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# Retinal Protein *O*-GlcNAcylation and the Ocular Renin-Angiotensin System: Signaling Cross-Roads in Diabetic Retinopathy

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## Abstract

It is well established that diabetes and its associated hyperglycemia negatively impact retinal function, yet we know little about the role played by augmented flux through the hexosamine biosynthetic pathway (HBP). This offshoot of the glycolytic pathway produces UDP-*N*-acetyl-glucosamine, which serves as the substrate for post-translational *O*-linked modification of proteins in a process referred to as *O*-GlcNAcylation. HBP flux and subsequent protein *O*-GlcNAcylation serve as nutrient sensors, enabling cells to integrate metabolic information to appropriately modulate fundamental cellular processes including gene expression. Here we summarize the impact of diabetes on retinal physiology, highlighting recent studies that explore the role of *O*-GlcNAcylation-induced variation in mRNA translation in retinal dysfunction and the pathogenesis of diabetic retinopathy (DR). Augmented O-GlcNAcylation results in wide variation in the selection of mRNAs for translation, in part, due to *O*-GlcNAcylation of the translational repressor 4E-BP1. Recent studies demonstrate that 4E-BP1 plays a critical role in regulating *O*-GlcNAcylation-induced changes in the translation of the mRNAs encoding vascular endothelial growth factor (VEGF), a number of important mitochondrial proteins, and CD40, a key costimulatory molecule involved in diabetes-induced retinal inflammation. Remarkably, 4E-BP1/2 ablation delays the onset of diabetes-induced visual dysfunction in mice. Thus, pharmacological interventions to prevent the impact of *O*-GlcNAcylation on 4E-BP1 may represent promising therapeutics to address the development and progression of DR. In this regard, we discuss the potential interplay between retinal O-GlcNAcylation and the ocular renin-angiotensin system as a potential therapeutic target of future interventions.

### Keywords

diabetes; retinopathy; O-GlcNAcylation; mRNA translation; renin-angiotensin system; oxidative stress; inflammation

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CONFLICT OF INTEREST

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# 1. DIABETIC RETINOPATHY

## 1.1. Introduction to Diabetes-induced Retinal Dysfunction

Diabetic retinopathy (DR) is the most frequent cause of new cases of blindness among adults aged 20–74 in developed countries. DR is a neurovascular complication that is observed in individuals with type 1 or type 2 diabetes. Almost everyone with type 1 diabetes, and most people with type 2 diabetes, develop some form of visual deficit within two decades of disease onset [1]. Furthermore, studies by multiple investigators have reported retinal defects [2, 3] and impaired visual function [4, 5] in pre-diabetic patients, as well as individuals with relatively normal glucose tolerances [6–8]. Despite decades of study, there remains a critical gap in our understanding of the initiating molecular events that lead to loss of retinal homeostasis and visual dysfunction in diabetes and prediabetes. As a result, there is a lack of effective therapeutic interventions to prevent the functional decline in vision that is observed in most diabetic individuals. Herein we review the impact of diabetes on retinal physiology, highlighting the role of *O*-GlcNAcylation-induced variation in mRNA translation in retinal dysfunction and the pathogenesis of DR. We also discuss the potential interplay between retinal O-GlcNAcylation and the ocular renin-angiotensin system as it was recently demonstrated that inhibition of the angiotensin-converting enzyme suppresses retinal protein *O*-GlcNAcylation *via* a signaling axis that is initiated by Ang1–7 and the Mas receptor.

## 1.2. Retinal Anatomy & Physiology

Located in the posterior segment of the eye, the retina is responsible for converting light into an electrochemical signal to relay an image to the brain. The retina contains ~50 distinct cell types arranged into three layers of nerve cell bodies interspersed by two plexiform layers of synapses [9]. In the classical model of phototransduction, photons of light are absorbed by photosensitive pigments in the outer segments of the rods and cones (Fig. 1). This leads to hyperpolarization of photoreceptors, and in turn, electrochemical stimulation of second-order neurons, including primarily bipolar and horizontal cells whose cell bodies reside in the inner nuclear layer (INL) [10, 11]. The signal is subsequently relayed to amacrine and retinal ganglion cells (RGCs) *via* either excitatory or inhibitory connections [12]. Axons of RGCs form the nerve fiber layer (NFL) that enters the optic nerve and ultimately synapses with the brain [13].

The retina is among the highest energy-consuming tissues, with sensory neurons depending almost entirely on oxidative metabolism [14]. Situated just outside the neurosensory layers, a retinal pigmented epithelium (RPE) monolayer and underlying vasculature known as the choroid provides oxygen and nutrients to the outer retina. Three layers of capillary networks are located in the NFL (superficial vascular plexus) and along both the inner and outer border of the INL (intermediate and deep capillary plexus, respectively). Stellate-shaped astrocytes are mostly confined to the NFL; however, the presence and distribution of astrocytes are also correlated with the distribution of retinal blood vessels [15, 16]. This correlation reflects the close association astrocytes have with endothelial cells of the retinal vasculature. Astrocytes exert supportive functions for the retinal vasculature:

ensheathing blood vessels, producing trophic factors (including vascular endothelial growth factor (VEGF)), and maintaining the integrity of the blood retinal barrier (BR-B) [17, 18]. As an immune-privileged site, the retina relies on resident microglia to respond to inflammatory insults. Microglia represent the retinal macrophage population; they undergo rapid morphological and functional transformation into phagocytes upon activation by a stimulus. Additional roles for retinal microglial homeostasis include control of neuronal apoptosis, the guidance of retinal vasculature growth, and aid in forming neuronal connections [19–21].

The most common glial cell type in the retina are Müller cells, which are situated transversally to all nuclear and plexiform layers of the retina. Longitudinal cytoplasmic projections of Müller cells contact retinal blood vessels, neurons, and other glial cells. Müller cells play a critical role in supporting and maintaining homeostasis of the entire retina. The function of Müller cells can generally be grouped into three categories: *1*) metabolic support and nutrient synthesis, *2*) handling of neurotransmitters and ions, and *3*) maintenance of the BRB [22, 23]. Structurally, Müller cells help to minimize intraretinal light scattering by providing light a direct path to focus onto photoreceptors, thereby improving visual acuity [24]. Given the intimate associations formed with other retinal cell types and their geographic domination, Müller cells are critical for maintaining healthy retinal function and homeostasis.

### 1.3. Clinical Manifestations of DR

The intimate associations formed between neuronal, endothelial, and glial cells of the retina support a model wherein different retinal cell types cooperate to form a properly functioning neurovascular unit [25–27]. It has long been accepted that DR adversely impacts the retinal microvasculature. More recently, the concept has evolved to suggest a more accurate representation of diabetic pathophysiology in the retina that encompasses defects in the retinal neurovascular unit [28, 29]. DR manifests clinically in the form of retinal hemorrhaging, neovascularization, leakage of fluid into the retina, and formation of cotton wool spots and hard exudates. However, loss of neurovascular coupling, gliosis, neuroinflammation, and neurodegeneration, can precede and even predict visible signs of microvascular disease in diabetic patients [30, 31].

It is estimated that 42% of patients with type 1 diabetes and 8% of patients with type 2 diabetes have a vision-threatening form of DR [32, 33]. While diabetic macular edema (DME) is most closely associated with visual acuity loss; impaired contrast sensitivity, visual field deficits, and diminished color vision can develop earlier in disease progression and cause significant difficulties, particularly in low light conditions [26]. Thus, it is critical to better understand the early molecular events that occur prior to the overt vascular manifestations of DR that are visible *via* fundus examination (Fig. 2). Within the first months of disease onset, adaptive biochemical changes in the retina include a reduction biosynthetic and electrical activity, likely to reset the normally high metabolic activity of the retina [26, 34]. Müller cells become reactive and exhibit morphological and functional alterations shortly after the development of diabetes, as they attempt to counter-regulate the changing metabolic environment [23]. Their activation in response to mounting deleterious

stimuli is referred to as gliosis. Microglial activation also contributes to the pathogenesis of retinal degenerative disease through the release of pro-inflammatory [35]. After prolonged hyperglycemia, adaptive changes begin to fail, and retinal neurons and pericytes are lost to apoptosis [36, 37]. Impairment of the retinal neurovasculature manifests clinically in the form of non-proliferative DR (NP-DR). Over time, the disintegration of the retinal neurovascular unit worsens, resulting in the most severe stage of the disease, proliferative DR (PDR), which is characterized by pre-retinal neovascularization [26]. Ultimately, vision loss occurs from either unrestrained proliferation of new blood vessels or from leakage of fluid through the retinal vasculature (*i.e.* DME) [38].

### 1.4. Current Interventions and the Molecular Pathogenesis of DR

Current therapeutic interventions for PDR or DME include laser-mediated ablation of the peripheral retina, surgical vitrectomy, or intraocular injections of steroids and VEGF inhibitors. Laser treatments and surgical approaches are accompanied by significant side effects (including diminished peripheral and night vision) and ultimately fail to alter the molecular events that cause the development of complications. More recently, VEGF inhibitors have emerged as the first clinical approach to address the molecular cause of DR. VEGF plays a central role in mediating the breakdown of the blood-retinal barrier, and examination of vitreous fluid from the eyes of patients with PDR demonstrates an increase in VEGF [39, 40]. Anti-VEGF therapies have dramatically improved treatment outcomes for DR; however, they reduce the severity of retinopathy in less than one-third of eyes treated for two years [41]. This limitation is likely due to the relatively poor correlation between VEGF levels and the extent of retinal edema [42], implying that additional factors are likely to play a role in disease progression. Indeed, the molecular pathophysiology of DR likely encompasses a complex interaction that includes pro-angiogenic signaling pathways, oxidative stress, inflammation, and endoplasmic reticulum (ER) stress [43–45].

A major underlying factor in the development and progression of DR is hyperglycemia [46, 47]. The Diabetes Control and Complications Trial (DCCT) for type 1 diabetes and the United Kingdom Prospective Diabetes Trial (UKPDS) for type 2 diabetes demonstrated that intensive glycemic control delays both the onset and progression of DR [48, 49]. An instigating factor of hyperglycemia-induced diabetic complications is hypothesized to be the overproduction of superoxide, a type of reactive oxygen species (ROS) produced by the electron transport chain of the mitochondria [50]. Although hyperglycemia systemically affects the body, differential effects may be seen in specific cell types such as retinal endothelium or mesangial cells of the kidney because they fail to restrict glucose transport into the cell in a hyperglycemic environment [51, 52]. Consequently, the large influx and subsequent oxidation of glucose increase the voltage gradient across the electron transport chain. Eventually, oxidative phosphorylation activity reaches a threshold, and the electron transport chain becomes bottlenecked, favoring the formation of superoxide [47, 53]. Downstream effects of ROS include activation of Protein Kinase C, which has several purported mechanisms of influencing blood flow in the retina, including elevated expression of VEGF [50, 54]. Additionally, downstream metabolites formed consequentially of ROS can feed into the polyol pathway which oxidizes glucose into sorbitol (consuming NADH and contributing to ROS susceptibility in the process) [55].

Finally, aberrant ROS production resulting from hyperglycemia can increase flux through the hexosamine biosynthetic pathway (HBP). The HBP is responsible for the production of uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), which serves as the substrate for the enzymatic glycosylation of the hydroxyl side chains of serine and threonine residues of proteins known as *O*-GlcNAcylation [56].

## 2. PROTEIN *O*-GLCNACYLATION

### 2.1. *O*-GlcNAcylation as a Nutrient Sensor

First described in 1984, *O*-GlcNAcylation is one of the most abundant post-translational modifications within the nucleocytoplasmic compartment [57]. *O*-GlcNAcylation is more reminiscent of protein phosphorylation than other classical forms of glycosylation, as it is a single monosaccharide (*N-acetyl* glucosamine) that is not modified by elongation or branching. Rather, *O*-GlcNAc modifications are dynamically (and reversibly) cycled in response to environmental stimuli by two proteins: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). OGT catalyzes the addition of the *O*-GlcNAc moiety while the hydrolase OGA catalyzes its removal. Traditional *N*-linked glycosylation occurs in the lumen of the ER and Golgi body [58]. However, proteins may be *O*-GlcNAcylated in either the cytoplasm or nucleus. *O*-GlcNAc modifications are widely abundant (~1,500 unique proteins [59]) and critical for fundamental cellular processes including gene expression, cell cycle control, apoptosis, inflammation, and nutrient sensing [60].

Global *O*-GlcNAcylation levels are highly sensitive to nutrient status within the cell, as the HBP requires from nearly every major metabolic pathway-glucose, amino acid, nucleotide, and fatty acid (Fig. 3). Typically, the HBP is a minor metabolic pathway, accounting for only 2–5% of glucose metabolism [61]. However, an overabundance of nutrients, as seen with diabetes and over-nutrition, can drastically augment HBP flux leading to enhanced protein *O*-GlcNAcylation. The HBP ultimately metabolizes the glycolytic intermediate fructose-6-phosphate (F-6-P) into the *O*-GlcNAc donor UDP-GlcNAc. Glutamine:fructose-6-phosphate aminotransferase (GFAT) catalyzes the first and rate-determining step in this pathway, which involves the irreversible transfer of the amino group from glutamine and the isomerization of F-6-P to produce glucosamine-6-phosphate. Variation in GFAT isoform expression and activity are critical determinants of protein *O*-GlcNAcylation in a given cell. Two GFAT isoforms are differentially expressed across tissues and unlike GFAT1, GFAT2 is insensitive to feedback inhibition from UDP-GlcNAc [62]. Thus, tissues with a predominance of GFAT2 are more likely to overcommit glucose to the HBP. Consistent with studies demonstrating that GFAT2 is the predominant isoform in the central nervous system [63], GFAT2 is likely the primary isoform responsible for committing F-6-P to the HBP in the retina. Notably, isoform-specific expression of the mRNA encoding GFAT2 is dramatically increased in the retina of mice fed a high-fat diet as compared to control chow [64].

### 2.2. *O*-GlcNAcylation-induced Abnormalities in DR

Several studies have confirmed *O*-GlcNAcylation is abnormally elevated in a variety of tissues in both diabetic humans and animals [61, 65–67]. Retinal protein *O*-GlcNAcylation is increased in both streptozotocin (STZ)-induced diabetic mice [68] and mice fed an

HFD [64]. These results are consistent with other reports indicatingthat retinal protein *O*-GlcNAcylation is elevated in 6-week-old diabetic Akita mice [69] and in the *db/db* mouse model of type 2 diabetes, in which *O*-GlcNAcylated retinal proteins localize to the ganglion cell layer, retinal pigmented epithelium, and inner plexiform layers [70].

Abnormalities in *O*-GlcNAc cycling are associated with glucose toxicity in diabetic individuals [71]. Additionally, the global elevation of *O*-GlcNAcylation, induced by inhibiting OGA can cause insulin resistance, even in the absence of hyperglycemia [72]. In addition to poor glycemic control, impaired insulin signaling is a primary agent responsible for the development of DR [34, 46, 73, 74]. OGT binds the insulin receptor substrate 1 (IRS1), where it catalyzes the addition of *O*-GlcNAc modifications on downstream effectors of the insulin signaling pathway. Direct *O*-GlcNAcylation of IRS1 attenuates the pro-survival effects of insulin thereby dampening insulin-stimulated Akt activation [72, 75–78]. Furthermore, enhancing intracellular *O*-GlcNAcylation levels *via* glucosamine (a metabolite that enters the HBP downstream of GFAT (Fig. 3) impairs activation of critical pro-survival proteins downstream of IRS1, including phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K), Akt, glycogen synthase kinase 3 (GSK3), and the transcription factor forkhead box protein O1a (FOXO1a) [79]. Systemic insulin administration reduces the rate of apoptosis in retinas of diabetic mice [36]. Furthermore, either systemic or local delivery of insulin prevents loss of retinal insulin receptor kinase activity [34]. However, exposure to high glucose cell culture medium and glucosamine attenuates the neuroprotective action of insulin in R28 cells, a model of retinal neurons [80]. Therefore, it is unclear if *in vivo* insulin administration and subsequent improvements in glucose homeostasis are sufficient to overcome hyperglycemia-induced retinal protein *O*-GlcNAcylation.

Aside from the neuroretina, enhanced protein *O*-GlcNAcylation also causes deleterious consequences for the retinal endothelial, epithelial, and glial cells. Abnormal O-GlcNAc cycling can induce early cell death of retinal pericytes, one of the earliest signs of DR [81]. Protein *O*-GlcNAcylation is elevated in both retinal endothelial cells and pericytes in Akita mice and in a model of oxygen-induced ischemic retinopathy during the neovascularization phase [69]. In cells exposed to the hyperglycemic culture medium, protein *O*-GlcNAcylation is higher in retinal pericytes as compared to retinal endothelial cells or astrocytes [81]. Hyperglycemic cell culture conditions also induce proliferation and migration of retinal endothelial cells with minimal apoptotic effects [81, 82]. Furthermore, enhanced protein *O*-GlcNAcylation in retinal endothelial cells contributes to angiogenic factor release and enhanced proliferation and migration [69, 70]. Elevation of intracellular *O*-GlcNAc is sufficient to upregulate VEGF in multiple retinal epithelial cell lines [83]. In an astrocyte cell line, *O*-GlcNAc modification of the cell cycle regulator p27 inhibits astrocyte migration while glucosamine-induced flux through the HBP causes acute activation of Akt and consequently increased ER stress [84, 85].

Retinal Müller cells are a particularly susceptible target of the glucotoxic effects of hyperglycemia. Müller cells are the only cell type in the retina to express GLUT2 transporters, which possess a high Michaelis constant ($K_m$ ~17 mM [86];) and a high maximum velocity for transporting glucose [87]. By contrast, GLUT1, 3, and 4 exhibit a much higher affinity for glucose ($K_m$ ~5 mM), and therefore saturated transport at much

lower blood glucose concentrations. Moreover, MIO-M1 Müller cells exhibit predominant expression of the GFAT2 isoform [64]. Thus, Muller glia exposed to hyperglycemic conditions have a particularly high capacity for shifting glucose molecules into the HBP. Recent evidence suggests that Müller cells are also particularly sensitive to dyslipidemia associated with type 2 diabetes [88, 89]. GFAT2 expression and *O*-GlcNAcylation levels are elevated in Müller cells exposed to the saturated fatty acid palmitate or the ceramide analog Cer6 [64]. Such findings are consistent with the nutrient-sensing role of the HBP that requires input of the fatty acid metabolite acetyl-CoA for UDP-GlcNAc synthesis (Fig. 3).

### 2.3. *O*-GlcNAcylation and Transcription

Protein *O*-GlcNAcylation plays a key role in nutrient regulation of gene expression. Many early studies investigating protein *O*-GlcNAcylation support a model wherein the *O*-GlcNAc modification alters the transcriptional activity, localization, and binding partners of multiple transcription factors [90–93]. Nearly all RNA polymerase II transcription factors are *O*-GlcNAc modified, and assembly of the pre-initiation complex necessary for transcription initiation requires *O*-GlcNAcylation of the C-terminal domain of RNA polymerase II [94–97]. OGA itself localizes to transcription start sites to catalyze the removal of the *O*-GlcNAc moiety from the C-terminal domain of RNA polymerase II in order for transcription elongation to proceed [98]. *O*-GlcNAc also plays an epigenetic role as it regulates both ubiquitination and methylation of histones [99–101]. A detailed overview of *O*-GlcNAc-mediated regulation of gene expression at the level of transcription has been reviewed [95].

Several transcription factors that play a role in the pathogenesis of DR are subject to *O*-GlcNAc modification. Specificity protein 1 (Sp1) is a transcription factor that facilitates upregulation of the VEGF gene in response to hyperglycemia. In retinal endothelial and pigmented epithelial cells in culture, *O*-GlcNAcylation of Sp1 resulted in upregulation of VEGF mRNA and protein [83]. Notably, depletion of cellular OGT prevents glucose-induced changes in levels of VEGF [83]. Similarly, *O*-GlcNAcylated Sp1 also mediates hyperglycemia-induced intracellular cell adhesion molecule 1 (ICAM-1), an important inflammatory molecule expressed in retinal endothelial cells [102]. NF-κB is a pleiotropic transcription factor with multiple roles in development, apoptosis, and inflammation [103]. Activation of NF-κB is associated with experimental and clinical diabetes [104]. After two months of streptozotocin-induced diabetes, *O*-GlcNAcylated NF-κB is enhanced in retinas from diabetic mice as compared to non-diabetic controls [68]. In fact, the level of *O*-GlcNAcylated NF-κB is elevated in apoptotic RGCs of diabetic mice [68]. These results indicate that diabetes-induced *O*-GlcNAcylation of NF-κB induces the activation of the transcription factor and contributes to RGC cell death that is associated with diabetes. Another important mediator of cell death is pro-apoptotic transcription factor p53. Primary cultures of retinal pericytes exposed to hyperglycemic culture medium display elevated levels of *O*-GlcNAc modified and total p53 [81]. Pharmacological inhibition of both GFAT and OGT attenuates the hyperglycemia-induced increase in total p53. Given these results, it is plausible that *O*-GlcNAcylation enhances the stability of p53, possibly by interfering with its proteasome-mediated degradation.

While there are several descriptive studies interrogating the role of *O*-GlcNAc in transcription, functional significance of *O*-GlcNAc modification in retinal gene transcription in the diabetic milieu remains to be fully elucidated. The limited studies mentioned herein suggest that *O*-GlcNAcylation of certain transcription factors affects their transcriptional activity. Further detailed studies identifying a potential molecular mechanism by which this regulation happens are needed to develop a more robust understanding of how nutrient-sensing *via* HBP flux and subsequent protein *O*-GlcNAcylation control gene expression at the level of transcription.

### 2.4. *O*-GlcNAcylation and mRNA Translation

Gene expression is ultimately the sum of events, including transcription, translation, and mRNA and protein degradation, that regulate cellular protein content. Translational control is a particularly important step in gene expression for short-term adaptation to changing metabolic conditions [105] and is thus likely to be a fundamental mechanism used in the early adaptive response of retinal cells to diabetes. In fact, mRNAs containing sequence elements associated with translational control are most enriched in the gene categories of synaptic transmission, angiogenesis, and cell adhesion [106], which are all key processes disrupted in the retina by diabetes. Mounting evidence suggests that the augmented protein O-GlcNAcylation level acts as a molecular switch that causes a fundamental shift in gene expression. In fact, many proteins are O-GlcNAcylated co-translationally in a manner that prevents their proteasomal degradation [107]. In retinal pericytes exposed to hyperglycemic culture conditions, *O*-GlcNAcylated proteins are most heavily represented in the functional category *Protein Synthesis and Processing* [81]. Nearly half of the ~80 ribosomal proteins are *O*-GlcNAcylated, and both OGT and OGA are tightly bound to cytosolic ribosomes [108]. In order to determine the extent to which *O*-GlcNAcylation affects retinal gene expression, next-generation sequencing was used to evaluate total mRNA abundances, and ribosome profiling was used to determine which mRNAs were actively being translated into protein. When protein O-GlcNAcylation levels are augmented, ~19% of the retinal transcriptome exhibits variation in ribosome density [109].

The rate at which a given mRNA is translated is limited by the recruitment of ribosomal subunits during the initiation phase (Fig. 4). The 5′-end of all eukaryotic mRNAs is capped by an $m^7$GTP structure. The cap-binding complex eIF4F (a heterotrimer composed of a helicase (eIF4A), a scaffolding protein (eIF4G), and a cap-binding protein (eIF4E)) assembles at the 5′-cap structure, and the eIF4F·mRNA complex recruits the small ribosomal subunit [110]. Interaction between initiation factors eIF4E and eIF4G is critical for cap-dependent translation initiation and is regulated by the eIF4E binding proteins (4E-BPs), which are direct targets of the master kinase mammalian target of rapamycin complex 1 (mTORC1). In addition, ~10% of mRNAs contain highly-structured nucleotide sequence elements, such as internal ribosomal entry sites (IRES), that enable ribosome recruitment independent of the 5′-cap, *i.e.* cap-independent translation initiation [111]. These sequences form complex structural elements that potentially hinder cap-dependent translation, due to the high unfolding energy required to facilitate 5′-UTR scanning. However, these same structural elements can facilitate translation initiation under adverse physiological conditions. Whereas eIF4E inhibits cap-independent translation by increasing competition

for initiation factors [112], sequestration of eIF4E by 4E-BP1 creates a cellular environment that potentially favors cap-independent translation. Importantly, eIF4E is the only canonical initiation factor differentially utilized in cap-dependent translation, but not cap-independent translation [113].

Diabetes is associated with both an increase in retinal 4E-BP1 protein *O*-GlcNAcylation [61, 65–67] and enhanced binding of 4E-BP1 to eIF4E [114]. Increasing *O*-GlcNAcylation levels by OGA inhibition promotes 4E-BP1 interaction with eIF4E, independent of a change in 4E-BP1 phosphorylation status [115]. In rodents, retinal 4E-BP1 protein expression is elevated shortly after the onset of diabetes-induced hyperglycemia or OGA inhibition [114, 116]. Pharmacological induction of glucosuria in diabetic mice to lower serum glucose concentrations reduces *O*-GlcNAcylation of 4E-BP1 in the retina and normalizes 4E-BP1 expression [116]. This hyperglycemia- or *O*-GlcNAc-mediated induction of 4E-BP1 expression is attributed to a reduced rate of degradation [116]. Unlike 4E-BP2, 4E-BP1 contains a PEST motif that is associated with targeting proteins for degradation through the ubiquitin-proteasome pathway [117]. Disrupting a key phosphorylation site at Thr-82 within the PEST motif of 4E-BP1 prevents polyubiquitination and degradation of the protein [116]. That same study demonstrated a critical role for 4E-BP1/2 in the pathology that leads to diabetes-induced vision loss. Remarkably, in the retina of mice deficient for 4E-BP1/2, diabetes-induced visual dysfunction is delayed [116]. This protective effect of 4E-BP1/2 ablation on visual function is likely mediated by preventing a diabetes-induced change in mRNA translation. Indeed, 4E-BP1 O-GlcNAcylation has been implicated in promoting the translation of mRNAs that encode key proteins involved in pro-angiogenic signaling, oxidative stress, and inflammation, which are all critical in the pathogenesis of DR.

As described above, the role of VEGF as a therapeutic target for DR is well established, however the specific mechanisms that upregulate VEGF protein levels in DR are less well explored. While VEGF expression is often thought of as being regulated transcriptionally (*i.e.* by hypoxia-inducible factor 1alpha), more recent studies indicate that it is also regulated at the level of mRNA translation [118–120]. The VEGF mRNA transcript has an exceptionally long G-C base pair rich 5′-untranslated region (UTR) that contains alternative start sites and stop codons in frame with the classical start site. Two independent IRES sequences within the UTR control start codon selection [121] and allow the VEGF mRNA to be translated independently of its 5′-cap [118]. A series of studies support that hyperglycemic conditions promote VEGF mRNA translation *via* 4E-BP1 action [114, 122, 123]. In both the retina of diabetic mice and in cells exposed to hyperglycemic culture conditions, 4E-BP1/2 is necessary for upregulated VEGF expression [114]. In cells exposed to culture media containing either increased glucose concentrations or glucosamine, the activity of the VEGF 5′-UTR is enhanced, and 4E-BP1/2 deletion is sufficient to prevent increased activity of the VEGF 5′-UTR upon exposure to hyperglycemic conditions [122, 123].

4E-BP-dependent translational control is also implicated in the development of diabetes-induced oxidative stress in the retina. Mitochondrial dysfunction and oxidative stress are critical factors in the development and progression DR. In the whole retina, translation of mRNAs encoding ~50 mitochondrial proteins is sensitive to OGA inhibition, and at least,

a subset of these changes (*e.g.* suppressed translation of SOD2) required 4E-BP1/2 [109]. *O*-GlcNAcylation alters the translation of mRNAs encoding mitochondrial proteins involved in oxidative phosphorylation, mitochondria-specific tRNA synthetases, and subunits of the translocase of the inner membrane (TIM)/translocase of outer membrane (TOM) complex. The functional outcome of BP1/2-dependent variation in mRNA translation is an increase in mitochondrial superoxide levels [109]. Whereas diabetic wild-type mice exhibit an increase in retinal ROS levels, 4E-BP1/2-deficient mice do not.

Bioinformatic analysis demonstrates that retinal mRNAs exhibiting altered translation in response to enhanced *O*-GlcNAcylation are most heavily represented in the “acute-phase response” pathway, which includes a range of pro-inflammatory cytokines, as well as the co-stimulatory molecule cluster of differentiation 40 (CD40) [109]. CD40 expression in retinal Müller glia is necessary to induce expression of other critical immunomodulatory molecules, including NOS2 (nitric oxide synthase 2) [124]. In a RiboTag mouse model [125] that expresses a hemagglutinin (HA)-tagged variant of ribosomal protein Rpl22 specifically in Müller cells, diabetes or administration of an OGA inhibitor promotes CD40 mRNA translation in Müller cells (Dierschke *et al*, 2020). In Müller cells in culture, expression of a 4E-BP1 variant that constitutively sequesters eIF4E is sufficient to promote cap-independent CD40 mRNA translation driven by the CD40 5′-UTR (Dierschke *et al*, 2020). *Cd40* mRNA translation, CD40 protein expression, and *Nos2* mRNA abundance are upregulated in the retina of wild-type diabetic mice, but not in the retina of diabetic 4E-BP1/2-deficient mice (Dierschke *et al*, 2020). Collectively, these findings support a model wherein *O*-GlcNAc-mediated sequestration of eIF4E by 4E-BP1 serves to promote retinal inflammation.

Other core translation initiation factors have also been reported as being *O*-GlcNAc modified. eIF2α is a major mediator of the unfolded protein response (UPR), a mechanism induced by ER stress that inhibits global translation initiation, with the exception of a few specific, stress-related mRNAs. *O*-GlcNAcylation of eIF2α inhibits its phosphorylation at a critical serine residue necessary to carry out the UPR, and thereby prevents ER stress-induced apoptosis [126]. *O*-GlcNAcylation of eIF4G enhances its stability [127]. In human HEK293T cells, *O*-GlcNAcylation of eIF4G promotes its interaction with poly(A)-binding protein (PABP) and poly(A) mRNA, which facilitates the interaction of the 5′-cap and 3′-end of mRNA to form a closed loop and stimulate translation by enabling terminating ribosomes to come into close proximity with the start codon [128, 129]. In MEF cells, the *O*-GlcNAc modification on eIF4G facilitates the preferential translation of stress mRNAs during the heat shock response, a signaling pathway that results in rapid induction of genes encoding molecular chaperones necessary for recovery from cellular damages [130]. Heat stress-induced eIF4G *O*-GlcNAcylation not only suppresses cap-dependent translation, but simultaneously enables eIF4G to preferentially bind specific mRNAs and initiate translation *via* a cap-independent mechanism [131]. Li and colleagues also demonstrated that the core translation factor eIF4A, an ATP-dependent RNA helicase, is also *O*-GlcNAc modified [128]. *O*-GlcNAcylation of eIF4A disrupts assembly of the eIF4F complex and inhibits the duplex unwinding activity of eIF4A, thereby resulting in an overall inhibition in protein synthesis and cellular proliferation [128].

Given the differential effects of *O*-GlcNAcylation on core translation initiation proteins, it is likely that the modification exists to fine-tune protein synthesis in response to various biological cues and fluctuations in nutrient status. Protein synthesis is a tightly regulated process that is fundamental to cell growth and proliferation. While many studies have begun to uncover the role the nutrient- and stress-responsive post-translational *O*-GlcNAc modification may play in mRNA translation, more studies are necessary to fully appreciate this mechanism whereby the cell integrates nutritional status with the fundamental cellular process of synthesizing proteins.

## 3. THE RENIN-ANGIOTENSIN SYSTEM (RAS)

### 3.1. A Role for the Ocular RAS in DR

The pathogenesis of DR encompasses dysregulation of a multitude of signaling pathways. However, recent evidence indicates dysregulation of the renin-angiotensin system (RAS) may play a prominent role in the disruption of the retinal neurovascular unit [132]. The RAS is classically defined by its systemic physiological functions, but it is becoming increasingly evident that local, tissue-specific RAS systems play an important role in disease pathologies, including DR. The RAS is one of the oldest studied hormone systems and is well known for its role in systemic vascular control and fluid homeostasis. The retina is one of several tissues that possess a local RAS, and it is well-established that all components of the RAS are expressed in the retina, including angiotensinogen, prorenin, renin, angiotensin I (Ang I), angiotensin II (Ang II), angiotensin-converting enzyme (ACE), Ang II type 1 receptor (AT1R), and Ang II type 2 receptor (AT2R) (reviewed in [133]). Secreted renin serves to cleave the pro-peptide angiotensinogen into Ang I (Fig. 5). ACE cleaves Ang I into the primary effector peptide of the RAS, Ang II. Ang II stimulates vasoconstriction, aldosterone synthesis and subsequent sodium resorption, fibrosis, and inflammation, mediated through binding to the AT1R G protein-coupled receptor [134]. The mechanisms induced by AT2R binding are less well understood; although, it is thought that activation of AT2R may mediate opposing actions of AT1R, such as stimulating vasodilation [135].

The role of Ang II in a healthy retina is likely related to the regulation of retinal blood vessel tonicity, glial function, and neuronal function [136]. In DR, Ang II formation is elevated, potentiates VEGF-mediated angiogenesis, and increases retinal blood vessel permeability (reviewed in [133]). Interest in further understanding the role of Ang II and other components of the RAS increased after clinical studies revealed that blocking either Ang II signaling with ARBs (Angiotensin Receptor Blockers) or preventing Ang II formation with ACE inhibitors (ACEi) reduced the risk for both incidence and progression of DR [137]. These intriguing clinical results prompted a question that would be best answered on a molecular level: how do ARBs and ACEi mediate beneficial effects in the retina?

The hypothesis that was subsequently developed to answer that question involved the action of the more recently discovered arm of the RAS that is mediated by the effector peptide angiotensin- [1–7] (Ang1–7). Ang1–7 is generated by the cleavage of Ang II by the enzyme ACE2 or by the cleavage of Ang I by various endopeptidases. Ang I may be alternatively degraded by ACE2 to generate angiotensin- [1–9], which in turn may be cleaved by neutral

endopeptidase or ACE to generate Ang1–7. Ang1–7 binds to the G protein-coupled receptor Mas to elicit systemic vasodilatory and antihypertensive effects. A handful of recent studies have provided evidence that some of the positive cardiometabolic effects observed in rodents administered RAS blockers are due to increased circulating levels of Ang1–7 [138–140]. In the case of ACEi, the basis for this rise in Ang1–7 is due to decreased degradation of Ang1–7 to angiotensin- [1–5] by ACE and increased Ang I resulting from a reduction in Ang II formation [141, 142]. Importantly, the positive effects of Ang1–7 thus far reported are independent of changes in blood pressure [143, 144].

Pharmacological blockade of the RAS also has beneficial effects on visual function. Administration of the ACEi perindopril in diabetic rodents improved both photoreceptoral and inner retinal function comparable to that of nondiabetic rodents [145]. ACEi also reduces VEGF expression and vascular permeability in the retinas of diabetic rodents [146, 147]. Systemic administration of the ACEi captopril enhances the expression of Ang1–7 in the retina with a concomitant decrease in diabetes-induced Ang II expression [148]. Intravitreal delivery of adenoviral encoded Ang1–7 also ameliorates hallmark characteristics of DR, including diabetes-induced retinal vascular permeability, the formation of acellular capillaries, infiltration of inflammatory cells, and oxidative damage in diabetic rodents [149]. These pioneering descriptive studies have done much to demonstrate the existence of an ocular RAS and have established a convincing line of evidence that targeting the ACE2/Ang1–7/Mas axis of the RAS in the retina could have promising therapeutic effects in ameliorating the deleterious consequences of DR.

### 3.2. Evidence for Interplay between Protein *O*-GlcNAcylation and the RAS

A number of studies provide evidence for the interplay between protein *O*-GlcNAcylation and the RAS. However, little is known about the dynamic between these two pathways in DR. In fact, the impact of *O*-GlcNAc modified proteins on ocular RAS signaling is completely unexplored. In mesangial and proximal tubular cells, exposure to either hyperglycemic culture conditions or glucosamine stimulates angiotensinogen gene expression [150–152]. Other studies suggest that enhanced protein *O*-GlcNAcylation modulates hypertrophic pathways associated with cardiovascular dysfunction in diabetes. Activation of the HBP in cardiomyocytes isolated from diabetic *db/db* mice resulted in an attenuated hypertrophic response upon exposure to Ang II as compared to cardiomyocytes isolated from nondiabetic control mice [153]. Inhibiting the HBP partially restored the hypertrophic signaling response in diabetic cardiomyocytes [153]. In adipose tissue, attenuation of protein *O*-GlcNAcylation resulted in a concomitant decrease in angiotensinogen, AT1R, OGT, and GFAT transcripts [154]. Similarly, infusion of glucosamine increased the expression of angiotensinogen in adipose tissue [155]. Taken together, these studies provide evidence to support a role for *O*-GlcNAc-mediated regulation of local and systemic RAS. However, studies examining specific signaling cascades that generate these results are lacking.

A recent study provides evidence that the ocular RAS modulates protein *O*-GlcNAcylation in the retina [156]. In the retina of mice fed an HFD, retinal protein *O*-GlcNAcylation is enhanced relative to chow-fed control mice [64]. However, when mice fedan HFD

were administered the ACEi captopril, retinal protein *O*-GlcNAcylation is attenuated relative to mice fed an HFD alone [156]. Thus, ACE inhibition is sufficient to attenuate retinal protein *O*-GlcNAcylation. This observation supports a previous report that pharmacological blockade of the RAS prevented the diabetes-induced increase in global *O*-GlcNAcylation in the kidney [157]. Administration of both captopril and A779, a Mas receptor antagonist, reverses the captopril-mediated attenuation of HFD-induced retinal protein *O*-GlcNAcylation, indicating that binding of Ang1–7 to the Mas receptor is necessary for the effect to occur [156]. In Müller cells in culture, Ang1–7 signals *via* a cAMP/EPAC (Exchange Proteins Activated by cAMP)/Rap1 axis to prevent an increase in *O*-GlcNAcylation (Fig. 5). Surprisingly, the suppressive effect of this signaling axis on *O*-GlcNAcylation is not overcome by glucosamine supplementation, indicating that suppression is mediated downstream of the typical rate-limiting enzyme GFAT [156]. Indeed, OGT activity is attenuated in Müller cells exposed to Ang1–7 [156]. Given the integral role of both protein *O*-GlcNAcylation and the RAS in DR, defining the specific molecular events associated with the intersection of these pathways may represent novel therapeutic targets for DR.

## CONCLUSIONS

The retina has the unique function of transmitting visual information to the brain to elicit a behaviorally useful response. Retinal glial, endothelial, and neuronal cells cooperate to form a retinal neurovascular unit. The retina has one of the highest metabolic demands of the body, and therefore is particularly susceptible to hyperglycemia-mediated tissue damage. Early adaptive changes of the retina to the diabetic metabolic environment involve attenuation of biosynthetic pathways. However, common signaling pathways in the retina regulate both biosynthesis and cell survival (283, 310), so a loss of biosynthetic activity is part of a larger disruption of retinal metabolism that results in retinal cell death as adaptive changes become maladaptive. This process most likely occurs in diabetic patients before impairments in the retinal vasculature are clinically visible. At this early stage of the disease, defects in the neuroretina, such as electroretino-graphic responses and visual field sensitivity, manifest (311, 312). Therefore, there is a need to understand what mediates the early metabolic adaptations the retina undergoes, and to understand how those alterations ultimately become maladaptive.

The global re-programming of retinal metabolism is mediated, in part, *via* increased flux through the HBP and consequent enhancement in protein *O*-GlcNAcylation. The HBP exists to integrate nutritional signals and fine-tune cellular responses to biological cues. The retina is particularly dependent upon such fine-tune regulation given the sophisticated coordination required of the retinal neurovascular unit that must exist to visually process the outside world. Recent studies demonstrate that enhanced retinal protein *O*-GlcNAcylation primarily alters gene expression at the level of mRNA translation. Further evidence supports the hypothesis that *O*-GlcNAcylation enhances the interaction between 4E-BP1 and eIF4E to promote angiogenic cytokine production, enhance mitochondrial superoxide production, and initiate retinal inflammation. Importantly, augmented *O*-GlcNAcylation may be attenuated *via* Ang1–7/cAMP-mediated attenuation of OGT activity. While further studies are needed,

addressing the defect in retinal protein *O*-GlcNAcylation may prove to be beneficial in preventing the development of visual dysfunction in diabetes.

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## FUTURE INSIGHTS

Many descriptive studies have identified O-GlcNAc modified transcription factors, but studies investigating the effects of this modification on protein synthesis are lacking. Therefore, O-GlcNAc-mediated control of gene expression at the level of mRNA translation is a potentially underappreciated phenomenon that could have far-reaching clinical implications. Indeed, O-GlcNAc-mediated control of gene expression in the retina could contribute noteworthy findings to the wider field of retinal physiology. To build upon this foundation of O-GlcNAc-mediated control of gene expression *via* 4E-BP, future studies should focus on mechanisms underlying the translation of mRNAs that contain internal, highly-structured elements in the context of diabetes and enhanced O-GlcNAcylation in the retina. Further defining these mechanisms and the RNA binding proteins involved in the regulation of protein synthesis, a better understanding of which messages get translated into a retinal protein will aid in developing novel therapies that can target early biochemical defects in the retina. Clinically available interventions for DR are not universally effective at reversing nor preventing vision loss. These therapeutic interventions for DR and associated diabetes-related ocular pathologies are targeted at consequences of the disease, rather than underlying molecular abnormalities. Therefore, while further mechanistic studies are needed, addressing abnormalities in retinal protein *O*-GlcNAcylation may prove to be beneficial in preventing the development of visual dysfunction in diabetes.

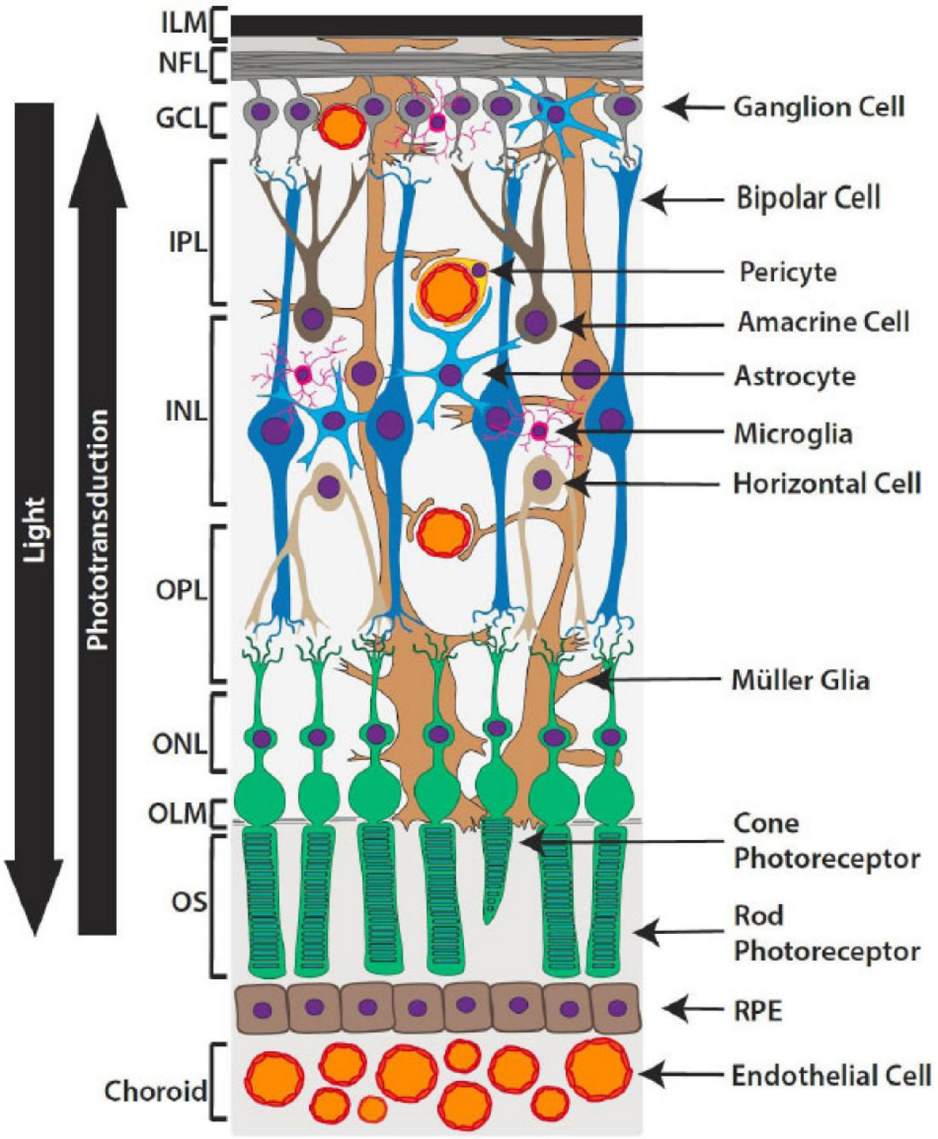


**Fig. (1). Diagram of the retinal neurovasculature.**

The retina contains a diverse array of cell types arranged into 9 neurosensory layers: 1. inner limiting membrane (ILM), 2. nerve fiber layer (NFL), 3. ganglion cell layer (GCL), 4. Inner plexiform layer (IPL), 5. inner nuclear layer (INL), 6. outer plexiform layer (OPL), 7. outer nuclear layer (ONL), 8. outer limiting membrane (OLM), 9. outer segments (OS) of photoreceptors. Situated just outside the neurosensory layers, a retinal pigmented epithelium (RPE) monolayer and underlying vasculature known as the choroid, provides oxygen and nourishment. Photons of light travel through the inner retinal layers and are absorbed by visual pigments in the outer segments of rods and cones. Phototransduction relays this input from the photoreceptors to bipolar and horizontal cells, with the signal subsequently being passed to amacrine and ganglion cells. The axon of a ganglion cell leaves the retina to ultimately transmit the message to the brain.

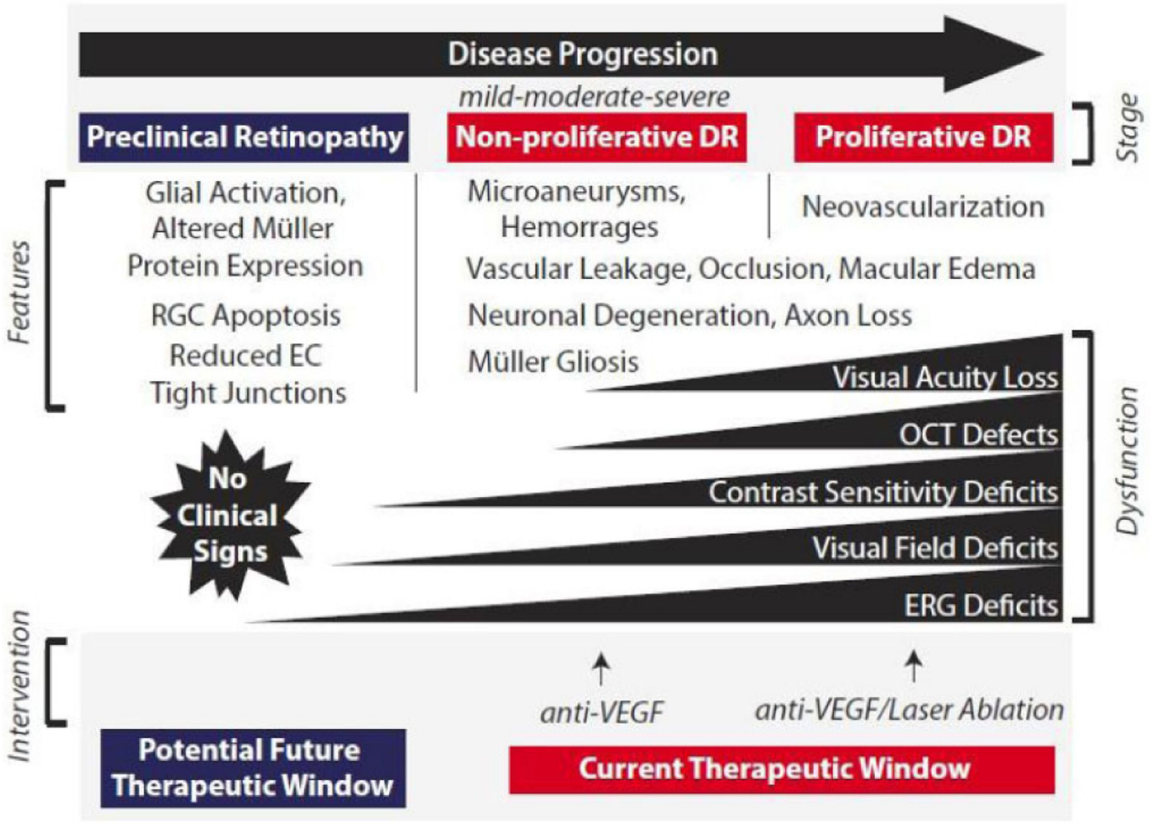


**Fig. (2). Model for the impact of diabetes on retinal neurovasculature leading to a decline in visual function.**

Adaptive and maladaptive changes in the neuroglial precede the microvascular dysfunction and neovascularization that characterizes the later stages of DR progression. Macular edema is most closely associated with vision loss, however, defects in visual function likely develop prior to overt clinical signs of disease. There is presently an absence of interventions that prevent the onset of disease or arrest its progression prior to a clinical diagnosis based on the detection of microvascular defects. DR, diabetic retinopathy; EC, endothelial cell; RGC, retinal ganglion cell; OCT, optical coherence tomography; ERG, electroretinogram; VEGF, vascular endothelial growth factor.

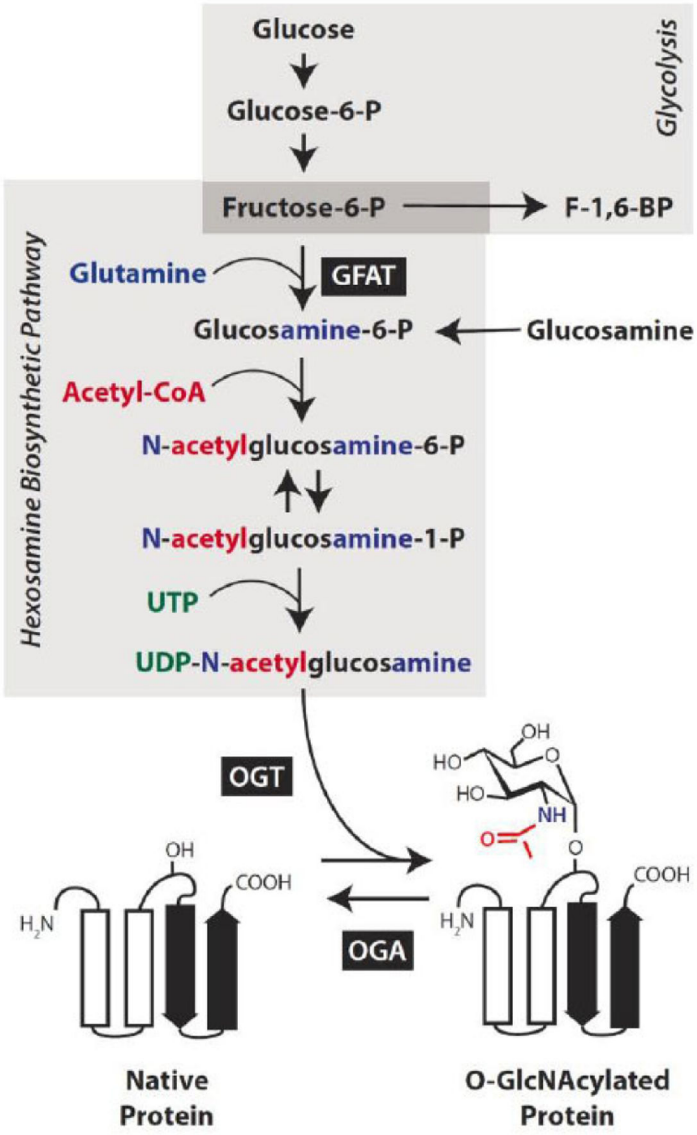


**Fig. (3). Glucose is metabolized by the hexosamine biosynthetic pathway to facilitate protein *O*-GlcNAcylation.**

In addition to glucose, the synthesis of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) requires input from major metabolic pathways, including amino acid (blue), fatty acid (red), and nucleotide (green) metabolism. The first and rate-limiting step in this nutrient sensitive pathway involves the isomerization of fructose-6-phosphate to produce glucosamine-6-phosphate and is catalyzed by the enzyme glutamin-fructose-6-phosphate amidotransferase (GFAT). Alternatively, glucosamine enters the pathway downstream of this rate-limiting step. Protein *O*-GlcNAcylation involves the enzymatic *O*-linked addition of the GlcNAc moiety *via* the Ser/Thr hydroxyl side chain of a nucleocytoplasmic protein (shown as ribbon diagram) and is catalyzed by the enzyme *O*-GlcNAc transferase (OGT). The enzyme *O*-GlcNAcase (OGA) catalyzes the removal of the *O*-GlcNAc modification.

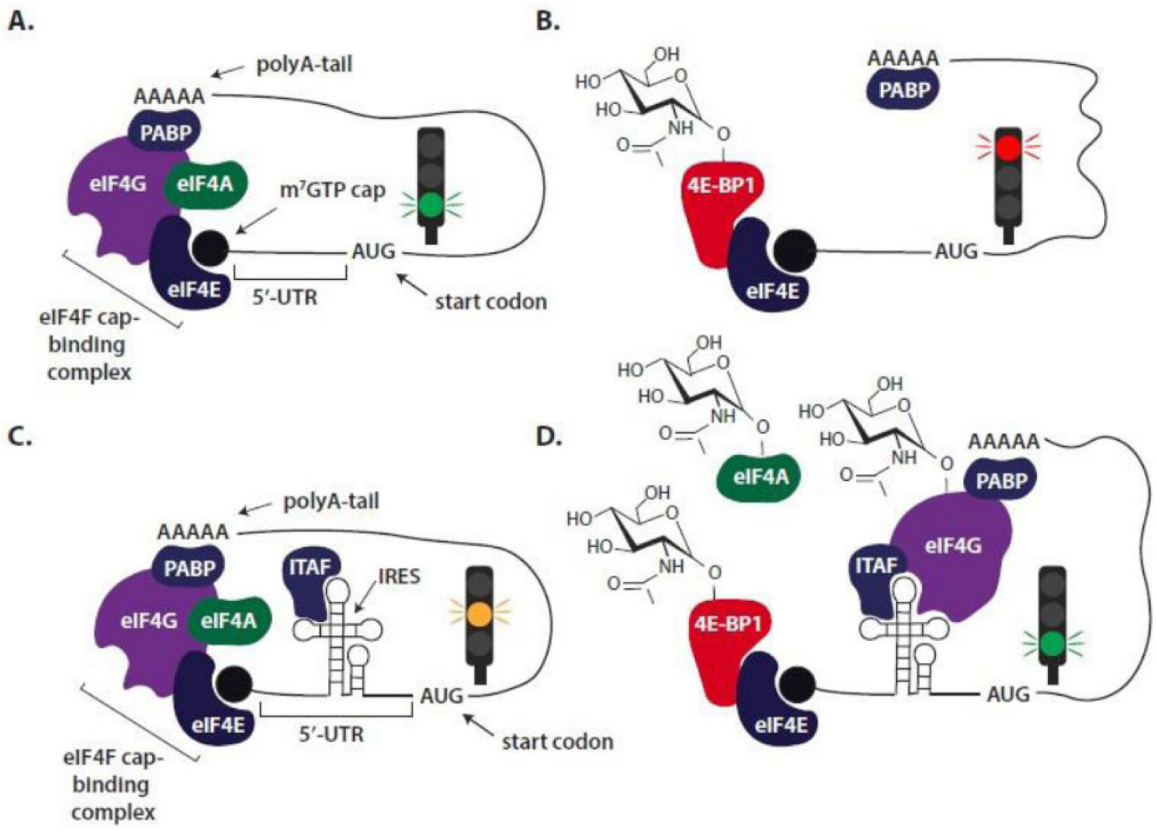


**Fig. (4). Diabetes-induced *O*-GlcNAcylation of the translational repressor 4E-BP1 mediates a shift from cap-dependent to cap-independent mRNA translation.**

Translation initiation represents a key rate-controlling step in gene expression. All eukaryotic mRNAs include an $m^7$GTP cap that is recognized by the cap-binding protein eIF4E. According to the traditional model of cap-dependent mRNA translation, recruitment of ribosomes is initiated by the assembly of the eIF4F complex consisting of eIF4E, eIF4G and eIF4A at the $m^7$GTP cap (*A.*). An interaction between eIF4G and polyA-binding protein (PABP), which associates with the 3′-polyA tail, leads to circularization of the mRNA and facilitates translation initiation. Sequestration of eIF4E by 4E-BP1 prevents eIF4F assembly and represses cap-dependent translation (*B*). A small percentage of mRNAs, including the mRNA that encodes VEGF, contain unique sequence elements in their 5′-untranslated region (UTR) such as internal ribosome entry sites (IRES). These complex structural elements potentially hinder cap-dependent translation (*C.*). However, diabetes-induced sequestration of eIF4E by 4E-BP1 creates a cellular environment that favors cap-independent translation (*D.*). While there is significant diversity in the mechanisms utilized by IRES, cap-independent translation of specific mRNAs may be facilitated by IRES-transacting factors (ITAF), as well as *O*-GlcNAcylation of eIF4A and eIF4G.

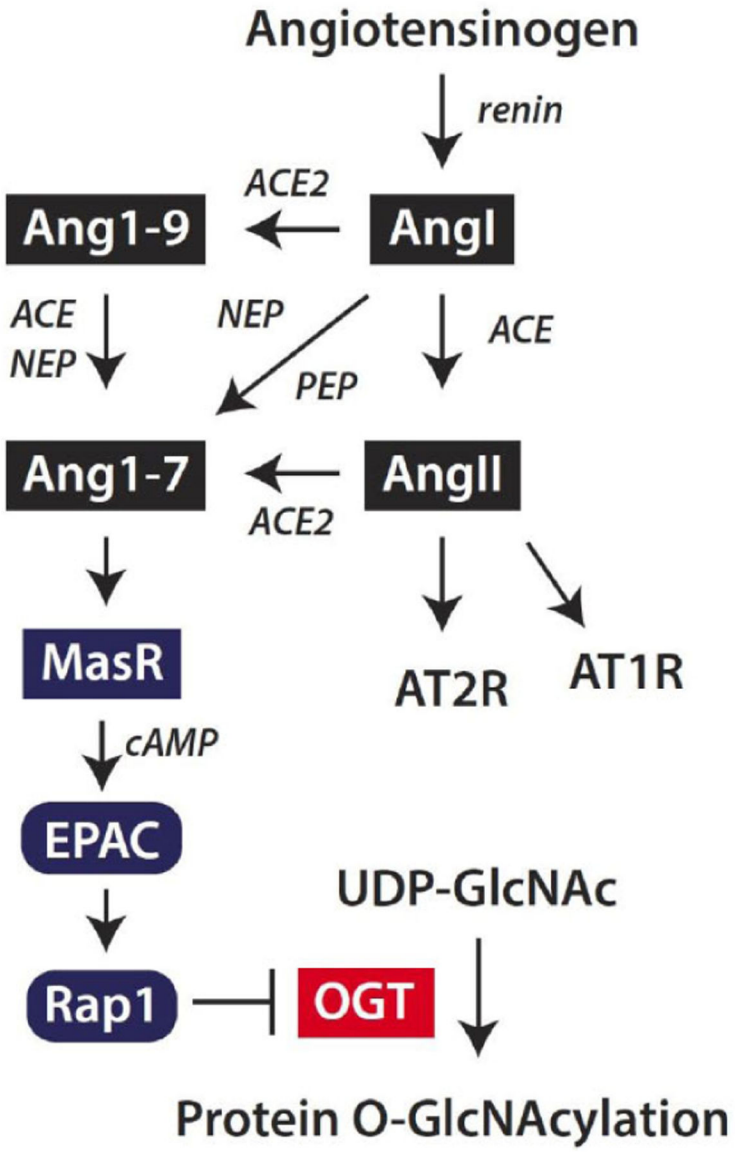


**Fig. (5). Angiotensin-(1–7) signaling attenuates protein *O*-GlcNAcylation in the retina.**

The classic renin-angiotensin system is initiated by cleavage of the pro-peptide angiotensinogen by renin to form Angiotensin I (AngI). AngI may be cleaved by angiotensin-converting enzyme (ACE) to generate angiotensin II (AngII), which acts *via* $AT_1$ and $AT_2$ receptors (AT1R and AT2R, respectively). Alternatively, Ang II may be cleaved by ACE2 to generate Angiotensin-(1–7) **(**Ang1–7). ACE2 also cleaves AngI to generate Ang1–9, which may be degraded by ACE or neutral endopeptidase (NEP) to produce Ang1–7. Finally, AngI may be degraded by endopeptidases such as NEP or prolyl endopeptidase (PEP) to directly form Ang1–7. Ang1–7 acts *via* the Mas receptor (MasR) to enhance cyclic AMP (cAMP) levels and suppress protein *O*-GlcNAcylation in retinal cells. Ang1–7 acts to suppress OGT activity *via* a signaling axis that includes the exchange protein activated by cAMP (EPAC) and Rap1.