymphangiogenesis (83; 101).

1.4.4 VEGF in retinal development

Development of the retina begins from the center of the optic nerve and radiates outward, reaching the periphery of the retina just before birth (102; 103). Astrocytes, originating from the accession point at the optic nerve, are guided by the ganglion cell axons into a tentative neural network along which the new blood vessels develop (104-107). Capillaries sprout from the inner retina, forming more
dense networks deeper within the retina. After the groundwork vasculature is laid, remodeling and pruning of the new vessels occurs around arterioles, mediated by apoptotic factors secreted from astrocytes and inflammatory cells within the retina (108). In the developing rat and cat retina, studies have shown that primary angiogenesis is driven by VEGF synthesis and secretion by Müller cells and astrocytes (77; 109). VEGF and VEGF-R expression correlates well with the leading edge of neovascularization (71; 109; 110). Differential expression of VEGF receptors, both spatially and temporally, as well as utilization of the distinct isoforms of VEGF, allow for tight positive and negative regulation during development (111-113). During adulthood, the expression of VEGF receptors decreases and most of the vasculature remains quiescent, except for angiogenesis occurring in the cycling ovary and during pregnancy in the placenta (114). However, endothelial cells of the vasculature maintain the ability to respond to angiogenic signals, especially during hypoxic stress or inflammation, and become activated during wound healing (114).

1.4.5 VEGF in diabetic retinopathy

Early breakdown of the blood-retinal-barrier has been attributed to enhanced expression of VEGF mRNA and protein (87; 115-118). Increased permeability correlates well with increased VEGF expression in the retina of diabetic rats. Additionally, the rate of blood-retinal-barrier breakdown is higher with VEGF immunoreactivity than without (118). The increase in both VEGF expression and
vascular permeability can be prevented by VEGF neutralizing receptors (119). One hypothesis is that VEGF may disrupt cell:cell interactions via modulation of the endothelial tight junctional protein occludin-1 observed in diabetic rats (120-122). Small molecule inhibitors of VEGF (123), dominant negative VEGF (124), humanized anti-VEGF antibodies (125); and soluble VEGF receptors (119) have all been utilized in clinical trials for the suppression of VEGF-induced neoangiogenesis and vascular permeability. While these treatments all effectively reduce VEGF expression, they are not isoform-specific, leading to global downregulation of VEGF signaling, which may have negative implications for wound healing and normal vascular pruning. While the development of isoform-specific antisense constructs adds specificity, the efficiency of repression is relatively low (126). Thus better methodology is needed for more efficient treatment of VEGF-related diseases.

Although the expression of VEGF has been attributed to the pathological changes associated with diabetic retinopathy, VEGF may exert a protective role during early stages of the disease. VEGF has been shown to increase proliferation and outgrowth of neuronal cell cultures. VEGF can also promote neuronal survival by preventing TNFα-induced apoptosis via VEGF-induced expression of the anti-apoptotic factor, Bcl-2 (127-129). Finally, upregulated expression of VEGF during hypoxia mediates increased endothelial cell and vessel survival (130). Thus disruption of VEGF signaling may contribute to earlier neuronal apoptosis. Therefore, a better understanding of the differential roles VEGF mediates in diabetic retinopathy will lead to improved treatment outcomes.
1.4.6 Transcriptional regulation

VEGF is the main contributor to hypoxia-mediated stimulation of angiogenesis. Hypoxia dramatically increases the expression of VEGF mRNA. This upregulation occurs in a relatively specific manner, as other members of the PlGF family, and FGF-2 expression are unaffected. The vegf gene contains an upstream HRE enhancer with a consensus sequence for the oxygen tension-sensitive transcription factor, hypoxia inducible factor (HIF)-1α (131). In addition to contributing to increased mRNA expression, hypoxia is known to increase the half-life of the VEGF mRNA considerably through binding of the factor HuR to the stability element within the 3’-untranslated region (UTR) (132). In addition to HIF-1α, VEGF mRNA expression has been shown to be regulated by STAT-3, c-myc, and TNF-α.

1.4.6.1 HIF-1α

HIF-1α is a member of the basic helix-loop-helix family of transcription factors. While it was originally identified as a transcription factor for the induction of erythropoietin expression during hypoxia, since then HIF-1α has been shown to regulate a number of substrates under a variety of stress conditions including: VEGF during hypoxia (133; 134); BiP during heat shock (135); cat-1 during amino acid starvation (135); c-myc during apoptosis (135); and ODC during G2/M phase induction (135). HIF-1α contains 2 primary subunits; subunit 1β is expressed constitutively, whereas 1α is hypoxia-inducible (136). In the presence of oxygen, 1α
is hydroxylated on proline residues, 402 and 564, by PHD-containing proteins, which target 1α for proteasomal degradation (137-140). While 1α mRNA is constantly expressed, protein levels under normoxic conditions are undetectable and the protein has a half-life of less than 5 minutes (141). During low oxygen tension, when prolyl hydroxylase is inhibited, 1α is rapidly synthesized and dimerizes with 1β. After association, the complex translocates to the nucleus where it binds to HREs in the DNA and upregulates hypoxia-responsive genes, such as the vegf gene, as well as those involved in oxygen transport and glucose metabolism (Figure 2) (132; 142; 143). Flt-1 also contains an HRE and is upregulated during hypoxia (132; 142; 143).

1.4.6.2 STAT-3

The vegf promoter contains STAT-3 binding elements. Mutation of these sites can lead to a repression of transcription and defective cardiac angiogenesis (144; 145). The STAT-3 pathway increases VEGF mRNA expression in vitro and in vivo via inflammatory pathways and production of ROS (144; 146-148). Additionally, VEGF signal transduction can activate STAT-3 in retinal endothelial cells in response to oxidative stress and production of peroxynitrite, providing for a feed-forward loop (149). Expression of a dominant negative form of STAT-3 decreases peroxynitrite-induced increase in VEGF mRNA. Inhibitors of transcription, but not translation inhibit this process (149). On the other hand, constitutive STAT-3 activity increases
VEGF expression and tumor angiogenesis (144), which may be abrogated by mutation of the STAT-binding region in the \textit{vegf} promoter.

1.4.6.3 Additional transcription factors

The \textit{vegf} gene was cloned and sequenced and consensus sequences within its promoter were identified for the SP-1, AP-1 and AP-2 transcription factors. PKC and PKA also have been shown to influence gene expression (62). Many cytokines and growth factors can affect VEGF mRNA expression or protein secretion, including PDGF, PGC-1\(\alpha\) (150), TNF-\(\alpha\), TGF-\(\beta\), FGF, IGF-1, IL-1\(\beta\), and IL-6, many of which are altered during hyperglycemia and diabetic retinopathy (151; 152).

1.4.6.4 mRNA stability

Hypoxia has been shown to increase the expression of VEGF mRNA, and increase rates of VEGF synthesis and secretion (153; 154). In addition to upregulating VEGF mRNA expression, hypoxia mediates increased stability of the VEGF mRNA, extending its half-life from 30 minutes to nearly 8 hours in human RPE cells (153; 154). DRBP, which binds to the 3’-UTR of the VEGF mRNA, plays a significant role in increasing mRNA stability during hypoxia. Decreased expression of this protein via RNAi prevents hypoxia-induced stabilization of the VEGF mRNA (155). The toll-like receptor Tec kinase, Bmx, is also capable of increasing LPS-induced VEGF mRNA half-life to 4 hours (156).
1.4.7 Translational regulation

While VEGF is classically thought to be regulated in a transcriptional manner, recent studies have indicated that VEGF can also be regulated at the translational level as well. VEGF has an exceptionally long 5’-untranslated region, at 1038bp, and contains alternative start sites and stop codons in frame with the classical start site, which may contribute to alternative splicing (157). This region is highly stable with over 80% G-C content and known to undergo complimentary binding and form complex secondary structures. Complex messages such as these are typically inefficiently translated under normal cap-dependent conditions (158).

1.4.7.1 Translation initiation

Of the steps involved in mRNA translation, translation initiation is the most regulated, and therefore the limiting step of the process (Figure 3) (159-162). The first step of initiation involves the hydrolysis of guanine triphosphate (GTP) on eIF2 and the assembly of the ternary complex, composed of eIF2-GTP•Met-tRNAi. In the second step the ternary complex binds to the 40S ribosomal subunit in conjunction with eIF1, eIF1A and the large scaffolding protein eIF3, forming the 43S pre-initiation complex. The eIF4F complex, composed of eIF4E, the mRNA cap-binding protein, eIF4G, a scaffolding and mRNA circularization factor, eIF4A, an RNA-dependent helicase and its associated factor eIF4B, and eIF4H, assembles upon the 5’-cap and unwinds structures within the 5’-UTR in the third step (163). eIF4E is
rate-limiting for assembly of the eIF4F complex and as such is limiting for translation initiation. In fact, absence of eIF4E is inconsistent with survival. Availability of eIF4E is mediated by a family of inhibitory 4E-BPs, which bind to and sequester eIF4E from the eIF4F complex. 4E-BPs are regulated by the mTORC1 pathway, and as such are responsive to nutrient availability and changes in energy status of the cell (164; 165). During starvation, when mTORC1 signaling is repressed, 4E-BP is hypophosphorylated and available to associate with eIF4E. eIF4E is also phosphorylated in response to cellular stress. Phosphorylation on S209 may result in a reduced affinity of eIF4E for the 5′-cap. This event is mediated by the eIF4E kinase Mnk1/2, which is regulated through MAPK/ERK signaling pathway. During the fourth step eIF4F and eIF3 load the mRNA onto the 43S pre-initiation complex and initiate scanning in a 5′-3′ direction for the AUG initiation codon. Finally, upon codon-anticodon pairing, GTP hydrolysis is triggered, requiring eIF2 and eIF5, and then the tRNA is released. eIF5B-GTP facilitates binding of the 60S subunit, allowing for elongation to ensue. Once the mRNA is loaded onto the 40S ribosome, the message is scanned until the met-tRNAi anticodon pairs with the initiator codon, causing the release of the inorganic phosphate from the eIF2·GDP·Pi complex (166), thus allowing for the dissociation of the initiation factors and the recruitment of the 60S ribosomal subunit to form the translationally-active 80S ribosome. As eIF2·GDP does not bind met-tRNAi, eIF2B, a guanidine exchange factor (GEF) for eIF2, is required to enhance the exchange of GDP bound to eIF2 for GTP. Phosphorylation of eIF2α on S51, mediated by 4 eIF2α kinases under a variety of cell stresses, allows for stable association with eIF2B, thus blocking its function.
and effectively inhibiting translation initiation. This series of events has been described as the scanning ribosome method (167). Since this description, an alternate mode of translation initiation has been identified, a method independent of the 5′-cap, thus termed cap-independent translation.

### 1.4.7.2 IRESs

Internal ribosome entry sites (IRESs), located in proximity to the initiation start site, contain sequence similarity to 18S rRNA, and as such recruit the 43S pre-initiation complex directly (Figure 4). This process eliminates the need for the rate-limiting assembly of the eIF4F complex or a 5′-m7GTP cap, as well as the need for upstream scanning, which becomes nearly impossible with complex secondary structure. IRESs were first discovered in picornavirus mRNAs, which are translated fairly efficiently even without the presence of the 5′-m7GTP cap of cellular mRNAs (168-170). Translation via internal initiation reduces the requirement for translation initiation factors. While eIF4A, eIF4G, eIF4B, eIF2, and eIF3 are the only factors required by some picornaviruses (171; 172), others have fewer requirements. While the need for this mechanism in viruses is apparent, the presence of similar sequences in capped cellular mRNAs was unclear. Cellular and viral IRESs are quite similar. Both have complex secondary structure, sequence similarity to 18S rRNA, location immediately upstream of start codons, and high G-C content. In fact, many of the cellular mRNAs suspected to have IRESs share similar 5′-UTR features, including long, highly structured and highly stable UTRs that would be inhibitory for
translation under physiological conditions. These cellular mRNAs encode key regulatory proteins, growth factors, proto-oncogenes and apoptotic factors, all of which require very tight regulation under specific conditions. Some of the mRNAs suspected to contain IRESs include FGF-2 (173), IGF-II (174), PDGF-2 (175), eIF4G (176; 177), HIF-1α (178) and the chaperone BiP (179). In addition to containing a long and structured 5'-UTR, VEGF has been shown to contain a uORF, 4 in frame upstream CUG initiation codons, and 2 putative IRESs (157; 180), each followed by one or more initiation codons (181). These features appear to be advantageous for efficient translation of the VEGF mRNA during hypoxia in vivo when cap-dependent translation is repressed (182). IRES A controls utilization of the classic AUG initiation codon, while IRES B regulates translation initiation from the first upstream CUG (183-186). Several regulatory pathways have been identified in the utilization of cellular IRESs, including phosphorylation of eIF2α in response to viral infection and nutrient starvation, overexpression of eIF4E during tumorigenesis, and enhanced sequestration of eIF4E during hypoxia.

1.4.7.2.1 eIF2α phosphorylation

eIF2α controls delivery of the methionyl-tRNA to the 43S pre-initiation complex, a step required for translation initiation. Phosphorylation of eIF2 on S51 of its α subunit by one of 4 eIF2α kinases results in an inability of eIF2B to exchange GDP, and inhibits translation (Figure 5) (187). Phosphorylation of eIF2α has been correlated with viral IRES utilization (188-190). cat-1 IRES utilization is also
dependent upon phosphorylation of eIF2α (188-190). During differentiation, global rates of protein synthesis are repressed, yet translation of PDGF, VEGF, and c-myc persists through IRES utilization. The reduction in protein synthesis correlates with eIF2α phosphorylation; whereas overexpression of a dominant negative form of the eIF2α kinase PKR or a phosphorylation mutant of eIF2α results in decreased IRES utilization of these mRNAs (191).

**1.4.7.2.2 Overexpression of eIF4E**

Overexpression of eIF4E induces neoplastic transformation of cells (192), promotes tumorigenesis (193; 194), and has been noted in many human tumors (195). siRNA-mediated knockdown of eIF4E suppresses the tumorigenic qualities of cancer cell lines (196). Studies have indicated that the translation of complex messages is highly dependent upon the availability of the eIF4F complex and translation of these messages may be selectively enhanced by overexpression of eIF4E (197-199), messages including the VEGF mRNA (200; 201). In support of this idea, overexpression of eIF4E in Chinese hamster ovary (CHO) cells results in a 130-fold induction of VEGF secretion, without a change in VEGF mRNA. This increase was due to an enhanced association of the VEGF message with heavy polysomes (202). Importantly, secretion of VEGF from CHO-4E cells increases the growth of endothelial cells in culture (203; 204), demonstrating a functional outcome of the VEGF increase.
Phosphorylation status of eIF4E also appears to be an important factor in eIF4E-mediated IRES utilization (204; 205). Inhibition of Mnk1, a kinase for eIF4E, results in decreased eIF4E phosphorylation, which dramatically reduces HSV-1 viral replication and expression of viral proteins, providing an alternate mechanism by which viruses can mediate translation initiation (206).

1.4.7.2.3 Sequestration of eIF4E

Cap-dependent translation depends upon assembly of the eIF4F complex, which recognizes and binds to the 5’-m7GTP cap (207; 208). During mitosis, hypoxia or apoptosis, eIF4E is dephosphorylated and 4E-BP1 is also in a hypophosphorylated state, allowing it to bind to and sequester eIF4E (209-212). Under these conditions, there is a switch to cap-independent translation, making mRNAs that are normally inefficiently translated more competitive for translation (180; 213-216). 4E-BP1 is overexpressed in many breast cancers and has been suspected to regulate a hypoxia-activated switch from cap-dependent to cap-independent mRNA translation (175). Overexpression of this translational repressor results in increased tumor angiogenesis, cell growth and increased VEGF mRNA translation. Conversely, overexpression of a deletion mutant of 4E-BP1 that is unable to bind to eIF4E fails to increase VEGF translation in response to hypoxia. Importantly, overexpression of wildtype or the mutant form of 4E-BP1 has no impact on VEGF IRES utilization under normoxic conditions (175), suggesting that this is a hypoxia-specific mechanism.
1.4.7.3 ITAFs

Cellular proteins have been shown to mediate the function of viral IRESs (217). Acting as RNA chaperones, IRES transactivating factors (ITAFs) bind to and remodel the mRNA, altering secondary structures. Their cell-specific location can drive localized activity of IRESs and competing ITAFs can regulate “on-off switches.” hnRNP A1 and PTB activate the EMCV IRES (218-220). PTB also participates in the activation of the BiP (220) and the APAF-1 IRES (221). While hnRNP A1 binds to and activates the FGF-2 IRES (135; 222; 223), p53 inactivates both cap-independent and cap-dependent translation of FGF-2 via a conformational change of its 5’-UTR (135; 222). HNRNP L and IFN-γ serve as ITAFs for VEGF, mediating a stress-responsive IRES switch (224).

1.4.7.4 miRNAs

miRNAs are small, non-coding RNAs, 18-24nt in length, that regulate gene expression by targeting mRNAs for degradation (225). First discovered in C. elegans during development, these sequences were proposed to bind to complementary sites within the 3’-UTR of target mRNAs and incite translational repression (226; 227). Since then, these short inhibitory RNAs have been identified in a wide variety of mammalian species and have been implicated in the development of a variety of disease states, particularly cancer, modulating cell proliferation, apoptosis, and transformation (225). miRNAs are first transcribed as long precursor transcripts.
(pri-miRNAs) by RNA polymerase II, then are capped and polyadenylated. These sequences may be found within intronic sequences of protein coding genes (228); one transcript may encode several miRNAs (229; 230). The miRNA sequences undergo imperfect complimentary binding to form stem-loop hairpin structures and then are processed by the RNase-III, Drosha and DGCR8/Pasha (231-235), which excise the miRNA within a 60-80nt transcript. The pre-miRNA is then exported to the cytoplasm where it undergoes a second cleavage by the RNase-III, Dicer, which generates the double-stranded mature miRNA (236-239). The miRNA is guided to its target mRNA by RISC, a component of the Argonaute protein complex (240-242). Upon binding of the miRNA to complimentary sequences within the 3’-UTR the mRNA is either cleaved or silenced, depending upon the complimentarity of binding. One miRNA can regulate hundreds of targets, and more than one can bind to a target at a time. miRNA expression during tumorigenic angiogenesis has received a lot of attention. Further, Dicer knockdown increases the expression of VEGF and Flt-1 (243-245), suggesting that miRNAs are involved in VEGF and VEGF-R regulation. There are many miRNA candidates for both direct and indirect VEGF regulation. miRNAs let7-f, 27b, and 130a have been identified as pro-angiogenic, whereas miR-221 and 222 inhibit endothelial cell proliferation, migration and angiogenesis. miR-378 and the miR-17-92 cluster mediates tumor angiogenesis. miR-155, 21, and 126 have been implicated in vascular inflammation and angiogenic diseases (246; 247). Finally, miR-15, 16, 20a and 20b are downregulated during hypoxia resulting in increased VEGF expression (248).
1.5 4E-BP1

Of the members of the 4E-BP family, 4E-BP1 is the most studied and most well understood. Because of the implications that the increased expression of 4E-BP1 exerts on translational control, precise regulation is important; thus 4E-BP1 is regulated at both the transcriptional and post-transcriptional levels (165; 210). A number of transcription factors, responsive to a variety of physiological triggers, contain binding consensus sequences within the eif4ebp1 gene. These include, among others, the insulin-sensitive, FoxO1, and the stress-sensitive, ATF4 (249-251). At the post-transcriptional level, 4E-BP1 is rapidly modified by both phosphorylation and ubiquitination (165; 252). 4E-BP1 also has been predicted to contain miRNA consensus sequences, although there has been little effort focused in this area (253).

1.5.1 Transcriptional regulation

1.5.1.1 FoxO1

FoxO1, a member of the Forkhead family of transcription factors, contains a canonical Fox box DNA binding domain with the addition of a unique 5 amino acid insert within the domain. This insert alters the binding consensus sequence, and thus the substrates recognized by this subfamily, allowing for specification (254). FoxO proteins contain three conserved phosphorylation sites that are regulated by
Akt, T24, S256, and S319 (255-259). These sites are unique to the FoxO family, making them the only members of the family to be regulated by the insulin signaling pathway. Phosphorylation of these sites facilitates FoxO association with 14-3-3 proteins (258; 260; 261), resulting in the exclusion of FoxO from the nucleus and inhibition of FoxO-mediated transcriptional regulation, and eventually ubiquitination (Figure 6) (262). 4E-BP1 contains a consensus sequence for FoxO1 (249; 251; 263). Expression of a constitutively active FoxO1 increases the expression of 4E-BP1 via direct binding to 4E-BP1 promoter, and subsequently, increases its association with eIF4E. Results in vivo utilizing FoxO1++/+ mice have had similar outcomes (264).

1.5.1.2 ATF4

ATF4, also known as CREB-2, belongs to the AP-1 family of CREB-like transcription factors. These proteins contain leucine zipper regions, which mediate protein:protein interactions, and a stretch of basic amino acids, which mediates DNA binding (265; 266). The multiple uORFs present in the ATF4 mRNA allow for increased expression during cellular stresses, such as ER stress, oxidative stress, and amino acid deprivation, when eIF2α phosphorylation is increased (267-269). ATF4 expression regulates genes encoding proteins involved in amino acid transport, metabolism, and oxidative stress (270). *eif4ebp1* has been shown to be a direct target of ATF4, containing 2 potential binding sites within the first intron (250). Endoplasmic reticulum (ER) stress-mediated induction of ATF4 results in an
increase in 4E-BP1 expression (271). This phenomenon is inhibited by the transcriptional inhibitor, actinomycin D, suggesting that 4E-BP1 is being regulated at the transcriptional level. Expression of a dominant negative form of ATF4 suppresses thapsigargin-induced 4E-BP1 upregulation, whereas overexpression of WT ATF4 increases 4E-BP1 after thapsigargin treatment (271).

### 1.5.2 Post-translational regulation

#### 1.5.2.1 Phosphorylation

Activation of the mTORC1 pathway by nutrients and a subset of growth factors induces the phosphorylation of the downstream targets of mTORC1, including S6K1 and 4E-BP1. Phosphorylation of 4E-BP1 is affected by mTORC1 activity, and thus influenced by energy status and nutrient availability (272). 4E-BP1 is phosphorylated on a set of 7 conserved threonine and serine residues, 4 of which are thought to be regulated by mTORC1-T37, T46, T70, and S65 (Figure 7) (210; 211; 273; 274). Some studies have indicated that S65 phosphorylation is required for the release of 4E-BP1 from eIF4E (275), however, another study indicates that phosphorylation on this site alone is insufficient (276). Prior phosphorylation on T46, then T37, is required for subsequent phosphorylation on S65 and T70, creating a hierarchal phosphorylation cascade (210; 211; 277; 278). 4E-BP1 is dephosphorylated by PP1/PP2A (278).
1.5.2.2 Ubiquitination

Recent studies have also suggested that 4E-BP1 is ubiquitinated and degraded in response to phosphorylation (Figure 6) (212; 279). Treatment of cells with the proteasomal inhibitors, lactacystin and MG-132, results in an accumulation of 4E-BP1 (204; 206; 212). Apparent dephosphorylation of 4E-BP1 occurs by a mechanism of 4E-BP1 synthesis, and degradation of phosphorylated 4E-BP1 (212; 279). Treatment with calyculin A, a protein phosphatase inhibitor, decreases the half-life of the protein, indicating that phosphorylated 4E-BP1 may be a trigger for protein degradation (252; 280); phosphorylation on T37/46 appears to be particularly important for this process. MG-132 treatment increases the expression of high molecular weight forms of 4E-BP1, corresponding to polyubiquitination of the protein, which bind poorly to eIF4E (212; 279). Ubiquitination represents an additional and rapid post-translational modification that will be an important consideration for future studies of 4E-BP1 expression.
1.6 Figures and legends

Figure 1: The hexosamine biosynthetic pathway.
Fructose-6-phosphate, an intermediate of glycolysis is converted to glucosamine-6-phosphate in the first and rate-limiting step of the HBP, catalyzed by GFAT. Glucosamine-6-phosphate is then converted to UDP-GlcNAc, the endproduct of HBP, which is added to serine and threonine residues of substrate proteins by OGT, and removed by OGA.
**Figure 2: Regulation of eIF2.**
eIF2 is responsible for delivery of met-tRNA to the PIC, a step that requires GTP. As eIF2·GDP does not bind met-tRNAi, eIF2B is required to enhance the exchange of GDP bound to eIF2 for GTP. Phosphorylation of eIF2α on S51 allows for stable association with eIF2B, thus blocking its function and effectively inhibiting translation initiation.
**Figure 3: Regulation of HIF-1α.**
Under conditions of high oxygen tension, HIF-1α is hydroxylated on proline residues and targeted for ubiquitination and proteasome-mediated degradation. Under conditions of low oxygen tension, HIF-1α protein is stabilized, and translocates to the nucleus where it interacts with HIF-1β and binds to HREs in the DNA, upregulating the transcription of hypoxia-responsive genes.
Translation initiation can be divided into several stages, formation of the 43S PIC, recruitment of the PIC to the 5’ end of the mRNA, scanning of the 5’-UTR and recognition of the AUG initiator codon, and assembly of the 80S ribosome and initiation of translation. In the first stage, the met-tRNAi, in complex with eIF2·GTP, is delivered to the 40S ribosome. eIF3 is required for the recruitment of mRNA to the 43S PIC due to its interaction with the eIF4F complex at the 5’ cap structure. eIF4F is composed of eIF4E, the cap-binding protein, eIF4G, the mRNA-ribosome bridge and mRNA circularization protein, and eIF4A, an RNA-dependent helicase. This complex is inhibited by the 4E-BP1, which completes with eIF4G for the binding site on eIF4E. Once the mRNA is loaded onto the 40S ribosome, the message is scanned until the met-tRNAi anticodon pairs with the initiator codon, causing the release of the inorganic phosphate from the eIF2-GDP-Pi complex, thus allowing for the dissociation of the initiation factors and the recruitment of the 60S ribosomal subunit to form the translationally-active 80S ribosome. As eIF2-GDP does not bind met-tRNAi, eIF2B is required to enhance the exchange of GDP bound to eIF2 for GTP. Phosphorylation of eIF2α on S51 allows for stable association with eIF2B, thus blocking its function and effectively inhibiting translation initiation.
Figure 5: IRES utilization.
During cap-dependent translation initiation, the ribosomal complex assembles at the 5'-m7GTP cap and scans along the message for pairing with the AUG initiation codon. IRES utilization is a method of cap-independent translation in which the PIC is directly recruited to region immediately upstream of the 5'-cap, eliminating the requirement for the eIF4F complex and prolonged scanning.
Figure 6: FoxO1 nuclear translocation.
FoxO1 regulates the expression patterns of many genes, including those involved in the response to DNA damage and oxidative stress, such as Gadd45 and p300 (MnSOD). FoxO1 shuttles in and out of the nucleus based on the activation of Akt and the insulin/IGF-1 signaling pathway.
Figure 7: Regulation of 4E-BP1.
Phosphorylation on a number of sites, and subsequent inhibition of 4E-BP1 is mediated by mTORC1 signaling. Phosphorylation then serves as a signal for ubiquitination and degradation of the protein.
1.7 Introduction of dissertation project

1.7.1 Statement of the problem

Appropriate expression of VEGF is required for proper development; deletion of even a single VEGF allele is incompatible with survival beyond the first few weeks of life. Furthermore, reduced VEGF expression after development results in growth arrest and increased mortality. VEGF also confers neuroprotective actions. Upregulation during hypoxia and cellular stress protects cells from apoptosis, whereas altered expression results in a neurodegenerative syndrome similar to ALS. However, overexpression of VEGF can have negative consequences, especially within the retina. In models of oxygen-induced retinopathy and diabetic retinopathy, enhanced expression of VEGF results in increased vascular permeability and pathogenic angiogenesis. Therefore, strict regulation of VEGF expression is crucial for cellular perpetuation. VEGF is thus capable of being regulated on multiple levels. While VEGF transcriptional regulation by hypoxia has been well studied, less attention has been given to its translational control. The VEGF mRNA is complex, containing a uORF, 4 upstream CUG initiation codons, and 2 IRESs. Translational upregulation of VEGF under stress conditions such as hypoxia and diabetes has been documented, but the factors involved have not been identified. Further, a time course for VEGF upregulation is also lacking, thus providing only an incomplete picture of the progression of diabetic retinopathy.
Overexpression of 4E-BP1 in breast cancer cell lines results in increased VEGF IRES utilization. 4E-BP1 expression is also upregulated in the retina after the induction of diabetes during the same time course as VEGF upregulation. Ablation of 4E-BP1 results in a decrease in diabetes-induced expression of VEGF in the mouse retina, implicating 4E-BP1 in VEGF regulation. Understanding the mechanism by which 4E-BP1 expression is altered during diabetes thus could serve to identify important targets for altering VEGF expression during diabetic retinopathy. Therefore, the following aims have been proposed:

1.7.2 Aims

1) To determine the time course of VEGF protein expression upregulation in the retina of diabetic rodents, and to identify factors involved in the upregulation (Chapter III)

2) To determine the mechanism by which 4E-BP1 expression is increased in Müller cells under hyperglycemic conditions (Chapter IV)
CHAPTER II

MATERIALS AND METHODS
**Animals**

All animals were housed in The Pennsylvania State University College of Medicine Animal Facility in accordance with the Institutional Animal Care and Use Committee guidelines. All animals were maintained on a 12hr light-dark cycle and were given ad libitum water and a standard chow diet [Harlan-Teklad Rodent Chow 8604 (281)].

**STZ-induced diabetic rats**

Male Sprague-Dawley rats, weighing 120-180g (Charles River Laboratories, Wilmington, MA, USA) were rendered diabetic by a single i.p. injection of STZ (Sigma-Aldrich, St. Louis, MO, USA), as previously described (282). Rats exhibiting blood-glucose concentrations above 250mg/dL, measured 24hrs after the drug administration, were considered diabetic.

**STZ-induced diabetic mice**

Wildtype and *Eif4ebp1;Eif4ebp2* double knockout mice on a Balb/C background were kindly provided by Dr. Nahum Sonenberg (McGill University) (283). At 4 weeks of age, mice were made diabetic according to the AMDCC, “Low-Dose Streptozotocin Induction Protocol (Mouse).” Mice with blood-glucose concentrations exceeding 250mg/dL when measured once per week were
considered diabetic; those that did not meet this threshold were excluded from the study.

**Ins2^{Akita} Mice**

Male C57BL/6J Ins2^{Akita} heterozygote mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred in The Pennsylvania State College of Medicine Juvenile Diabetes Research Foundation Diabetic Retinopathy Center Animal Core, in accordance with The Pennsylvania State College of Medicine Institutional Animal Care and Use Committee guidelines. At 4.5 weeks of age mice were genotyped and tested for a diabetic phenotype, i.e. a blood glucose concentration exceeding 250mg/dL; WT littermates were used as controls (284). For high fat diet studies, wildtype and Ins2^{Akita} mice, 4 weeks of age, were fed either a normal chow diet or a high fat content diet for 4 weeks.

**Alloxan-induced diabetic rats**

Male Sprague-Dawley rats, weighing 120-180g (Charles River Laboratories, Wilmington, MA, USA) were fasted or fed ad libitum for 18hrs prior to the experiment. A subset of fasted rats was fed ad libitum for 45min prior sacrifice. Diabetes was induced in postabsorptive rats by i.v. injection of alloxan (48 mg/kg body weight in 0.155 mol/L saline) (Sigma-Aldrich, St. Louis, MO, USA). A urine sample was taken the day prior to the experiment to confirm the presence of
hyperglycemia (0.025 mol/L glucose), blood-glucose concentrations were assessed prior to sacrifice (250mg/dL is considered diabetic); rats that failed to meet or exceed this threshold were excluded from the experiment. Approximately 54hrs following the alloxan injection, rats were fasted overnight, and at 72hrs, rats were either sacrificed or refed for 45min prior to sacrifice. Mean weight of food consumed during the refeeding period was comparable between groups.

**Cell culture**

TR-MUL, [kindly provided by Dr. Ken-ichi Hosoya (Toyama Medical and Pharmaceutical University)], MIO-M1 [kindly provided by Dr. Steve Abcouwer (The Pennsylvania State University College of Medicine)], and HepG2 cells were maintained in low glucose DMEM (5.6mM) (Gibco, Carlsbad, CA, USA) and low serum (2% heat-inactivated FBS) and grown to subconfluence before seeding. TR-MUL cells were maintained at 33°C and MIO-M1 and HepG2 cells were maintained at 37°C, all in a humid 5% CO₂ atmosphere. Cells (500,000) were seeded into 6-well dishes and grown to approximately 75% confluence, at which time the culture medium was aspirated, cells were washed with sterile PBS, and experimental medium was added. For studies of the effects of hyperglycemic conditions, cells were either replenished with low glucose medium supplemented with 25mM mannitol (for osmotic control) or 25mM glucose and 10% heat-inactivated FBS for 10hrs, which was determined to be the time point of maximal stimulation for 4E-BP1 protein upregulation. In studies utilizing pharmaceuticals, cell culture medium
was supplemented with the following for 10hrs: 40µM PUGNAc, dissolved in medium (Carbogen AMCIS Ltd., Manchester, UK), 3mM glucosamine (Sigma-Aldrich, St. Louis, MO, USA), dissolved in medium, 10µM MG-132 (Calbiochem, San Diego, CA, USA), dissolved in DMSO, 100µM apocynin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in DMSO, 20µM azaserine (Sigma-Aldrich, St. Louis, MO, USA) dissolved in DMSO, 10µM DPI (Sigma-Aldrich, St. Louis, MO, USA) dissolved in DMSO, or 100µM H₂O₂ in H₂O; control cells were treated with an equal volume of solvent for the times indicated.

**Protein quantitation**

At the indicated time points, retinas or liver samples were isolated and flash-frozen in liquid nitrogen. Retinas were later sonicated in 500µL of Stuart extraction buffer (285), supplemented with inhibitors (microcystin, sodium vanadate, benzamidine, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 12,000rpm for 10min at 4°C. Liver samples were homogenized in 7 volumes of 4E-HB (20mM HEPES, 2mM EGTA, 50mM NaF, 100mM KCl, 0.2mM EDTA, 50mM β-glycerophosphate, pH 7.4), supplemented with inhibitors, and centrifuged at 1,000rpm for 3min at 4°C. Supernatant was collected, a fraction of which was added to 1x Laemmli buffer and boiled for 5min. For Western blot analysis, 60µg of retinal protein and 10µg of liver protein, were loaded per lane. Protein was separated by SDS-PAGE on 7.5%, 10%, 12.5%, and 15% gels, or 4-15% and 4-20% gradient Criterion gels (BioRad Laboratories, Hercules, CA, USA), and transferred to a PVDF
membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in 5% milk in TBST (0.05 M % tris, pH 7.6, with 0.9% NaCl and 0.1% Triton X-100) (except for when probing for O-GlcNAc, in which case the membranes were blocked in 5% BSA in TBST), washed, and then incubated overnight at 4°C with the primary antibody. All antibodies (Table 1), except the following, were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA): anti-VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), anti-phospho eIF2α S51 (BioSource International, Inc., Camarillo, CA, USA), anti-4E-BP1 (Bethyl Laboratories, Inc., Montgomery, TX, USA), anti-p70S6K1 (Bethyl Laboratories, Inc., Montgomery, TX, USA), anti-HIF-1α (Novus Biologicals, LLC, Littleton, CO, USA), polyclonal anti-Mnk1/2, anti-FGF-2 (Upstate, Billerica, MA, USA), anti-PDGF (R&D Systems, Minneapolis, MN, USA), anti-O-GlcNAc (Covance, Princeton, NJ, USA), anti-PEDF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-ubiquitin [a kind gift from Dr. Vincent Chau (The Pennsylvania State University College of Medicine)], and anti-GFAT [a kind gift from Dr. Cora Weigert (University of Tuebingen, Germany)]. Monoclonal antibodies to the α-subunit of eIF2, eIF4E, and eIF4G were prepared in our laboratory as described previously (286-288). The blots were incubated with horseradish peroxidase-conjugated secondary antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA), washed, and incubated for 1 hr at a 1:10,000 dilution, and then developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). Quantitation was performed using a GeneGnome HR imaging system with GeneTools software (SynGene, Cambridge, UK). VEGF secretion into the medium was analyzed using a Quantikine Rat VEGF
Immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

**Quantitation of total 4E-BP1 protein expression**

Tissue or cells were harvested, then sonicated, in 200-500µL of Lambda phosphatase buffer (289), supplemented with inhibitors (with the exception of the phosphatase inhibitors microcystin and sodium vanadate) and combined with 4µL (1,600U) of Lambda phosphatase (New England BioLabs, Ipswich, MA, USA) for 1hr at 37°C. Samples were centrifuged at 1,000g for 3min, and the supernatant was removed and boiled for 3min, centrifuged again, combined with 2x Laemmli buffer, boiled again for 5min, and subjected to Western blot analysis.

**eIF4E and O-GlcNAc immunoprecipitation**

BioMag anti-mouse IgG magnetic beads (BioClone Inc., San Diego, CA, USA) (1 mL/sample) were washed 3 times in low salt buffer (LSB) (20mM Tris HCl, 5mM EDTA, 150mM NaCl, 0.5% Triton X-100, 0.1% β-mercaptoethanol in 1L H2O, pH 7.4) then resuspended in half of the original volume in LSB. Beads were combined with 175µL PBS, 12.5µL Triton X-100, and 4µg monoclonal anti-eIF4E antibody (per sample) and incubated on an orbital rocker overnight at 4°C. Retinas were dounce homogenized in 500µL 4E-HB plus inhibitors, cells were harvested, then sonicated in 4E-HB, plus inhibitors, and 2.5% Triton X-100 and 0.25% deoxycholate. Lysates
were subjected to centrifugation for 3min at 1000g and then were incubated for 1hr at 4°C with 200µL of the bead mixture. After incubation, the bead mixture was washed twice with LSB, once with high salt buffer (HSB) (50mM Tris HCl, 500mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 0.04% β-mercaptoethanol in 400mL H2O, pH 7.4), and then resuspended in 100µL/sample 1x Laemmli buffer and boiled for 5min. After the beads were removed, 20µL of sample were loaded onto a 4-15% Criterion gel and subjected to Western blot analysis.

**4E-BP1 immunoprecipitation**

BioMag anti-mouse IgG magnetic beads (1.5mL/sample) were washed 3 times in LSB then resuspended in 1/5 of the original volume in LSB. Beads were combined with 175µL PBS, 12.5µL Triton X-100, and 4µg monoclonal anti-4E-BP1 antibody (per sample) and incubated on an orbital rocker overnight at 4°C. Cells were harvested in 4E-HB, plus inhibitors, and 2.5% Triton X-100 and 0.25% deoxycholate. Lysates were subjected to centrifugation for 3min at 1,000g and then incubated for 1hr at 4°C with 200µL of bead mixture. After incubation, the bead mixture was washed twice with LSB, once with HSB, and then resuspended in 50µL/sample 1x Laemmli buffer and boiled for 5min. After the beads were removed, 20µL of sample were loaded onto a 4-15% Criterion gel and subjected to Western blot analysis.
Quantitative real-time PCR

RNA was isolated from the retinas or cells, following a standard TriZol protocol (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. Total RNA, at least 500ng, was reverse transcribed in a 20µl reaction using an ABI High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). For quantitation of mRNA, qRT-PCR was performed using Taqman RT-PCR Assay Kit (Applied Biosystems, Foster City, CA, USA) or QuantiTech SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). Taqman (R) Gene Expression Assay primers were designed to detect the following sequences:

- β-actin (CTTCCTTCTGGGTATGGAATCCTG)
- VEGFA (CTCCACCATGCCAAGTGGTCCAGG), designed to detect all VEGFA isoforms,
- FoxO1 (TCTCTAATTCTTGAGGGGTGGTTGC),
- 4E-BP1 (CACCCCCGGAGGAACCAGAATCATC),
- Gadd45 (GCTGGTGACGAACCCACATTCATC),
- p300 (AGCGATGGCACAGATTTTGGTTCAC), and
- angiopoietin2 (GAACAGCTTCCTAGAAAAGAAAAGTG). The following QuantiTech Primer Assay kits (Qiagen) were used:

- β-actin (Rn_Actb_1_SG QuantiTect Primer Assay NM_031144)
- VEGFA (Rn_RGD:619991_1_SG QuantiTect Primer Assay NM_001110335), and
- 4E-BP1 (Rn_Eif4ebp1_1_SG QuantiTect Primer Assay NM_053857).
Statistical Analysis

For statistical analysis, data from the control samples were compared by ANOVA or Student's t-test to the experimental samples with the aid of GraphPad Prism version 4. Where appropriate, data were log-transformed for normality; the data are presented as a percentage of the control mean ± SEM.
2.1 Figures and legends

Figure 8: Weight and blood-glucose concentrations over time in control and STZ-induced diabetic rats.

(A) *Body weight of control and STZ rats.* Rats were weighed each week, to the nearest gram body weight. The study was repeated four times. Values represent the mean ± SEM of 51 control and 71 diabetic rats at 1 week, 25 control and 30 diabetic rats at 2 weeks, 10 control and 17 diabetic rats at 4 weeks, and 10 control and 14 diabetic rats at 6 weeks; control (open circles) and diabetic (closed circles).

(B) *Blood-glucose concentration measurement in control and STZ rats.* Blood was collected at 1, 2, 4, and 6 weeks after the induction of diabetes, and the glucose concentration was measured as described under Materials and Methods; control (white bars) and diabetic (black bars). * p<0.001 vs control from the same time point.
Figure 9: Blood-glucose concentrations over time in control and STZ-induced diabetic mice.

At 4 weeks of age, *Eif4ebp1;Eif4ebp2* DKO mice were rendered diabetic as described under Materials and Methods; age-matched controls were injected with citrate buffer. Mice with blood-glucose concentrations measured weekly exceeding 250mg/dL were considered diabetic. The study was repeated 3 times and the aggregate data are shown. Values represent the mean ± SEM for 20 WT control (white squares), 29 WT diabetic (white circles), 18 DKO control (black squares), or 30 DKO diabetic (black circles) mice. At all time points the STZ groups blood-glucose concentrations were significantly elevated over the control groups. *p<0.001* wildtype diabetic vs control at the same time point. †*p<0.001* DKO diabetic vs control from the same time point.
Figure 10: Blood-glucose concentrations over time in WT and Ins2\textsuperscript{Akita} mice. 4.5 week male C57BL/6J Ins2\textsuperscript{Akita} heterozygous mice were genotyped and tested for a diabetic phenotype, that of a blood glucose concentration exceeding 250mg/dL; littermates homozygous for the wildtype Ins2 gene, were used as controls. Values represent the mean ± SEM of 16 control and 16 diabetic mice at 6 weeks, 20 control and 14 diabetic mice at 16 weeks, and 13 control and 15 diabetic mice at 24 weeks; control (white bars) and diabetic (black bars). * p<0.001 vs wildtype at the same time point.
Table 1: Antibodies used for protein quantitation.

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All antibodies were used at the dilution suggested by the manufacturer.
CHAPTER III

ABLATION OF 4E-BP1/2 PREVENTS DIABETES-INDUCED
UPREGULATION OF VEGF PROTEIN EXPRESSION IN THE
RODENT RETINA
3.1 Abstract

Among the causes implicated in the development of diabetic retinopathy, both abnormal neovascularization and increased vascular permeability have been linked to enhanced expression of the angiogenic factor, VEGF. Expression of VEGF can be regulated through both transcriptional and translational mechanisms. The present study was designed to examine VEGF protein and mRNA expression in the retina in response to experimentally induced diabetes. Retinas were isolated from control and STZ-treated rats over a range of time points following the induction of diabetes and analyzed for VEGF expression and biomarkers of various translational control mechanisms. While the relative abundance of VEGF mRNA remained constant through 12 weeks, VEGF protein expression was upregulated over the controls as early as 2 weeks following the induction of diabetes. Immunoprecipitation of eIF4E revealed significantly more 4E-BP1 bound to eIF4E in the retina of diabetic compared to control rats. The increased association of 4E-BP1 with eIF4E would be expected to repress global rates of protein synthesis due to decreased cap-dependent translation, while permitting cap-independent translation, such as translation through an internal ribosome entry site, to proceed unimpaired. Enhanced association was due to decreased relative phosphorylation and increased expression of 4E-BP1. The diabetes-induced upregulation of VEGF protein expression was completely prevented in Eif4ebp1;Eif4ebp2 double-knockout mice, suggesting that 4E-BP1 expression is required for diabetes-induced VEGF upregulation. The relationship between 4E-BP1 expression and VEGF upregulation
in response to diabetes was observed in 3 models of Type 1 diabetes, STZ-induced diabetes in the rat and mouse, and the Ins2Akita diabetic mouse. Overall, the data indicate that diabetes-induced signaling, resulting in elevated 4E-BP1 expression and increased binding of 4E-BP1 to eIF4E, may act to repress global rates of mRNA translation while selectively enhancing the translation of a subset of mRNAs including the mRNA encoding VEGF.

### 3.2 Introduction

Diabetic retinopathy is the leading cause of acquired blindness among Americans under the age of 65, affecting 5.3 million people, or 2.5% of the population (290). Diabetic retinopathy is characterized by abnormal vascular flow, increased permeability, and the eventual nonperfusion of capillaries. Early in the progression of the disease there is a change in the structure and cellular composition of the microvasculature. The endothelial cells that maintain the blood-retinal-barrier become damaged resulting in breakdown of the barrier and subsequently, increased vascular permeability and accumulation of extracellular fluid in the macula (291). Changes in permeability and vascular structure lead to capillary occlusion, causing localized retinal ischemia, which stimulates abnormal neovascularization mediated by angiogenic factors, in particular, VEGF. Finally, hemorrhaging of the new leaky, immature vessels into the vitreous may lead to retinal detachment and eventually loss of vision (292).
In many cases of pathological vascularization, such as that observed during tumorigenesis (293), polycystic ovary syndrome (294), and age-related macular degeneration (295), the tight regulation of anti- and pro-angiogenic factors that controls the angiogenic process is disrupted and VEGF expression is higher than that observed during normal vascular development. Similarly, upregulation of VEGF protein expression has been observed in the retina in many animal models of diabetes and ischemia (71; 296-298), and in the vitreous of diabetic retinopathy patients (79; 115; 299). Additional evidence has shown that overexpression of functional VEGF in normal tissue results in vascular changes similar to those seen in diabetic retinopathy (300; 301). In contrast, inhibition of VEGF function in the retina by VEGF-neutralizing agents such as anti-VEGF antibodies (293), overexpression of dominant negative or chimeric Flt-1/VEGF receptor (302; 303), or inhibition of receptor tyrosine kinases (304) has been shown to attenuate the abnormal vascular changes associated with the disease.

The vegf gene is regulated by oxygen tension through expression of the transcription factor HIF-1α (305). During periods of low oxygen tension (e.g. ischemia) HIF-1α is stabilized by interaction with HIF-1β, preventing its proteasome-mediated degradation, and allowing it to translocate to the nucleus, bind to DNA, and facilitate the transcription of specific genes, such as vegf (303; 306). Translational regulation of VEGF is influenced by the architecture of the 5’-UTR of the VEGF mRNA. The VEGF mRNA belongs to a class of mRNAs with long 5’-UTRs, containing stretches of stable G-C pairing, and areas of complimentarity that
act to repress translation of the coding region under normal physiological conditions. Translation of such mRNAs is thought to be mediated in a cap-independent manner (307). The VEGF message also contains two putative IRESs that allow for cap-independent translation under conditions of global translational repression (180; 184; 185; 308).

Translation initiation is a tightly regulated process mediated by modifications of initiation factors. Phosphorylation of the α subunit of eIF2 is a mechanism for regulating cap-independent translation, such as through utilization of an IRES (191). eIF2, when GTP-bound, is responsible for the delivery of the initiator methionyl-tRNA to the pre-initiation complex, where it facilitates scanning along the message for the initiator codon. When eIF2 is phosphorylated on its α subunit it forms a stable complex with eIF2B, the factor that facilitates the exchange of GDP for GTP on eIF2, and maintains the pool of active eIF2 available for initiation. The phosphorylated eIF2:eIF2B complex prevents the recycling of eIF2, thus reducing the amount available to reinitiate translation, and subsequently, repress global rates of protein synthesis. Alternatively, control of eIF4E, the mRNA cap-binding protein, regulates utilization of cap-dependent translation. The ability of eIF4E to participate in assembly of the eIF4F complex, composed of eIF4E, eIF4G, the mRNA-ribosome bridge and mRNA circularization protein, and eIF4A, an RNA-dependent helicase, is regulated by a class of 4E-BPs, which inhibit translation via sequestration of eIF4E (207). In the hypophosphorylated state, 4E-BPs compete with eIF4G for binding to eIF4E and prevent eIF4F complex assembly (209; 309).
When hyperphosphorylated, a state mediated by mTORC1-mediated signaling, 4E-BPs release from eIF4E and may be degraded by the proteasome (165; 310; 311).

In the present study we show that at early time points of STZ-induced diabetes in rats, a model of Type 1 diabetes, VEGF protein expression in the retina is upregulated. The upregulation occurs despite constant expression of VEGF mRNA and in the absence of increased HIF-1α expression, suggesting that the alteration occurs independently of transcriptional regulation and may be a result of increased translational efficiency of the VEGF mRNA. 4E-BP1 protein expression is also upregulated in the absence of a change in its mRNA abundance. This alteration leads an increase in sequestration of eIF4E, expected to result in suppression of cap-dependent translation and consequently a shift toward cap-independent translation. This mechanism is supported by the observation that the absence of 4E-BP1 and 4E-BP2 in diabetic mice prevents the diabetes-induced increase in VEGF expression. Overall the results indicate that diabetes-induced alteration in 4E-BP1 expression, perhaps through protein stabilization, leads to a shift in the mRNA selection pattern for translation, leading to the enhanced translation of the VEGF mRNA.
3.3 Results

3.3.1 Upregulation of VEGF protein, but not mRNA, expression after the induction of diabetes

VEGF protein was analyzed by immunoblot of retinal homogenates prepared from control and diabetic rats 1, 2, 4, 6, and 12 weeks after induction of diabetes. Whereas VEGF protein expression was increased significantly in the retinas of diabetic rats over the controls at 2, 4, and 6 weeks (Figure 11A), the relative abundance of VEGF mRNA remained constant (Figure 11B). There was a slight trend toward a decrease in VEGF expression in the retina of diabetic rats at 12 weeks, however, this difference was not significant group contained a smaller number of animals, nearly half the size of other groups as we focused on the robust changes of the younger animals. In addition to unchanged VEGF mRNA, protein expression of the transcription factor, HIF-1α, did not differ between the groups (data not shown), suggesting that the mechanism for VEGF protein upregulation is post-transcriptional. There was no change in the expression of FGF-2, PDGF, or PEDF protein expression (data not shown), members of similar growth factor families encoded by mRNA with highly structured UTRs, indicating a selective mechanism for VEGF upregulation. For additional data, see Appendix A.
3.3.2 Increased association of 4E-BP1 with eIF4E is a result of decreased relative phosphorylation and increased expression of 4E-BP1 in the retina of diabetic rats

As there did not appear to be a change in the expression of the VEGF mRNA, translational control mechanisms were explored that might explain its upregulated expression. Sequestration of eIF4E by 4E-BP1 leads to a repression in rates of global protein synthesis. Sequestration of eIF4E is regulated by the phosphorylation status of 4E-BP1, which reflects changes in the pattern of mRNAs selected for translation (165). When 4E-BP1 is in a hypophosphorylated state, it binds to eIF4E and prevents assembly of the eIF4F complex, essential for cap-dependent translation (209; 309). However, when 4E-BP1 is phosphorylated on multiple sites, a response mediated by mTORC1-mediated signaling, it releases from eIF4E and is degraded (165; 310; 311). Enhanced association of 4E-BP1 and eIF4E has been shown to regulate VEGF IRES utilization in breast cancer cell lines. Therefore, the association between 4E-BP1 and eIF4E was assessed. In the present study, a significant increase in 4E-BP1 associated with eIF4E was detected (Figure 12A). There was no change in the amount of 4E-BP2 bound to eIF4E (Figure 12A). The increased association was attributed to a reduction in the relative phosphorylation of 4E-BP1 on T37/46, a key regulatory phosphorylation site (Figure 12B), and increased expression of 4E-BP1 (Figure 12C). 4E-BP1 mRNA remained constant (Figure 12D). See Table 2 for a summary of the data.
3.3.3 VEGF protein and 4E-BP1 protein expression are coordinated in a genetic model of type 1 diabetes

The Ins2\textsuperscript{Akita} mutation is a single amino acid substitution (C96Y) in the insulin 2 gene that disrupts disulfide bond formation between the A and B chain, resulting in misfolding of proinsulin. The accumulation of misfolded protein leads to ER stress and ultimately, \( \beta \)-cell apoptosis (284). Male heterozygous Ins2\textsuperscript{Akita} mice become diabetic, exhibiting hypoinsulinemia and hyperglycemia at about 4 weeks of age (284). These mice have been used in recent studies to investigate diabetic complications, in light of the similar disease progression and characteristics to that of the established STZ-induced diabetic models, including increased retinal vascular permeability and blood-retinal-barrier breakdown (284). Thus, to further evaluate the correlation between VEGF upregulation and 4E-BP1 expression observed in the STZ-induced diabetic rat model as a common mechanism, retinas were harvested from Ins2\textsuperscript{Akita} mice and analyzed for VEGF and 4E-BP1 protein and mRNA expression. The time course for VEGF upregulation in the Ins2\textsuperscript{Akita} mice was delayed compared to that of the STZ diabetic rat, but the trends in VEGF and 4E-BP1 protein (Figure 13A-B) were similar to the STZ diabetic rat (Table 3). VEGF and 4E-BP1 mRNA expression were unchanged (data not shown).
3.3.4 Ablation of 4E-BP1 prevents diabetes-induced VEGF protein upregulation

If the diabetes-induced increase in 4E-BP1 expression is responsible for the upregulated expression of VEGF, then it would be reasonable to predict VEGF expression would be attenuated in the absence of 4E-BP1. To test this hypothesis, retinas from control and STZ-induced diabetic, WT and DKO Eif4ebp1;Eif4ebp2, mice were analyzed. While retinal 4E-BP1 protein was detected in Eif4ebp1;Eif4ebp2 WT mice, the protein was undetectable in the retinal extracts from Eif4ebp1;Eif4ebp2 DKO mice, confirming lack of 4E-BP1 expression (Figure 14A). After 5 weeks of STZ-induced diabetes, 4E-BP1 and VEGF protein expression was significantly higher in the retinas of the diabetic compared to the control Eif4ebp1;Eif4ebp2 wildtype mice, however, there was a slight trend toward a decrease in VEGF expression between the control and diabetic Eif4ebp1;Eif4ebp2 double knockout mice (Figure 14B-C), despite severe hyperglycemia. VEGF protein expression was comparable between control WT and DKO mice. This result suggests that increased 4E-BP1 expression is necessary for diabetes-induced upregulation of VEGF mRNA translation.
3.4 Discussion

VEGF upregulation is a cardinal biochemical response in human and animal models of diabetic retinopathy, but the mechanisms underlying this repose *in vivo* have been obscure. In the present study VEGF protein expression was increased in the retina of diabetic compared to control rats after 2 weeks of STZ-induced diabetes. This expression had returned to basal levels by 12 weeks, posing the question of how this early and non-sustained expression can contribute to the chronic alterations observed in this disease. Most studies have documented that the vascular pathology associated with diabetic retinopathy, such as vascular leakage (291), appearance of acellular capillaries (297), and endothelial apoptosis (312), occur after prolonged periods, 3-8 months, of diabetes in rodents. However, there is some evidence that changes may be occurring within the first few weeks. One study reported blood-retinal-barrier leakage in response to a near 2-fold increase in VEGF expression after only 8 days of diabetes. The increase in permeability could be entirely prevented by treatment with a chimeric VEGF receptor (313). So it is entirely possible that the increased VEGF expression observed in this study within the first few weeks of diabetes may in fact contribute to early blood-retinal-barrier breakdown, although investigation of this possibility was beyond the scope of this study. Alternatively, VEGF expression has shown to serve a protective role especially during stress, including ischemia. Additionally, VEGF increases proliferation and outgrowth of neuronal cell cultures, and can induce neuronal survival through the upregulated expression of Bcl-2 to prevent TNF-α mediated
apoptosis (127-129). Therefore it is also possible that the increased expression of VEGF in this model, early in the progression of the disease, may serve to protect the retina from neuronal apoptosis. The decrease in expression after 12 weeks may reflect failure of a homeostatic mechanism of protection, leading to increased TUNEL staining observed at this time (314).

In the present study, expression of VEGF protein was increased, without increased VEGF mRNA expression, in the retina of three animal models of Type 1 diabetes: STZ-induced diabetic rats and mice, and Ins2Akita mice, compared to age-matched control animals. These findings suggest that VEGF expression is regulated in a post-transcriptional manner and the upregulation is not due to increased production or stability of the message. Although other groups have reported an increase in VEGF mRNA expression (87; 315), under the conditions utilized in the present study no difference was detected. However, the qRT-PCR primers used in this study are designed to detect all isoforms of VEGF-A, so there is a possibility of a change in the mRNA expression of a particular isoform that would not be detected using a global primer set. Additionally, there has been little study into differential mRNA expression within retinal cell subsets, as most studies have focused on whole retinal homogenates. It is likely that VEGF-A expression patterns differ among the various cell types and alterations occur only within certain cells rather than globally.

Concomitant increases in 4E-BP1 protein expression lead to enhanced sequestration of eIF4E and inhibition of eIF4F complex assembly and cap-dependent translation
(165), possibly providing a mechanism by which cap-independent translation, and translation of the VEGF mRNA, is favored. Upregulated expression of 4E-BP1 appears to be necessary for the increased expression of VEGF, as the absence of 4E-BP1/2 prevents diabetes-induced VEGF expression after 5 weeks of diabetes. Lack of changes in 4E-BP1 mRNA and in FoxO1 expression and localization, suggest, as is the case for the VEGF protein expression, regulation at the post-transcriptional level. While the effect of phosphorylation on function of 4E-BPs has received substantial attention (165), 4E-BP degradation is not well understood. However, evidence for proteasome-dependent degradation of 4E-BP1 has been shown by accumulation of the protein after proteasome inhibition (205) and by ubiquitin immunoprecipitation (316); thus phosphorylation of 4E-BP1 may have a dual role in inhibiting the function of the protein and in targeting it for ubiquitination (205; 252). Additionally, the protein becomes stabilized in its hypophosphorylated state, as treatment with the pro-apoptotic cytokine, TRAIL, leads to hypophosphorylation and accumulation of the protein possibly through inhibition of the proteasome (252). In the present study, increased binding of 4E-BP1 to eIF4E in the diabetic retina suggests decreased ubiquitination of 4E-BP1, as studies by Constantinou and Clemens (316) have shown inefficient binding of ubiquitinated 4E-BP1 to eIF4E. Studies have shown that oxidative stress in the retina leads to covalent modification of active site sulfhydryls contained within the E1 and E2 enzymes involved in the ubiquitin-proteasome pathway (317). Treatment of RPE or whole bovine retinas with H2O2 resulted in a dose-dependent increase in oxidized glutathione and a greater ratio of oxidized to reduced glutathione, indicative of oxidative stress, and
subsequently a dose-dependent decrease in levels of E1 and protein:ubiquitin conjugates. Additionally, the ability to form de novo ubiquitin conjugates was impaired and the activity of both E1 and E2 enzymes was reduced 60–80% (317). Taken together with the current data, this allows for the possibility of a mechanism in diabetes by which the 4E-BP1 protein is stabilized through inhibition of its degradation via the diabetes-induced production of ROS. Studies described in Chapter IV were performed to elucidate the mechanism behind the diabetes-induced upregulated expression of 4E-BP1.

While a shift in the distribution of VEGF mRNA between the nonpolysomal and polysomal fractions within sucrose density fractions was not detected in this study (data not shown), it is possible that the VEGF message is shifting within the polysomal fraction, from light to heavy polysomes. Treatment of MCT proximal tubule epithelial cells with angiotensin II causes such a shift in the VEGF message, without precipitating changes in message expression or stability, an effect dependent upon the expression of wildtype 4E-BP1 (318). Other studies have detected increased IRES utilization in cells overexpressing 4E-BP1 (175). While further studies are required to determine the exact mechanism of 4E-BP1-induced regulation of VEGF, it is likely that diabetes-induced 4E-BP1 upregulation leads to increased VEGF IRES utilization, accounting for the enhanced expression of the protein.
The use of multiple measurable outputs and models is important in establishing molecular mechanisms of human disease in animal models. In the present study, three rodent models of Type 1 diabetes yielded strikingly similar results in the upregulation of VEGF and expression of 4E-BP1, although the time course was shifted in the Ins2Akita model, perhaps due to differences in susceptibility or to the initial delayed onset of the disease. Together, the evidence from these models strongly suggests that 4E-BP1 is a key modulator of translational control of VEGF expression in the retina during diabetes. Further details about the regulation of VEGF expression may lead to means to control abnormal vascular permeability in diabetic retinopathy.
3.5 Figures and legends

**A**  
Western blot analysis of VEGF expression. 60µg of protein were loaded per lane. Protein was analyzed from 13 control and 14 diabetic rats at 1 week, 12 control and 14 diabetic rats at 2 weeks, 12 control and 12 diabetic rats at 4 weeks, 13 control and 13 diabetic rats at 6 weeks, 5 control and 7 diabetic rats at 12 weeks. Values represent the mean expressed as a percentage of control samples ± SEM; control (white bars) and diabetic (black bars). Representative blots are shown as an inset.

**B**  
Quantitative RT-PCR of VEGF. Retinal RNA was extracted following a standard TriZol protocol from 6 control and 9 diabetic rats at 4 weeks, 3 control and 6 diabetic rats at 6 weeks, and 10 control and 10 diabetic rats at 12 weeks. Values represent the mean expressed as a percentage of internal β-actin control ± SEM; control (white bars) and diabetic (black bars). * p<0.05.

**Figure 11:** VEGF protein is increased in the retina of diabetic rats in the absence of changes in VEGF mRNA.

(A) Western blot analysis of VEGF expression. 60µg of protein were loaded per lane. Protein was analyzed from 13 control and 14 diabetic rats at 1 week, 12 control and 14 diabetic rats at 2 weeks, 12 control and 12 diabetic rats at 4 weeks, 13 control and 13 diabetic rats at 6 weeks, 5 control and 7 diabetic rats at 12 weeks. Values represent the mean expressed as a percentage of control samples ± SEM; control (white bars) and diabetic (black bars). Representative blots are shown as an inset.

(B) Quantitative RT-PCR of VEGF. Retinal RNA was extracted following a standard TriZol protocol from 6 control and 9 diabetic rats at 4 weeks, 3 control and 6 diabetic rats at 6 weeks, and 10 control and 10 diabetic rats at 12 weeks. Values represent the mean expressed as a percentage of internal β-actin control ± SEM; control (white bars) and diabetic (black bars). * p<0.05.
Figure 12: Enhanced association of 4E-BP1 with eIF4E in the retina of diabetic rats is a result of decreased relative phosphorylation and increased total expression of 4E-BP1.

(A) eIF4E immunoprecipitation. eIF4E immunoprecipitation in the retinas of 4-week control and STZ rats was carried out as described in Materials and Methods. 20μL of sample were loaded per well. 4E-BP1 expression was normalized to eIF4E and displayed as a percentage of the control mean ± SEM (n=8); control (white bars) and diabetic (black bars).

(B) Western blot analysis of 4E-BP1 phosphorylation. 4E-BP1 phosphorylation on T37/46 was normalized to total 4E-BP1 measured from 12 control and 12 diabetic rat retinal extracts 4 weeks after the induction of diabetes; values are displayed as a percentage of the control mean ± SEM; control (white bars) and diabetic (black bars).

(C) Western blot analysis of 4E-BP1 protein expression. Retinal lysates were subjected to Lambda phosphatase treatment as described in Materials and Methods. 60μg of protein were loaded per lane. Blots are representative of at least 3 independent sets of animals; control (white bars) and diabetic (black bars).

(D) Quantitative RT-PCR of 4E-BP1 mRNA. Retinal RNA was...
extracted following a standard TriZol protocol from 6 control and 9 diabetic rats at 4 weeks, 3 control and 6 diabetic rats at 6 weeks, and 10 control and 10 diabetic rats at 12 weeks. Values represent the mean expressed as a percentage of internal β-actin control ± SEM; control (white bars) and diabetic (black bars). Representative blots are shown as an inset. * p<0.05, ** p<0.01, *** p<0.001
Table 2: Summary of protein and mRNA quantitation from the retina of STZ-induced diabetic rats.

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“No,” indicated that the value was unchanged, “yes,” indicates that a change was detected; ⬆️ represents an increase in the retina of STZ-induced diabetic rats, ⬇️ represents a decrease. Hatching indicates that no measurement was made.
Figure 13: VEGF expression increases concomitantly with 4E-BP1 expression in Ins2Akita mice.

(A) Western analysis of 4E-BP1 in Lambda phosphatase-treated retinal extracts. 60µg of protein was loaded per lane. Data is representative of retinas from 7 WT and 4 Ins2 mice at 3mon, and 3 WT and 3 Ins2 mice at 8.5mon. (B) Western analysis of VEGF expression. 60µg of protein were loaded per lane. Values represent the mean expressed as a percentage of control samples ± SEM; control (white bars) and diabetic (black bars). Representative blots are shown as an inset. * p<0.01, ** p<0.001.
Table 3: Summary of protein and mRNA quantitation from the retina of Ins2<sup>Akita</sup> mice.

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“No,” indicated that the value was unchanged, “yes,” indicates that a change was detected; † represents an increase in the retina of STZ-induced diabetic rats, ↓ represents a decrease. Hatching indicates that no measurement was made.
Figure 14: Ablation of 4E-BP1/2 in mice attenuates the diabetes-induced upregulation of VEGF expression.

(A) Western analysis of 4E-BP1/2 in the retina of 5-week WT and DKO control and STZ-induced diabetic mice. 60µg protein were loaded per lane. Protein was analyzed from 6 WT and 6 DKO mice.

(B) Western analysis of VEGF expression in the retina of 5-week WT and DKO control and STZ-induced diabetic mice. 60µg of protein were loaded per lane. Protein was analyzed from 12 control and 12 diabetic WT mice, and 7 control and 8 diabetic DKO mice. Values represent the mean expressed as a percentage of control samples ± SEM; control (white bars) and diabetic (black bars). Representative blots are shown as an inset. * p<0.05.
Hyperglycemic conditions induce O-GlcNAcylation of 4E-BP1 in retinal Müller cells in culture
4.1 Abstract

The studies described in Chapter III demonstrated that VEGF protein, but not mRNA expression, increases in the retina of diabetic rodents. The upregulation of VEGF expression requires 4E-BP1, an inhibitory factor that represses global rates of protein synthesis. In the present study, the mechanism that accounts for upregulated 4E-BP1 expression was examined in cultures of retinal Müller cells exposed to hyperglycemic conditions with the hypothesis being that increased flux through the hexosamine biosynthetic pathway (HBP) leads to the modification and stabilization of 4E-BP1. Hyperglycemic conditions maximally enhanced 4E-BP1 expression after 10hrs of exposure. Paralleling the earlier in vivo studies, 4E-BP1 was less phosphorylated on T37/46, an inactivating phosphorylation site, corresponding to increased association with eIF4E. These events led to the enhanced secretion of VEGF in both Müller cells and in WT MEFs, an effect that was completely inhibited in Eif4ebp1;Eif4ebp2 DKO MEFs, suggesting that 4E-BP1 is required for VEGF production in vitro, as it is in vivo. Hyperglycemic conditions induced O-GlcNAcylation of 4E-BP1, which did not interfere with its ability to bind to eIF4E. However, this modification did prohibit the ubiquitination of 4E-BP1, which would be expected to reduce its sequestration and degradation, leading to its enhanced stability and increased ability to bind to and inhibit eIF4E. To the best of our knowledge this study constitutes the first report of O-linked modification of 4E-BP1.
4.2 Introduction

In Chapter III, we have demonstrated that 4E-BP1 protein expression is increased in the retina of diabetic rodents. The increased expression of 4E-BP1 is required for the upregulation of VEGF, an important factor that has been linked to the increased vascular permeability and neoangiogenesis associated with diabetic retinopathy. 4E-BP1 is a member of a class of binding proteins that bind to and inhibit eIF4E, the mRNA cap-binding protein, and thus suppress cap-dependent translation. As an important modulator of translation initiation, 4E-BP1 expression must be tightly regulated. Phosphorylation of 4E-BP1 on multiple sites, mediated by the mammalian target of rapamycin complex 1 (mTORC1), leads to the inhibition of the protein, hindering its ability to bind to and sequester eIF4E. Recent studies have also suggested that phosphorylation serves to target 4E-BP1 for ubiquitination, leading to its proteasome-mediated degradation. In our previous study, relative phosphorylation of 4E-BP1 was decreased in the retina of STZ-induced diabetic rats, relieving repression of the protein. One possibility for reduced phosphorylation is the presence of a competitive modification, such as O-GlcNAcylation, on or near important regulatory phosphorylation site(s). Therefore the present study was designed to test the hypothesis that hyperglycemia-induced flux through the hexosamine biosynthetic pathway (HBP) leads to the O-GlcNAcylation and decreased ubiquitination of 4E-BP1 in retinal Müller cells.
The HBP converts glucose, through a series of enzymatic reactions, to UDP-GlcNAc, the precursor for all amino sugars utilized in the synthesis of glycoproteins, glycolipids, and proteoglycans, and O-GlcNAc, a modification added to serine and threonine residues of substrate proteins (319). The first and rate-determining step in the pathway is the conversion of fructose-6-phosphate, an intermediate of glycolysis, to glucosamine-6-phosphate. This reaction involves the transfer of the amide group from glutamine to fructose-6-phosphate, which is catalyzed by the enzyme glutamine-fructose-6-phosphate-amidotransferase (GFAT). O-GlcNAcylation is a dynamic and reversible process, while O-GlcNAc transferase (OGT) catalyzes the addition of O-GlcNAc residues to substrates, O-GlcNAcase (OGA) removes the modification, in a relationship akin to kinases and phosphatases (18-20). In fact, this modification often occurs on or near phosphorylation sites, usually occurring in a mutually exclusive manner with phosphorylation (21-25).

While the HBP pathway, a relatively minor branch of glycolysis, constitutes only 2-3% of total glucose metabolism under normal conditions (29), both hyperglycemic conditions and diabetes have been shown to increase flux through the HBP and increase the concentration of its endproduct, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (30). While the presence of high glucose concentrations alone will present additional substrate for the pathway, hyperglycemia-induced inhibition of GAPDH, an important regulatory enzyme of glycolysis (9; 31; 32), and upregulation of GFAT, the rate-limiting enzyme of the HBP, expression and activity readily result in a build-up of both substrates for and intermediate metabolites of the pathway. O-GlcNAcylation has proven to be an
important regulatory modification, involved in modulating transcription, signal transduction, and degradation (21; 32).

Previous studies have shown that glutamine, a substrate utilized in the rate-limiting first step of hexosamine biosynthesis, is required for flux through this pathway (29; 33). As the only cell type present in the retina that contains glutamine synthetase (45; 46), the Müller cell is particularly susceptible to the metabolic effects of activation of the HBP. Additionally, it has been demonstrated that among the cell types present in the retina, Müller cells readily consume more glucose than other cells, especially when exposed to high glucose concentrations (41; 42). Their location within the retina, spanning the entire width, enveloping the retinal neurons and providing trophic factors to neurons and photoreceptors also makes them a prime target for the release of factors that affect neighboring cells. In fact many studies have implicated Müller cell activation in the pathogenesis of diabetic retinopathy, showing the Müller cell as an origination point for inflammatory cytokines and angiogenic factors, including VEGF (47-51; 79). Therefore, Müller cells in culture serve as an attractive model for studying the involvement of the HBP in the development of retinopathy.

In cells exposed to hyperglycemic conditions a larger proportion of total 4E-BP1 was O-GlcNAcylated when compared to cells maintained in low glucose, and this modification, unlike phosphorylation on certain sites, did not interfere with its ability to bind to eIF4E. Further, O-GlcNAcylation prevented phosphorylation and subsequent ubiquitination of 4E-BP1, suggesting that these events are mutually exclusive and may be competing for the same site. The results support the model in
which hyperglycemia-induced O-GlcNAcylation of 4E-BP1 confers enhanced stability, leading to increased expression of the protein. 4E-BP1 binds to and inhibits eIF4E, repressing cap-dependent translation, making cap-independently translated mRNAs more competitive for translation, such as the mRNA encoding VEGF.

4.3 Results

4.3.1 Hyperglycemia-induced upregulated 4E-BP1 expression leads to increased association of 4E-BP1 with eIF4E

To determine the time course for 4E-BP1 protein upregulated expression under hyperglycemic conditions, cells were exposed to high glucose from 4 to 18hrs. 4E-BP1 expression was significantly increased 4hrs after exposure to high glucose, and continued through 18hrs of treatment (Figure 15). As the maximally stimulating time point without inducing noticeable cell death, 10hrs of treatment was selected for all further studies. These results were also confirmed in the human Muller cell line, MIO-M1 (data not shown). While 4E-BP1 protein expression was significantly upregulated after 10 hours of hyperglycemia, the expression of 4E-BP1 mRNA was similar between groups (Figure 16A). Additionally, high glucose caused a decrease in the relative phosphorylation status of 4E-BP1 on T37/46, a site important for the regulation of 4E-BP1 function and ubiquitination (Figure 16B). Enhanced expression and decreased phosphorylation resulted in a significant increase in the
amount of 4E-BP1 bound to eIF4E, as measured in an eIF4E immunoprecipitation (Figure 16C). The increased association of 4E-BP1 with eIF4E would be expected to repress global rates of protein synthesis.

4.3.2 Upregulated 4E-BP1 expression is required for secretion of VEGF under hyperglycemic conditions

Enhanced expression of 4E-BP1 in the retina of diabetic rodents resulted in an increase in VEGF expression in our previous study. To determine if the increase in 4E-BP1 expression resulted in enhanced VEGF secretion in vitro, media was collected from cells after exposure to hyperglycemia. After 10hrs of high glucose treatment VEGF concentration was significantly elevated in the cell culture medium as measured by VEGFA ELISA (Figure 17A). There was no change in the expression of VEGF mRNA in the cell lysates, suggesting a post-translational mechanism for the enhanced production of VEGF (Figure 17B). VEGF secretion was also increased in MIO-M1 cells (Figure 17C).

Studies in Chapter III utilizing Eif4ebp1;Eif4ebp2 double knockout mice have shown that in the absence of 4E-BP1/2 the diabetes-induced upregulation of VEGF expression in the retina is suppressed. To confirm the dependence of VEGF production upon 4E-BP1 expression, Eif4ebp1;Eif4ebp2 wildtype and double knockout mouse embryonic fibroblasts (MEFs) were exposed to hyperglycemia for 10hrs. Expression of 4E-BP1 and 4E-BP2 was undetectable in the double knockout cells (data not shown). 4E-BP1 expression increased in wildtype MEFs during hyperglycemic conditions (data not
shown), and this led to a significant increase in VEGF secretion into the medium (Figure 17D). Secretion of VEGF from double knockout MEFs, on the other hand was decreased under hyperglycemic conditions, confirming the requirement for 4E-BP1 expression (Figure 17D). In fact, VEGF secretion from the DKO was hardly detectable, at the lowest range of detection under either condition. Thus data is presented as a percentage of control for comparative secretion between treatment groups along the same scale.

4.3.3 The upregulated expression of 4E-BP1 under hyperglycemic conditions is mediated through the hexosamine biosynthetic pathway

A number of studies have shown that flux through the HBP increases during diabetes and in response to hyperglycemic conditions, leading to the O-GlcNAcylation of a variety of proteins involved in transcription, signal transduction, and degradation (35-38). Glucosamine can be used to directly stimulate the HBP, bypassing GFAT, the rate-limiting enzyme of the pathway. As a result, this metabolite has been shown to be 40 times more potent than glucose in mediating insulin resistance in adipocytes (320). Additionally, our previous studies in R28 retinal neurons demonstrate that glucosamine treatment can increase the secretion of VEGF (321). Therefore, to test the hypothesis that the HBP can induce upregulation of 4E-BP1 expression and increase VEGF secretion in retinal Müller cells, M10-M1 cells were treated with 3mM glucosamine for 10hrs. Glucosamine treatment led to an increase in 4E-BP1 protein expression (Figure 18A) and an increase in VEGF secretion into the culture medium (Figure 18B). Conversely, co-
treatment of cells with high glucose and the GFAT inhibitor, azaserine, completely prevented the high glucose-induced upregulation of 4E-BP1 (Figure 18C) and VEGF (Figure 18D). In fact, treatment of MIO-M1 cells with high glucose resulted in a significant increase in GFAT expression (Figure 18E), which would be expected to increase flux through the HBP. Finally, exposure of cells to PUGNAc, a non-metabolizable analog of glucosamine that forms a stable complex with and inhibits O-GlcNAcase, the enzyme that catalyzes the removal of O-GlcNAc residues, results in increased 4E-BP1 expression (Figure 19) and a dramatic increase in VEGF secretion into the culture medium (Figure 19). These results suggest that the HBP plays a major role in the high glucose-induced upregulation of 4E-BP1 and VEGF expression.

4.3.4 4E-BP1 is O-GlcNAcylated during exposure to hyperglycemic conditions

Many proteins are O-GlcNAcylated in response to hyperglycemic conditions and diabetes. O-GlcNAcylation of IRS-1, Akt, and FoxO1 alter their function and lead to downregulated insulin signaling (18; 33; 34). Immunoprecipitation of 4E-BP1 revealed a significant increase in O-GlcNAcylation in cells cultured under hyperglycemic conditions (Figure 20A). Additionally, exposure of cells to PUGNAc resulted in a similar increase in O-GlcNAcylation of 4E-BP1 (Figure 20A). Immunoprecipitation of O-GlcNAc and immunoblot for 4E-BP1 yielded similar results (Figure 20B). These results were replicated in MIO-M1 cells (Figure 20C), confirming this general phenomenon in both cell lines.
O-linked modifications often occur on or near sites of phosphorylation, usually in a mutually exclusive manner. As 4E-BP1 interaction with eIF4E is inhibited by phosphorylation, it was important to assess whether or not the presence of O-GlcNAc interferes with the ability of 4E-BP1 to associate with eIF4E. Immunoprecipitation of eIF4E followed by immunoblot of O-GlcNAc revealed an increased association of eIF4E with O-GlcNAc-modified 4E-BP1, suggesting that the O-GlcNAc modification did not prohibit the interaction of 4E-BP1 with eIF4E as phosphorylation does (Figure 20D).

4.3.5 The production of reactive oxygen species plays a role in high glucose-induced 4E-BP1 expression

The production of reactive oxygen species (ROS) plays a major role in hyperglycemia-induced retinal pathology (9-11). In fact, oxidative stress has been strongly implicated in VEGF upregulation, as VEGF requires superoxide production for its angiogenic properties (14). Additionally, diabetes-induced vascular dysfunction and VEGF expression can be prevented by inhibition of ROS production. To determine the extent of the participation of ROS in high glucose-mediated 4E-BP1 upregulation, TR-MUL cells were treated with H2O2. H2O2 treatment resulted in an increase in 4E-BP1 protein expression to a similar extent to that of high glucose (Figure 21A), and a substantial increase in VEGF secretion (Figure 21B). A significant contributor of ROS in the retina, and in retinal vasculature is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Exposure of cells
to the NADPH oxidase inhibitors apocynin and DPI prevented high glucose-induced increase in 4E-BP1 expression (Figure 21C). Importantly, apocynin treatment also reduced O-GlcNAcylation of 4E-BP1 (Figure 21D). The results suggest that ROS may play a significant role in the upregulation of 4E-BP1 under these conditions.

4.3.6 Hyperglycemic conditions reduce ubiquitination of 4E-BP1

The relative phosphorylation status of 4E-BP1 on T37/46, a site essential for ubiquitination (252), is reduced in the retina of diabetic rodents in our previous studies. Therefore, the reduction in phosphorylation in this model may contribute to decreased ubiquitination and possibly leading to decreased degradation of the protein. To test this hypothesis, TR-MUL cells were exposed to high glucose, after which immunoprecipitation was performed to detect ubiquitinated 4E-BP1. After 10hrs of exposure to high glucose, the ubiquitination of 4E-BP1 decreased significantly (Figure 22A).

Reduced phosphorylation on T37/46 may be the result of competing O-GlcNAcylation occurring on or near that site. If the T37/46 site hosts both ubiquitination and O-GlcNAcylation of 4E-BP1, or if the presence of one sterically hinders the other, these modifications should be mutually exclusive. To test this possibility, high-glucose treated and PUGNAc-treated cells were subjected to O-GlcNAc immunoprecipitation and co-immunoprecipitation of ubiquitin and 4E-BP1 was assessed. Ubiquitin was undetectable in the O-GlcNAc immunoprecipitates, suggesting that only non-ubiquitinated 4E-BP1 co-immunoprecipitated with O-
GlcNAc (Figure 22B). These results confirm that O-GlcNAcylation could prevent ubiquitination of 4E-BP1.

Overall, the data support the model that hyperglycemic conditions increase O-GlcNAcylation of 4E-BP1 via increased flux through the HBP. O-GlcNAcylation prevents the phosphorylation and ubiquitination at T37/46, perhaps stabilizing the protein and allowing for its interaction with and suppression of eIF4E (Figure 23).

4.4 Discussion

Results from in vivo studies on retinal tissue are often difficult to interpret due to the heterogeneity of cell types and subtle changes in subsets of cell types are impossible to detect utilizing whole retinal homogenates. Müller cells serve as an attractive target cell for studying retinopathies, as they are the primary glial cell of the retina, important not only for maintaining the structural integrity of the retina, with endfeet spanning the entire depth, but also in providing trophic factors for retinal neurons and in removing potentially damaging excess excitatory neurotransmitters (42; 322). Many studies have indicated that Müller cells may be the primary cell producing and secreting VEGF in the retina, shown by VEGF co-localization with the Müller cell marker, GFAP in immunohistochemical studies (39; 40; 47-51). Recently it has been shown that Müller cells, when compared to both endothelial cells and retinal neurons from the same donors, consume significantly more glucose, especially during exposure to high glucose concentrations. Additionally, while endothelial cells were capable of responding to cytokines, there
was relatively no response to hyperglycemic conditions, suggesting that other cells, such as Müller cells, may release factors, such as IL-1β, in response to hyperglycemic conditions that affect retinal endothelial cells (323).

Previously we have shown that VEGF expression increases in the rodent retina during diabetes. The upregulated expression of VEGF is dependent upon 4E-BP1 expression, which is also increased during diabetes. In the current studies, hyperglycemic conditions caused an increase in 4E-BP1 expression and the secretion of VEGF. Ablation of 4E-BP1/2 prevented the hyperglycemia-induced VEGF secretion, recapitulating the in vivo results, in Müller cells in vitro. The confirmation of the results in two distinct retinal Müller cell lines, representing both rat and human cells, lends credence to the proposed model. The results of these studies suggest that high glucose-treated Müller cells are a useful model for studying the molecular mechanisms of diabetic retinopathy in vitro.

Hyperglycemic conditions have been shown to increase O-GlcNAcylation of multiple proteins affecting glucose utilization and insulin sensitivity, having significant implications in the development of diabetes-related complications. Thus far O-GlcNAcylation has been shown to alter signal transduction, degradation and transcription. Here we report modulation of translation through the modification of a key regulator of the initiation of mRNA translation. Treatment of Muller cells with glucosamine results in a similar increase in 4E-BP1 expression as high glucose treatment, an effect that can be completely abrogated by the use of the GFAT
inhibitor, azaserine, suggesting that high glucose-mediated 4E-BP1 upregulation may occur through this pathway. Further, treatment of Muller cells with PUGNAc, an inhibitor of O-GlcNAcase, results in the accumulation of O-GlcNAc residues on a variety of proteins, including 4E-BP1. To the best of our knowledge, this is the first report of O-GlcNAcylation of 4E-BP1, providing an additional means for differential regulation of the protein.

The production of ROS has been shown to mediate a majority of hyperglycemia-induced pathological pathways. In the present studies, treatment of cells with \( \text{H}_2\text{O}_2 \) alone results in increased 4E-BP1 protein expression and VEGF secretion, suggesting that ROS production may play a major role in the expression of these proteins. Further, inhibition of NADPH oxidase, a major contributor to ROS production in the retina, completely abrogates both the high glucose-induced upregulated expression and O-GlcNAcylation of 4E-BP1. These results suggest that ROS production through NADPH oxidase may regulate the HBP under hyperglycemic conditions in retinal Müller cells. Therefore, NADPH oxidase serves as an attractive target in studying hyperglycemia-induced retinal pathology in diabetic retinopathy, because the production of ROS represents an overlap for many pathways activated by hyperglycemia (9).

We have also shown that O-GlcNAcylation and ubiquitination of 4E-BP1 is mutually exclusive, indicating that these modifications may compete for the same site or possibly that the O-GlcNAc modification prohibits phosphorylation and subsequent
ubiquitination through other means such as steric hindrance or induction of a conformational change. Further studies are required for the precise identification of the O-GlcNAcylation site(s).

Previously and in the present study we have demonstrated the importance of 4E-BP1 expression in the upregulation of VEGF expression, a key mitogenic factor in diabetic retinopathy. While ablation of 4E-BP1 alleviates the increased expression of VEGF, the consequences of removing a protein integrally related to mRNA translation have not been well studied. Therefore, by targeting a specific pathway pathologically upregulated during the disease state, such as the HBP, or a specific modification, such as O-GlcNAc, the off-target effects of altering more global processes such as translation, signal transduction, or degradation may be avoided.
Figure 15: 4E-BP1 protein expression is increased during hyperglycemic conditions.
TR-MUL cells were exposed to either low (5mM; white bars) or high glucose (25mM; black bars). Cells were harvested at the indicated time points. Lysates were subjected to Lambda phosphatase treatment and then subjected to Western blot analysis for 4E-BP1 protein quantitation. Values represent the mean expressed as a percentage of the control ± SEM. Representative blots are shown as an inset. *p<0.05, **p<0.01, ***p<0.001.
Figure 16: Relative phosphorylation of 4E-BP1 on T37/46 is reduced, leading to enhanced association with eIF4E.  
(A) Quantitative RT-PCR of 4E-BP1 mRNA. RNA was extracted from TR-MUL cells exposed to low (white bar) and high glucose (black bar) conditions for 10hrs following a standard TriZol protocol. Values represent the mean of 4E-BP1 mRNA expression normalized to an internal β-actin control expressed as a percentage of control ± SEM. (B) Western blot analysis of 4E-BP1 phosphorylation on T37/46. TR-MUL cells exposed to low (white bar) and high glucose (black bar). 30µg of protein were subjected to Western blot analysis for 4E-BP1 T37/46 phosphorylation. Values represents the mean expressed as a percentage of the control ± SEM. (C) Analysis of 4E-BP1:eIF4E association. Lysates from eIF4E immunoprecipitation were subjected to Western blot analysis for the co-immunoprecipitation of 4E-BP1 from cells exposed to low (white bar) and high glucose (black bar). Values represent the mean expressed as a percentage of the control ± SEM.
expressed as a percentage of the control ± SEM. Representative blots are shown as an inset. *p<0.05.
Figure 17: Hyperglycemic conditions enhance the secretion of VEGF in retinal Müller cells, but not in Eif4ebp1;Eif4ebp2 MEFs.

(A) Analysis of VEGF secretion. Medium was collected from TR-MUL cells exposed to low (white bar) and high glucose (black bar) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM.

(B) Quantitative RT-PCR of VEGF mRNA. RNA was extracted from TR-MUL cells exposed to low (white bars) and high glucose (black bars) following a standard TriZol protocol. Values represent the mean of 4E-BP1 mRNA expression normalized to an internal β-actin control expressed as a percentage of control ± SEM.

(C) Analysis of VEGF secretion in the human Müller cell line, MIO-M1. Medium was collected from TR-MUL cells exposed to low (white bar) and high glucose (black bar) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM.

(D) Analysis of VEGF secretion. Medium was collected from Eif4ebp1;Eif4ebp2 WT
and DKO MEFs exposed to low (white bars) and high glucose (black bars) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM. * p<0.001, † p<0.01 when compared to DKO cells maintained in low glucose medium.
Figure 18: The hexosamine biosynthetic pathway regulates high-glucose mediated induction of 4E-BP1 expression.

(A) Western blot analysis of 4E-BP1 expression. TR-MUL cells were exposed to low glucose (white bar) or low glucose supplemented with 3mM glucosamine (black bar) for 10hrs. Lysates were subjected to Lambda phosphatase treatment and 30µg protein were loaded per lane. Blots, shown as an inset, are representative of at least 3 independent experiments. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM. (B) Analysis of VEGF secretion. Medium was collected from TR-MUL cells exposed to low glucose (white bar) and low glucose supplemented with 3mM glucosamine (black bar) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM. (C) Western blot analysis of 4E-BP1 expression. TR-MUL cells were exposed to low glucose (white bar), high glucose (black bar), or high glucose supplemented with 20µM azaserine (gray bar) for 10hrs. Lysates were subjected to Lambda phosphatase treatment and 30µg protein were loaded per lane. Blots, shown as an inset, are representative of at least 3 independent experiments. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM. (D) Analysis of VEGF secretion. Medium was collected from TR-MUL cells exposed to low (white bar), high glucose (black bar), and high glucose supplemented with 20µM azaserine (gray bar) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM. (E) Western blot analysis of GFAT expression. MIO-M1 cells were exposed to low (white bar) or high glucose (black bar) for 10hrs. 30µg protein were loaded per lane. Blots, shown as an inset, are representative of at least 3 independent experiments. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM. † p<0.05 (versus 5mM), ** p<0.01, ***p<0.001.
Figure 19: PUGNAc, a nonmetabolizable analog of glucosamine, increases 4E-BP1 protein expression and VEGF secretion.

Analysis of VEGF secretion. Medium was collected from TR-MUL cells exposed to low glucose (white bar) and low glucose supplemented with 40µM PUGNAc (black bar) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM. *p<0.05, ** p<0.01. (Inset) Western blot analysis of 4E-BP1 expression. Cells were exposed to low glucose (white bar) or low glucose supplemented with 40µM PUGNAc (black bar) for 10hrs. Lysates were subjected to Lambda phosphatase treatment and 30µg protein were loaded per lane. Blots, shown as an inset, are representative of at least 3 independent experiments.
Figure 20: 4E-BP1 is O-GlcNAcylated in response to hyperglycemic conditions.

(A) Western blot analysis of O-GlcNAcylated 4E-BP1. TR-MUL cell lysates from 4E-BP1 immunoprecipitation were subjected to Western blot analysis for the co-immunoprecipitation of O-GlcNAc after exposure to low (white bar) or high glucose (black bar), and after treatment with 40µM PUGNAc (gray bar). Blots, shown as an inset, are representative of at least 3 independent experiments. Values represent the mean expressed as a percentage of the control ± SEM.

(B) Western blot analysis of O-GlcNAcylated 4E-BP1. TR-MUL cell lysates from O-GlcNAc immunoprecipitation were subjected to Western blot analysis for the co-immunoprecipitation of 4E-BP1 after exposure to low (white bar) or high glucose (black bar), and after treatment with 40µM PUGNAc (gray bar). Blots, shown as an inset, are representative of at least 3 independent experiments. Values represent the mean expressed as a percentage of the control ± SEM.

(C) Western blot analysis of O-GlcNAcylated 4E-BP1.
MIO-M1 cell lysates from 4E-BP1 immunoprecipitation were subjected to Western blot analysis for the co-immunoprecipitation of O-GlcNAc after exposure to low (white bar) or high glucose (black bar), and after treatment with 40µM PUGNAc (gray bar). Blots, shown as an inset, are representative of at least 3 independent experiments. Values represent the mean expressed as a percentage of the control ± SEM. (D) Analysis of O-GlcNAcylated 4E-BP1:eIF4E association. MIO-M1 cell lysates from eIF4E immunoprecipitation were subjected to Western blot analysis for the co-immunoprecipitation of O-GlcNAcylated 4E-BP1 after exposure to low (white bar) or high glucose (black bar), and after treatment with 40µM PUGNAc (gray bar). Data represent at least two independent experiments of 3-6 replicates. Blots, shown as an inset, are representative of at least 3 independent experiments. Values represent the mean expressed as a percentage of the control ± SEM. Representative blots are shown as an inset. *p<0.05, ** p<0.01.
Figure 21: Production of ROS by NADPH oxidase mediates high glucose-induced upregulation of 4E-BP1 expression.  

(A) Western blot analysis of 4E-BP1 expression. Cells were exposed to control or 100µM H₂O₂ for 10hr. Lysates were subjected to Lambda phosphatase treatment and 30µg protein were loaded per lane. Blots, shown as an inset, are representative of at least 3 independent experiments. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; control (white bar), H₂O₂ (black bar).

(B) Analysis of VEGF secretion. Medium was collected from TR-MUL cells exposed to low (white bar) and high glucose (black bar) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM.

(C) Western blot analysis of 4E-BP1 expression. Cells were exposed to low or high...
glucose, supplemented with DMSO, 100µM apocynin, or 10µM DPI for 10hr. Lysates were subjected to Lambda phosphatase treatment and 30µg protein were loaded per lane. Blots, shown as an inset, are representative of at least 3 independent experiments. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; low glucose (white bar), high glucose (black bar), apocynin (dark gray), DPI (light gray).  

**D) Analysis of O-GlcNAcylated 4E-BP1.** Western blot analysis of O-GlcNAc and 4E-BP1 expression after 4E-BP1 immunoprecipitation. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; low glucose (white bar), high glucose (black bar), apocynin (gray bar). Data are representative of at least 2 independent experiments of 3 replicates. Representative blots are show as an inset. * p<0.05, ** p<0.01, *** p<0.001.
Figure 2: O-GlcNAcylation of 4E-BP1 prevents its ubiquitination.

(A) Western blot analysis of ubiquitinated 4E-BP1. Müller cell lysates from 4E-BP1 immunoprecipitation were subjected to Western blot analysis for the co-immunoprecipitation of ubiquitin-modified 4E-BP1 after exposure to low and high glucose. 

(B) Western blot analysis of O-GlcNAc immunoprecipitates. TR-MUL cell lysates from O-GlcNAc immunoprecipitation were subjected to Western blot analysis for the co-immunoprecipitation of ubiquitin and 4E-BP1 after exposure to low and high glucose. Representative blots of at least two independent experiments of 3-6 replicates are shown.
Figure 23: Model of hyperglycemia-induced 4E-BP1 protein upregulation. Hyperglycemic conditions increase O-GlcNAcylation of 4E-BP1 via increased flux through the hexosamine biosynthetic pathway. The O-GlcNAcylation of 4E-BP1 prevents its phosphorylation and ubiquitination on T37/46, which may stabilize the protein, allowing for its interaction with, and suppression of, eIF4E.
CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS
VEGF expression was increased in the retina of diabetic rats after 2 weeks of diabetes, and returned to basal levels after 12 weeks of diabetes. While other groups have detected increased expression of VEGF mRNA, in the current study, VEGF protein expression, but not mRNA expression, was increased in the retina of diabetic rats. In total retinal homogenates it is not possible to detect changes in expression between retinal cells or cell layers. Additionally, through the use of global primer sets, it is not possible to detect changes in specific VEGF isoforms. Therefore it is possible that a change in mRNA expression was overlooked, however, the results are consistent across 3 rodent models of Type 1 diabetes and was replicated in vitro under hyperglycemic conditions in cultures of retinal Müller cells, so “total” VEGFA expression is unchanged.

While VEGF has been linked to increased permeability and abnormal angiogenesis in diabetic retinopathy, the effects of altered expression of VEGF on neuronal survival early in the progression of diabetes is not well understood. Many studies have shown that VEGF can prevent hypoxia-induced apoptosis of neuronal cells through the expression of anti-apoptotic factors. As neuronal apoptosis in response to diabetes has been documented after 3 months of diabetes (314), it is possible that VEGF expression protects retinal cells from apoptosis during early hyperglycemia, and upon reduction of VEGF expression, apoptosis becomes maximal. Due to the technical difficulties arising from the small size of the rodent retina, we were unable to determine the effects of VEGF upregulation on vascular permeability in this study. It is likely that increased VEGF expression would contribute to increased retinal
permeability and in the absence of 4E-BP1, which represses VEGF expression, this permeability would be reduced or reversed. However, the results are inconclusive and require further study. It is also unclear if the increased expression of VEGF is functional and activating the VEGF receptors.

4E-BP1 expression was increased concomitantly with VEGF expression, leading to increased association with eIF4E. In the absence of 4E-BP1, the diabetes-induced increase in VEGF expression is completely suppressed, suggesting that 4E-BP1 is required for diabetic- and high glucose-induced expression of VEGF. Overexpression of 4E-BP1 in breast cancer cell lines has been shown to increase IRES utilization of VEGF due to inhibition of cap-dependent mRNA translation. While the results of this study are consistent with suppression of cap-dependent translation and VEGF IRES utilization, protein synthesis was not assessed, nor was the translational efficiency of VEGF measured. Therefore studies are still required to determine if increased IRES utilization is the mechanism by which 4E-BP1 increases VEGF protein expression. Additionally, it is unclear if this represents a global mechanism by which complex messages are translated through IRES utilization, or whether VEGF is somehow selectively translated under these conditions. Changes in FGF-2, PDGF, and PEDF, all messages which contain IRESs and that are translated in a similar manner, were not detected in this study. Enhanced expression of ITAFs involved in VEGF IRES utilization could explain the specificity for VEGF, however, expression of hNRNP L and ProRS, ITAFs for VEGF, were not different between control and high glucose-treated Müller cells (data not
shown). It is possible that there may be an increase in DNA-binding in spite of constant expression. DNA-binding assays are required for proper assessment of this possibility. In addition to increased translational efficiency, enhanced protein stability may account for or contribute to increased VEGF expression. Further study is required to determine if diabetes or hyperglycemic conditions alter the half-life of VEGF.

Many proteins have been identified as substrates for modification by the HBP. O-GlcNAcylation affects multiple pathways within signaling transduction, degradation, and transcription, proteins including Akt, IRS-1, IRS-2, and FoxO1. Modification of these proteins leads to alterations in insulin signaling, insulin sensitivity, and glucose uptake. This study represents the first report of O-GlcNAcylation of a key regulatory protein in mRNA translation, 4E-BP1. O-GlcNAcylation represents yet another regulatory modification, in addition to phosphorylation and ubiquitination, for altering the expression and activity of this protein within the cell. This highlights the importance of its strict regulation, as well as leads to implications for its altered regulation in many pathological conditions in which O-GlcNAcylation may be affected. O-GlcNAc residues are added to threonine and serine residues of proteins, usually on the same site as or near important regulatory phosphorylation sites, making these two modifications mutually exclusive. T37/46 mediates both phosphorylation and ubiquitination of 4E-BP1, and was identified on an O-GlcNAcylation database as being a candidate for this modification. In accordance with this site as a candidate, in cells treated with high glucose O-GlcNAcylation of
4E-BP1 is upregulated and the relative phosphorylation on T37/46 is reduced. Altered phosphorylation could not be accounted for by changes in mTORC1 signaling, or PP2A expression, a phosphatase for 4E-BP1. These results could suggest that reduced activity toward a specific substrate, or more likely, the presence of a competing modification, such as O-GlcNAcylation.

Phosphorylation on T37/46 is required for ubiquitination of 4E-BP1. If 4E-BP1 is also O-GlcNAcylated on this site, then it should interfere with ubiquitination as well. In agreement with this idea, high glucose reduced ubiquitination of 4E-BP1. Additionally, co-immunoprecipitation experiments demonstrate that ubiquitin is undetectable in an O-GlcNAc IP, suggesting that only non-ubiquitinated 4E-BP1 is detected. Reduced ubiquitination of 4E-BP1 would be expected to increase the half-life of 4E-BP1 significantly, contributing to increased 4E-BP1 expression. While the observations are consistent with that model, studies examining the half-life of 4E-BP1 are still lacking. Additionally, these studies have not led to the conclusive identification of T37/46 as the site of O-GlcNAcylation; it is possible that O-GlcNAcylation of a nearby site may interfere with phosphorylation via steric hindrance or by altering the protein conformation. In fact, since O-GlcNAcylation does not inhibit 4E-BP1 association with eIF4E, as phosphorylation does, it may be more likely that this modification occurs on a nearby site. Therefore definitive identification site(s) of O-GlcNAcylation by mass spectrometry, followed by verification via mutagenesis studies, is essential for the understanding of the interaction between the post-translational modifications of 4E-BP1.
The production of ROS mediates many hyperglycemia-induced pathways. In the current study inhibition of NADPH oxidase by apocynin and DPI results in an attenuation of high glucose-induced 4E-BP1 expression. Additionally, apocynin reduces O-GlcNAcylation of 4E-BP1, indicating that ROS production by NADPH oxidase may be important in mediating hyperglycemic pathology. It has been suggested that high glucose-induced production of ROS inhibits glycolysis, thereby exacerbating the exposure of high glucose concentrations and increasing flux through the HBP (9; 10). In addition to decreased GAPDH expression, which results in inhibition of glycolysis, altered GFAT expression contributes to hyperglycemia-induced flux through the HBP. In Müller cells exposed to hyperglycemic conditions, GFAT expression increased, indicating that GFAT expression plays a role in high glucose-mediated HBP activation. Altered GFAT expression could account for differential response of cells and animals to acute versus chronic hyperglycemic conditions. Additionally, expression of GFAT has been shown to be affected by the production of ROS. Thus induction of GFAT expression may be the mechanism by which ROS affects 4E-BP1 expression. Cycling GFAT expression in response to spikes in blood-glucose may also explain the transient changes in 4E-BP1 and VEGF expression observed in vivo. Further studies are required to test this hypothesis.

In summary, the data are consistent with the model that hyperglycemia-induced O-GlcNAcylation of 4E-BP1 interferes with its phosphorylation and subsequent degradation, leading to the accumulation of 4E-BP1. 4E-BP1 sequesters eIF4E, preventing formation of the eIF4F complex, thereby inhibiting cap-dependent
mRNA translation. Upregulation of 4E-BP1 expression leads to enhanced expression of VEGF, possibly through enhanced translational efficiency or protein stabilization.
APPENDIX A

ADDITIONAL STUDIES IN VIVO
A.1 Supplemental data for Chapter III

A.1.1 Absence of changes in FoxO1 signaling in the retina of diabetic rats

Alterations in FoxO1 expression and signaling have been noted in skeletal muscle (324), liver (325), and β-cells (326; 327) of diabetic rats. Additionally, increased DNA binding and translocation to the nucleus was observed in the retinas of both STZ-induced diabetic and Zucker diabetic fatty rats (328). As FoxO1 is insulin-responsive and has been identified as a transcription factor for 4E-BP1, FoxO1 expression, localization, and target induction was investigated. There did not appear to be a change in FoxO1 expression, or in FoxO1 phosphorylation on S256, an Akt-mediated phosphorylation site (Figure 24A). To further assess FoxO1 activation, mRNA expression of the FoxO1 targets Ang2, Gadd45, and p300 were measured. At 4 and 6 weeks, expression of these mRNAs was similar between control and diabetic rats (Figure 24B). Additionally, if any change was present, a decrease in the intensity of FoxO1 was observed by immunohistochemistry (Figure 24C).
A.1.2 Absence of changes in eIF4E phosphorylation status in the retina of diabetic rats

Phosphorylation of eIF4E is thought to reduce the affinity for the 5'-m\(^7\)GTP cap (329-331), which may inhibit the rate of cap-dependent mRNA translation. Therefore, eIF4E expression and phosphorylation were assessed by Western blot analysis. There was no observable change in the expression of eIF4E or in the phosphorylation of eIF4E on S209 (Figure 25). Activation of the ERK signaling pathway and the eIF4E kinase Mnk1/2 was also evaluated. ERK, p38, and Mnk1/2 expression was similar between control and diabetic rats (Figure 25).

A.1.3 Absence of changes in eIF2α phosphorylation or mTORC1 signaling in the retina of diabetic rats

Stress-induced VEGF translation is thought to be mediated by increased utilization of the VEGF IRESs (182). Phosphorylation of eIF2α on S51, which globally represses protein synthesis, correlates with the increased utilization of some viral IRESs (188-190); therefore phosphorylation on this site was measured in the retinas of control and diabetic rats. There was no detectable difference in either expression of eIF2α or phosphorylation of eIF2α on S51 (Figure 26A), suggesting that eIF2α phosphorylation is not the mechanism of VEGF upregulation in this model. There was also no change in the relative expression of the catalytic subunit, ε, of eIF2B.

Alternatively, increased expression of the RNA-dependent helicase eIF4A can
increase the translation of complex mRNAs with highly stable secondary structure. However, there did not appear to be a change in the expression of eIF4A between control and diabetic rats (Figure 26A).

Alterations in the expression or activity of mTORC1 may account for changes in the expression and phosphorylation of downstream targets, such as 4E-BP1. mTOR expression and phosphorylation on S2448 and S2481 were assessed. Expression and phosphorylation of mTOR was constant between the retinas of control and diabetic rats (Figure 26B). Additionally, there was no detectable difference in phosphorylation of S6K1 (Figure 26B). AMPK activation can increase repression of mTORC1 through the upstream inhibitors TSC1/2. However, there was no change in the expression or phosphorylation of AMPK on T172 (Figure 26B). These results imply that altered expression of mTOR or altered activity of mTORC1 toward its downstream targets cannot account for the increased expression, or decreased relative phosphorylation of 4E-BP1.

A.1.4 Absence of changes in insulin signaling in the retina of diabetic rodents

Altered insulin signaling, which has been reported in many models of diabetes, can result in changes in 4E-BP1 transcription and post-translational modification. Akt expression and phosphorylation on S473 and T308 were assessed. There was no apparent change in Akt expression or phosphorylation on these sites (Figure 27). Additionally, there was no observable alteration in the expression of the downstream targets AS-160 or TRB-3 (Figure 27). IRS expression decreases in
several *in vivo* models of diabetes. However, there was no change in the expression or phosphorylation of IRS-1 in this study (Figure 27). While it is possible that Akt activity toward specific substrates may be altered, it is unlikely that 4E-BP1 expression is altered through this pathway.

**A.2. Additional *in vivo* studies**

**A.2.1 High fat diet feeding reduced VEGF expression in Ins2^Akita^ diabetic mice**

High fat diet feeding has been shown to induce insulin resistance and decrease glucose utilization. To assess the effect of administering a high fat diet to Ins2^Akita^ diabetic mice on VEGF and 4E-BP1 expression, 4 week-old WT and Ins2^Akita^ mice were fed a high fat content diet for 4 weeks. While there was no change in the expression of VEGF between chow fed and high fat diet fed wildtype mice, there was a significant decrease in VEGF expression in the high fat diet fed Ins2^Akita^ mice as compared to their chow-fed littermates (Figure 28). Additionally, there was a trend toward a decrease in the expression of VEGF between chow-fed WT and chow-fed Ins2^Akita^ mice (Figure 28). These results may suggest interplay between diabetes and nutrient excess in regulating expression of VEGF. Further investigation is needed to understand how high fat diet contributes to decreased VEGF expression.
A.2.2 Alloxan-induced diabetic rats

A.2.2.1 VEGF protein expression is increased in the retina of alloxan-induced diabetic rats

Treatment with alloxan, a toxic glucose analog that is taken up through GLUT-2 transporters and destroys β-cells in the pancreas, results in insulin-dependent diabetes in rodents (332; 333). This is a 3-day model of severe hyperglycemia; in the absence of exogenous insulin administration, these animals die in a relatively short time period. Expression of 4E-BP1 in the retina increased significantly 3 days after alloxan treatment (Figure 29A). Additionally, VEGF protein expression was increased (Figure 29B). These results are similar to those observed in the STZ-induced model of diabetes.

A.2.2.2 VEGF protein expression decreases in the liver upon re-feeding of alloxan-induced diabetic rats

4E-BP1 expression was assessed in the livers of fasted or refed alloxan-induced diabetic rats. Interestingly, 4E-BP1 expression increased during fasting but was comparable to control levels during feeding (Figure 20). These results may suggest that 4E-BP1 expression cycles in response to nutrient signaling. It is possible that mTORC1 expression or activity may change in this model of diabetes, despite a lack of change in the STZ-induced diabetes model. Alternatively, 4E-BP1 mRNA may be
influenced in this model by nutrient availability. Further studies are required to
delineate the mechanism by which feeding and fasting affects 4E-BP1 expression in
diabetic rats.

Altered VEGF expression in response to feeding and fasting presents an interesting
observation. In an alloxan model of diabetes VEGF expression was only increased in
the liver in fasted, and in the retina in freely fed animals, however, animals that had
been fasted then re-fed had a repression of VEGF induction. A similar result was
obtained in a high fat feeding study in 8-week-old Ins2\textsuperscript{Akita} mice. While an increase
in VEGF expression was not detected in the diabetic Ins2\textsuperscript{Akita} mice, it had not been
expected, as there was no change detected at that time in the previous studies.
However, there was a significant decrease in VEGF expression in the Ins2\textsuperscript{Akita} mice
on the high fat diet when compared to their chow-fed littermates. This phenomenon
was not observed between the WT groups, which suggests a specific interaction
between the nutritive state of the animal and the presence of diabetes.
A.3 Figures and legends

Figure 24: Expression and relative activity of FoxO1 toward its substrates are unaffected in the retina of STZ-induced diabetic rats.

(A) Western blot analysis of FoxO1 expression and phosphorylation. 60µg of protein were loaded per lane. Blots are representative of at least two independent sets of animals. (B) Quantitative RT-PCR of FoxO1 targets. Retinal RNA was extracted following a standard TriZol protocol from 6 control and 9 diabetic rats at 4 weeks, 3 control and 6 diabetic rats at 6 weeks, and 10 control and 10 diabetic rats at 12 weeks. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; ang2 (white bars), Gadd45 (hatched bars), p300 (black bars).
Figure 25: Absence of changes in eIF4G, eIF4E, Mnk, p38, and ERK protein expression and phosphorylation status.

Western blot analysis of retinal homogenates. 60μg of protein were loaded per lane. Blots are representative of at least three independent sets of animals.
Figure 26: Absence of changes in eIF2α, eIF2Bε, eIF4A, and the mTORC1 signaling pathway.

(A-B) Western blot analysis of retinal homogenates. 60µg of protein were loaded per lane. Blots are representative of at least three independent sets of animals.
Figure 27: Absence of changes in expression and phosphorylation status of proteins in the insulin signaling pathway.
Western blot analysis of retinal homogenates. 60µg of protein were loaded per lane. Blots are representative of at least three independent sets of animals.
Figure 28: High fat diet reduces VEGF protein expression in Ins2Akita diabetic mice.

Western blot analysis of retinal homogenates. Retinas were harvested from 11 WT chow-fed, 10 WT HFD-fed, 11 Ins2Akita chow-fed, and 10 Ins2Akita HFD-fed mice, 10-weeks of age. 60µg of protein were loaded per lane. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; control (white bars), diabetic (gray bars), high fat diet (hatched bars). Representative blots are shown as an inset. * p<0.05.
Figure 29: 4E-BP1 and VEGF protein expression is upregulated in the retina of alloxan-induced diabetic rats.

(A) Western blot analysis of 4E-BP1 expression. Retinas were harvested from 6 control, and 6 alloxan-induced diabetic rats. Lysates were subjected to Lambda phosphatase treatment and 60µg of protein were loaded per lane. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; control (white bar), diabetic (black bar).  

(B) Western blot analysis of VEGF expression. 60µg of protein were loaded per lane. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; control (white bar), diabetic (black bars). Representative blots are shown as an inset. * p<0.05.
Figure 30: VEGF expression decreases in the liver of fasted then re-fed alloxan-induced diabetic rats.

Western analysis of VEGF expression. Retinas were harvested from rats 3 days after alloxan treatment. 10µg of protein were loaded per lane. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM; fasted (white bars), refed (black bars), diabetic (hatched bars). Each group contained an n=3. Representative blots are shown as an inset. * p<0.05.
APPENDIX B

ADDITIONAL STUDIES IN VITRO
B.1 Supplemental Data for Chapter IV

B.1.1 Inhibition of the proteasome increases 4E-BP1 expression and VEGF secretion

Recent studies have demonstrated that 4E-BP1 is regulated by ubiquitination (205; 252; 280; 316). Inhibition of the 26S proteasome with the chemical MG-132 results in an accumulation of 4E-BP1 protein in Jurkat cells (252). Phosphorylation on T37/46 may serve as a priming event, as 4E-BP1 was simultaneously phosphorylated and ubiquitinated, and treatment with MG-132 increases unmodified, but not higher molecular weight phosphorylated forms of 4E-BP1 (252). In Müller cells also, 6hrs of exposure to MG-132 results in significant accumulation of 4E-BP1 (Figure 31A). Importantly, MG-132 treatment also led to an increase in VEGF secretion into the medium (Figure 31B), suggesting that 4E-BP1 stabilization can induce VEGF production and secretion from Müller cells.

B.1.2 Absence of changes in proteins involved in translational regulation and insulin signaling in response to high glucose treatment

In order to understand the mechanism of high glucose-induced expression of 4E-BP1, the expression of proteins involved in translational regulation and insulin signaling were measured after 10hrs of high glucose treatment. There were no
changes observed in the phosphorylation statuses of eIF4G, eIF4E, and eIF2α, nor was there a change in the phosphorylation statuses of S6 or S6K1 (Figure 32A). Additionally, there did not appear to be a change in the insulin signaling mediators, Akt, IRS-1, or IRS-2, in expression or phosphorylation status. Expression of 14-3-3, a protein involved in FoxO1 shuttling, and a target of Akt, was similar between control and high glucose-treated cells (Figure 32B). PKC, which has been shown to be activated in diabetes, was not altered (Figure 32B). Finally, expression of PP2A, a phosphatase for 4E-BP1 was measured. However, there was no difference in the expression of this protein between control and high glucose-treated cells (Figure 32B), suggesting that increased dephosphorylation of 4E-BP1 by PP2A could not account for the relative decrease in phosphorylation. Nor could altered insulin signaling account for altered 4E-BP1 or VEGF expression during high glucose treatment.

B.1.3 Expression of 4E-BP1 and VEGF is upregulated after high glucose treatment in HepG2 liver cells.

To recapitulate upregulation of 4E-BP1 and VEGF expression under hyperglycemic conditions in an independent cell line outside of the retina, HepG2 liver cells were utilized for exposure to high glucose. 4E-BP1 and VEGF expression were increased during high glucose treatment in HepG2 liver cells (Figure 33A-B), to a similar extent to that observed in the Müller cells. The results confirm that the response of
these proteins to hyperglycemic conditions represents a global response rather than pathology specific to the retina.
B.2 Figures and legends

Figure 31: Inhibition of the proteasome, by treatment with MG-132, results in an increase in 4E-BP1 protein expression and VEGF secretion.
(A) Western blot analysis of 4E-BP1 expression. TR-MUL cells were exposed to DMSO (white bar) or MG-132 (black bar) in low glucose for 6hrs. Lysates were subjected to Lambda phosphatase treatment and 30µg protein were loaded per lane. Blots are representative of at least 3 independent experiments. Values were normalized to β-actin control and expressed as a percentage of the control mean ± SEM. 
(B) Analysis of VEGF secretion. Medium was collected from TR-MUL cells exposed to DMSO (white bar) or MG-132 (black bar) in low glucose for 6hrs and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Values represent the mean expressed as a percentage of the control ± SEM. *p<0.05.
Figure 32: Absence of changes in proteins involved in translational regulation and insulin signaling in response to high glucose treatment. (A-B) Western blot analysis of cell lysates. 30µg of protein were loaded per lane. Blots are representative of at least 3 independent experiments.
Figure 3: Expression of 4E-BP1 and VEGF is upregulated after high glucose treatment in HepG2 liver cells.

(A) Western blot analysis of 4E-BP1 expression. 30µg protein were loaded per lane. Blots shown as an inset are representative of at least 3 independent experiments.

(B) Analysis of VEGF secretion. Medium was collected from TR-MUL cells exposed to low (white bar) or high glucose (black bar) and subjected to VEGFA ELISA. VEGF concentration was normalized to protein content in the cell lysates. Data are representative of at least 2 independent experiments of 3 replicates. Values represent the mean expressed as a percentage of the control ± SEM. * p<0.01, ** p<0.001.
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Presentations/Abstracts:
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