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The Translational Repressor 4E-BP1 Contributes to Diabetes-Induced Visual Dysfunction

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PURPOSE. The translational repressor 4E-BP1 interacts with the mRNA cap-binding protein eIF4E and thereby promotes cap-independent translation of mRNAs encoding proteins that contribute to diabetic retinopathy. Interaction of 4E-BP1 with eIF4E is enhanced in the retina of diabetic rodents, at least in part, as a result of elevated 4E-BP1 protein expression. In the present study, we examined the role of 4E-BP1 in diabetes-induced visual dysfunction, as well as the mechanism whereby hyperglycemia promotes 4E-BP1 expression.

METHODS. Nondiabetic and diabetic wild-type and 4E-BP1/2 knockout mice were evaluated for visual function using a virtual optomotor test (Optomotry). Retinas were harvested from nondiabetic and type 1 diabetic mice and analyzed for protein abundance and posttranslational modifications. Similar analyses were performed on cells in culture exposed to hyperglycemic conditions or an O-GlcNAcase inhibitor (Thiamet G [TMG]).

RESULTS. Diabetes-induced visual dysfunction was delayed in mice deficient of 4E-BP1/2 as compared to controls. 4E-BP1 protein expression was enhanced by hyperglycemia in the retina of diabetic rodents and by hyperglycemic conditions in retinal cells in culture. A similar elevation in 4E-BP1 expression was observed with TMG. The rate of 4E-BP1 degradation was significantly prolonged by either hyperglycemic conditions or TMG. A PEST motif in the C-terminus of 4E-BP1 regulated polyubiquitination, turnover, and binding of an E3 ubiquitin ligase complex containing CUL3.

CONCLUSIONS. The findings support a model whereby elevated 4E-BP1 expression observed in the retina of diabetic rodents is the result of O-GlcNAcylation of 4E-BP1 within its PEST motif.

Keywords: diabetes, retina, eukaryotic translation initiation, eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1), ubiquitylation (ubiquitination)

The primary cause of diabetic retinopathy is neurovascular complications that result from hyperglycemia.1 The Diabetes Control and Complications Trial demonstrated that intensive glycemic control is associated with a reduction in both the onset and progression of diabetic retinopathy,2 yet the molecular mechanisms of hyperglycemia-mediated neurovascular dysfunction remain incompletely understood. We recently demonstrated that hyperglycemia promotes translation of the mRNA encoding the proangiogenic cytokine vascular endothelial growth factor (VEGF) in the retina of diabetic mice.3 Vascular endothelial growth factor levels are elevated in the vitreous fluid from eyes of patients with diabetic retinopathy, and the cytokine is considered a key molecular role player in the neurovascular complications of diabetic retinopathy.4 Thus, altered selection of mRNAs, such as the one encoding VEGF, for translation represents an intriguing target for novel clinical therapies that move beyond addressing the symptoms of diabetes and attempt to target the underlying molecular mechanisms.

A potential molecular target for preventing hyperglycemia-induced upregulation of VEGF mRNA translation is the translational repressor 4E-BP1. Previous studies have shown that 4E-BP1 expression is elevated in the retina of diabetic mice, and in retinal cells in culture exposed to hyperglycemic conditions.5 Yet, in the retina of mice lacking 4E-BP1, VEGF expression remains unchanged in response to diabetes.5 4E-BP1 regulates the selection of mRNAs for translation through sequestration of the cap-binding protein eIF4E that recognizes the m7GTP cap structure found at the 5’-terminus of all eukaryotic mRNAs.6 Variation in availability of eIF4E serves as a critical regulator of gene expression patterns, as some messages, such as the one that encodes VEGF contain RNA elements that facilitate ribosome recruitment and translation independent of eIF4E and the 5’-cap structure (i.e., cap-independent translation).7 Diabetes-induced hyperglycemia mediates a 4E-BP1-dependent shift in gene expression that is driven by downregulation of cap-dependent translation concomitant with upregulation of cap-independent translation.8 Thus, the effects of hyperglycemia on 4E-BP1 not only alter global gene expression patterns during diabetes, but also function as a key modulator of the changes in growth factor expression that are causally linked to development of complications in the retina.9

The interaction of 4E-BP1 with eIF4E is negatively regulated by mammalian target of rapamycin (mTORC1)-dependent phosphorylation of serine and threonine residues on 4E-
Hyperglycemia Attenuates 4E-BP1 Degradation

Hyperglycemia-induced sequestration of eIF4E is in part due to attenuated mTORC1 signaling, as 4E-BP1 phosphorylation is reduced in both the retina of diabetic mice and in retinal cells in culture following exposure to hyperglycemic conditions. 3, 5 However, 4E-BP1 is also modified posttranslationally by the covalent addition of O-linked N-acetylglucosamine (O-GlcNAc) in a manner that promotes sequestration of eIF4E independent of 4E-BP1 phosphorylation. 11 Thus, hyperglycemia-driven O-GlcNAc modification of 4E-BP1 provides a mechanism in addition to phosphorylation to control its function.

In the retina of diabetic rodents, 4E-BP1 protein expression is transiently elevated 4 to 6 weeks after the induction of diabetes. 5 In the present study, we show that mice lacking 4E-BP1/2 were protected from diabetes-induced visual impairment during this early stage of the disease. Thus, we set out to establish the mechanism whereby diabetes promotes expression of 4E-BP1. Elevated 4E-BP1 protein expression in the retina of diabetic rodents occurs in the absence of changes in 4E-BP1 mRNA expression, 5 suggesting that a posttranscriptional control mechanism regulates its expression. Furthermore, 4E-BP1 mRNA has a relatively short 5' untranslated region (UTR) that appears to lack significant secondary structure or regulatory sequences necessary for translational regulation. We report that both hyperglycemic conditions and enhanced global O-GlcNAcylation are associated with attenuation of 4E-BP1 degradation. This finding supports previous reports that O-GlcNAcylation serves as a global mechanism for protecting proteins against proteasomal degradation. 12-15 In the present study, we identified a conserved PEST motif in the C-terminus of 4E-BP1, which includes Thr82, a residue critical for 4E-BP1 stability. 16 Thus, many PEST motifs are not recognized by the ubiquitin ligase system until they are tagged for degradation by phosphorylation. However, O-GlcNAc moieties, which can competitively occupy the same Ser or Thr residues that are targeted by phosphorylation, potentially block the phosphorylation events that target proteins for degradation. To evaluate the role of this motif in reduced 4E-BP1 turnover, we performed alanine substitution on residues within the PEST domain and found that these sites regulated 4E-BP1 polyubiquitination and interaction with the E3 ligase complex KLHL25-CUL3. Overall, the findings support a model whereby elevated 4E-BP1 expression in the retina of diabetic mice is the result of PEST motif O-GlcNAcylation.

Materials and Methods

Materials

Preparation of the 4E-BP1 and eIF4E antibodies has been previously described. 17, 18 4E-BP1 phosphospecific antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat anti-mouse and goat anti-rabbit IgG horseradish peroxidase–conjugated antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA). Anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody was purchased from Santa Cruz (Dallas, TX, USA), and all other antibodies were purchased from Cell Signaling Technology.

Animals

At ~4 weeks of age, male 4E-BP1/2 double knockout C57BL/6J mice received injections of 50 mg/kg streptozotocin (STZ) dissolved in sodium citrate buffer (pH 4.5) for 5 consecutive days to induce diabetes. Non-littermate wild-type mice were treated similarly as controls. Alternatively, male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing ~200 g received a single intraperitoneal injection containing 65 mg/kg STZ. Control rodents received an equivalent volume of sodium citrate buffer. Diabetic phenotype was confirmed by blood glucose concentrations > 250 mg/dL in freely fed animals. Glycemic control was achieved in diabetic mice by twice-daily subcutaneous injections of phlorizin (200 mg/kg) during the last 7 full days of the experiment, as well as 3 hours prior to tissue harvest. Where indicated, mice received 50 mg/kg Thiamet G (TMG) or phosphate buffer as a control via intraperitoneal injection. Retinas were harvested 4 weeks after STZ or 24 hours after TMG injections, flash-frozen in liquid nitrogen, and later sonicated in 250 μL extraction buffer as previously described. 3 The homogenate was centrifuged at 10,000 g for 5 minutes at 4°C, and the supernatant was prepared for analysis as described below.

All experimental procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Pennsylvania State College of Medicine Institutional Animal Care and Use Committee.

Behavioral Assessment of Visual Thresholds

A virtual optomotor system (Optomotry; Cerebral Mechanics, White Plains, NY, USA) was used to assess visual function in control and diabetic mice as previously described. 19 Briefly, four inward-facing liquid crystal display monitors were used to create the illusion of a virtual cylinder rotating around a mouse. Contrast sensitivity (CS) and spatial frequency thresholds (SF) were assessed using a video camera to monitor elicitation of the optokinetic reflex. Contrast sensitivity was assessed at a SF of 0.092 cyc/deg. Spatial frequency was assessed at 100% contrast. The CS and SF thresholds were identified as the highest values that elicited the reflexive head movement. Both thresholds were averaged over three trials on consecutive days.

Cell Culture

TR-MUL retinal Müller cells were provided by K. Hosoya (Toyama Medical and Pharmaceutical University). TR-MUL and HEK293E cell cultures were maintained in Dulbeco’s modified Eagle’s medium (DMEM) lacking sodium pyruvate and containing 5 mM glucose (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO, USA) and 1% penicillin/streptomycin (Gibco). Where indicated, cells were exposed to medium containing 25 to 30 mM glucose or 5 mM glucose supplemented with 20 to 25 mM mannitol to serve as an osmotic control. Transfections were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. 4E-BP1 tagged with HA was exogenously expressed in cells using the pCMV6-4E-BP1-HA plasmid. This construct was generated by adding a C-terminal HA tag to the pCMV6-4E-BP1 plasmid (purchased from Origene Technologies, Rockville, MD, USA), using the forward 5’-AATTGCCTGAGTCAACGGCTAATCTGAAATCATGTTGAGTAATGTTCTGCCTCCTGAAATTTGTGA-3’ and reverse 5’-TCCGGAAATTCGGGATAT-3’ primers and cloning the resulting PCR product back into pCMV6 using EcoRI and XhoI. Site-directed mutagenesis to generate 4E-BP1-T82A was previously described. 11 Additional 4E-BP1 variants were generated using Quick-Change Lightning (Agilent Technologies, Wilmington, DE, USA) and the primers listed in Supplementary Table S1. Where indicated, cells were treated with 1 μM cycloheximide (A.G. Scientific, Inc., San Diego, CA, USA), 50 nM TMG, 10 nM ST045849.
Hyperglycemia Attenuates 4E-BP1 Degradation

In the present study we directly assessed diabetes-induced visual dysfunction in 4E-BP1/2-deficient mice by measurement of the optokinetic response. Following 6 weeks of STZ-induced diabetes, wild-type mice exhibited a deficit in CS, whereas 4E-BP1/2-deficient mice failed to do so (Fig. 1A). Moreover, SF was attenuated in diabetic wild-type mice as compared to nondiabetic wild-type mice; however, diabetic and nondiabetic 4E-BP1/2-deficient mice exhibited similar SF (Fig. 1B). Notably, both blood glucose concentrations were similar in wild-type and 4E-BP1-deficient mice (Supplementary Fig. S1). The protective effects of 4E-BP1/2 ablation on CS were maintained at least 10 weeks after STZ administration (Fig. 1C); however, both wild-type and 4E-BP1/2-deficient mice exhibited reduced SF at this time point (Fig. 1D). Moreover, both CS and SF thresholds were reduced in wild-type and 4E-BP1/2-deficient mice 14 weeks after STZ administration (Fig. 1E).

RESULTS

Diabetes-Induced Visual Dysfunction Is Delayed in 4E-BP1/2-Deficient Mice

We previously demonstrated that 4E-BP1 protein expression is elevated in the retina of rodents as early as 4 weeks after the onset of diabetes and returns to basal levels by 12 weeks.5 To assess the role of hyperglycemia in elevated 4E-BP1 expression, diabetic mice were administered phlorizin to lower blood glucose concentrations by blocking intestinal glucose absorption and producing renal glucosuria.20 As previously observed,8 twice-daily administration of phlorizin reduced serum glucose concentrations in diabetic mice (Fig. 2A). Following 4 weeks of STZ-induced diabetes, total retinal 4E-BP1 expression was elevated in diabetic mice as compared to nondiabetic cohorts (Fig. 2B). Notably, phlorizin reduced 4E-BP1 expression in the retina of diabetic mice (Fig. 2B). Similarly, 4E-BP1 expression was modestly elevated in the retina of a previously described cohort of diabetic rats, whereas phlorizin-induced glycemic control reduced its expression to a value not significantly different from that in nondiabetic rats (Fig. 2C). To determine if elevated 4E-BP1 expression was associated with O-GlcNAcylation of the protein, 4E-BP1 was immunoprecipitated from retinas. Indeed, STZ-induced diabetes elevated O-GlcNAcylation of 4E-BP1 in the retina, whereas upon phlorizin treatment, levels of the covalent modification were not different from that in nondiabetic controls (Fig. 2D). To evaluate if O-GlcNAcylation alters retinal 4E-BP1 expression, mice were treated with TMG, which inhibits O-GlcNAc transferase (OGT), the enzyme that catalyzes the removal of O-GlcNAc residues from proteins. Thiamet G enhanced global protein O-GlcNAcylation (Fig. 2E), and produced an elevation in 4E-BP1 expression (Fig. 2F) concomitant with reduced phosphorylation of 4E-BP1 (Fig. 2G) in the retina.

O-GlcNAcylation Facilitates Hyperglycemia-Induced 4E-BP1 Expression

To further evaluate the mechanism whereby hyperglycemic conditions elevated 4E-BP1 expression, we employed rat Müller (TR-MUL) cells in culture exposed to medium containing either control (5 mM) or high (30 mM) concentrations of glucose. Müller cells are the main glial cells of the retina and a likely target of the pathologic effects of hyperglycemia.21,22 Moreover, diabetes-induced Müller cell abnormalities occur in temporal accord with the onset of visual deficiencies observed...
Following exposure to medium containing 30 mM glucose, 4E-BP1 expression and global GlcNAcylation levels were elevated in TR-MUL cells as compared to cells exposed to control medium containing 5 mM glucose (Fig. 3A). This observation supports a previous report showing that a similar effect of hyperglycemic conditions on 4E-BP1 expression was observed in TR-MUL cells in culture. To extend these observations, we evaluated the role of global GlcNAcylation on 4E-BP1 expression. When TR-MUL cells were exposed to medium containing TMG, both global protein GlcNAcylation and 4E-BP1 expression were elevated (Fig. 3B). To determine the necessity of GlcNAcylation in the effect of hyperglycemic conditions, TR-MUL cells were exposed to medium containing 30 mM glucose concentrations in combination with ST045849. ST045849 inhibits O-GlcNAc Transferase (OGT), the enzyme that catalyzes the addition of O-GlcNAc residues to the hydroxyl side chains of proteins. In the presence of ST045849, hyperglycemic conditions failed to produce an elevation in global GlcNAcylation levels or 4E-BP1 expression (Fig. 3C).

**Hyperglycemic Conditions and Thiamet G Elevate 4E-BP1 Expression via a Reduction in its Rate of Degradation**

To investigate the mechanism responsible for elevated expression of 4E-BP1, its rate of synthesis was assessed by incorporation of [35S]methionine into the protein in cells incubated under hyperglycemic or control conditions. There was no significant difference in incorporation of [35S]methionine into 4E-BP1 (Fig. 4A), suggesting that elevated expression of 4E-BP1 was not the result of enhanced synthesis. To evaluate the rate of 4E-BP1 degradation, cells were incubated in the presence of cycloheximide to inhibit protein synthesis. While the rate of 4E-BP1 turnover was relatively slow under hyperglycemic conditions, under control conditions the protein was almost completely degraded following prolonged exposure to cycloheximide (Fig. 4B). Together these findings suggest that the elevated expression of 4E-BP1 that is observed following exposure to hyperglycemic conditions is the result of attenuated degradation of the protein. To evaluate the effect of O-GlcNAcylation on the rate of 4E-BP1 degradation, cells were exposed to TMG to elevate global protein GlcNAcylation (Fig. 4C) and then cycloheximide was administered. In the presence of medium containing TMG, 4E-BP1 exhibited a reduced rate of degradation (Fig. 4D). To provide further support that GlcNAcylation reduces 4E-BP1 turnover, cells were labeled with [35S]methionine in the presence or absence of TMG. The [35S]methionine-containing medium was then replaced with medium containing an excess of nonradiolabeled methionine in the presence or absence of TMG, and 4E-BP1 was immunoprecipitated after 0 or 24 hours. In support of our finding with cycloheximide, the level of [35S]methionine-labeled 4E-BP1 was reduced in vehicle-treated cells after 24 hours, whereas the level of [35S]methionine-labeled 4E-BP1 was maintained in cells treated with TMG (Fig. 4E).

**Identification of a PEST Motif in the C-Terminus of 4E-BP1**

Unlike 4E-BP1, the 4E-BP2 isoform does not exhibit enhanced binding to eIF4E in the retina of diabetic rodents. Thus, we evaluated the effect of hyperglycemic conditions on 4E-BP2 expression and turnover. 4E-BP2 did not exhibit elevated expression or a differential rate of turnover upon exposure to hyperglycemic conditions (Fig. 5A). The amino acid sequences of 4E-BP1 and 4E-BP2 were then compared in silico using the Web-based algorithm epestfind (EMBOSS explorer, http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind [in the public domain]). This algorithm searches for PEST motifs...
**FIGURE 2.** Diabetes-induced hyperglycemia or the O-GlcNAcase inhibitor Thiamet G produces an elevation in 4E-BP1 expression in the retina. Diabetes was induced in mice (A, B) or rats (C, D) by STZ injection. Four weeks after the induction of diabetes, phlorizin (PHZ) was administered twice daily for 7 days to lower blood glucose concentrations. (B, C) Retina supernatant fractions were prepared and 4E-BP1 content was measured by Western blot analysis. Retina supernatant fractions were prepared and the content of 4E-BP1 was assessed by Western blot. (D) 4E-BP1 was immunoprecipitated (IP) from retina supernatant fractions and subjected to Western blot analysis to assess O-GlcNAcylation of the protein. Blood glucose concentrations and 4E-BP1 phosphorylation for rats were previously reported.3 (E–G) Global protein O-GlcNAcylation was enhanced in the retina by intraperitoneal injection of the O-GlcNAcase inhibitor, Thiamet G (TMG, 50 mg/kg). Global protein O-GlcNAcylation (E), 4E-BP1 content (F), and 4E-BP1 phosphorylation (G) were evaluated by Western blot. Values are means ± SE (n = 8 [A–D] and 4 [F–G]). Statistically significant differences (P < 0.05) are denoted by the presence of different letters above the bars on the graphs.

**FIGURE 3.** O-GlcNAcylation produces an elevation in 4E-BP1 expression. Rat TR-MUL Müller cell cultures were maintained in DMEM containing 5 mM glucose and supplemented with 10% heat-inactivated FBS. (A) Cells were exposed to medium containing 30 mM glucose. (B) Cells were exposed to the O-GlcNAcase inhibitor Thiamet G (TMG) to inhibit O-GlcNac cycling and elevate global O-GlcNAcylation levels. (C) Cells were exposed to medium containing 30 mM glucose in the presence of the O-GlcNAc Transferase inhibitor ST045849 (10 nM) to prevent hyperglycemia-induced protein O-GlcNAcylation. 4E-BP1 content as well as global O-GlcNAcylation was assessed in cell lysates by Western blot analysis relative to GAPDH. Values are means ± SE (n = 4). Statistically significant differences are denoted * versus control, P < 0.05.
characterized by hydrophilic stretches of amino acids with high concentrations of acidic and hydroxylated amino acids and the strict absence of positively charged amino acids. PEST motifs have long been associated with targeting proteins for degradation through the ubiquitin–proteasome pathway. Valid PEST motifs above a threshold score of 5.0 are of particular interest. A single strong PEST motif (±11.69) was assigned to the highly conserved residues 79 to 90 of 4E-BP1 (Fig. 5B). Notably, only poor PEST motifs were identified in 4E-BP2 (±1.30 to ±21.42), and the corresponding residues that were aligned with the PEST motif in 4E-BP1 were poorly conserved (Fig. 5C).

Identification of a Consensus GSK3 Site Within the PEST Motif

Since ω-GlcNAcylation of 4E-BP1 at Thr82 is a potential phosphorylation event, we evaluated the amino acid sequence around this residue to determine if it was a consensus phosphorylation site for any known kinases. Analysis of the sequences surrounding Thr82 in silico using Scansite (http://scansite.mit.edu [in the public domain]) identified Thr82 as a consensus GSK3 phosphorylation site. GSK3 phosphorylation-dependent proteolysis has been proposed as a global mediator of protein turnover. GSK3 is well known for its role in marking β-catenin for ubiquitination and proteolysis, but a similar effect has been reported for dozens of other proteins. To evaluate GSK3 as a regulator of 4E-BP1 stability, cells were exposed to medium containing either 25 mM glucose or 5 mM glucose supplemented with 20 mM mannitol for 16 hours. Cells were treated with 1 mM cycloheximide (CHX) and harvested at 3, 6, 24, and 30 hours post treatment. GSK3 subunit expression (Fig. 5F).

Thr82/Ser86 Regulate Ubiquitination and Degradation of 4E-BP1

The best-explored GSK3 substrates require priming phosphorylation at a residue located 4 amino acids C-terminally to the GSK3 target residue. Accordingly, GSK3-mediated phosphorylation of Thr82 potentially requires priming phosphorylation at Ser86. To evaluate a potential role for Thr82 and Ser86 in 4E-BP1 polyubiquitination, we coexpressed HA-tagged 4E-BP1 T82A, S86A, and the double alanine mutant T82A/S86A (AA) with FLAG-tagged ubiquitin; HA-tagged 4E-BP1 T82A, S86A, and AA all exhibited reduced polyubiquitination as compared to wild-type 4E-BP1 (Fig. 5G). Thus, disruption of the PEST motif via alanine substitution at Thr82 or Ser86 in 4E-BP1 turned over under hyperglycemic as compared to control conditions (Fig. 5H). However, as compared to wild type, the 4E-BP1 T82A variant exhibited enhanced stability in response to either hyperglycemic or control conditions (Fig. 5I). Notably, the rate of 4E-BP1 T82A turnover was not different under hyperglycemic as compared to control conditions.

Importantly, 4E-BP1 T82A stability was similar to that observed when all four lysines (Lys57, Lys69, Lys73, Lys105) were mutated to arginine to prevent polyubiquitination (Fig. 5J).
PEST Motif Regulates 4E-BP1 Interaction With the KLHL25-CUL3 Complex

A cullin-RING-based BCR (BTB-CUL3-RBX1) E3 ubiquitin-protein ligase complex has been previously implicated in the polyubiquitination of 4E-BP1 and its subsequent proteasomal degradation.29 The BTB domain-containing protein KLHL25 functions as a substrate-specific adaptor within this complex by binding to 4E-BP1. The scaffolding protein CUL3 links KLHL25 with the RING finger protein.30 Unlike eIF4E, which coimmunoprecipitated with both N-terminally and C-terminally HA-tagged 4E-BP1, CUL3 coimmunoprecipitated only with C-terminally tagged 4E-BP1 (Fig. 6A). Thus, the presence of a tag at the N-terminus of the protein interferes with its association with CUL3, and for subsequent studies C-terminally HA-tagged 4E-BP1 was used. Coimmunoprecipitation of eIF4E with C-terminally HA-tagged 4E-BP1 T82A/S86A was reduced as compared to that of wild-type 4E-BP1 (Fig. 6B). A similar reduction in the interaction with eIF4E was observed with both 4E-BP1 T82A and S86A (Fig. 6C). Surprisingly, coimmunoprecipitation of CUL3 with HA-tagged 4E-BP1 T82A/S86A was increased as compared to wild-type 4E-BP1 (Figs. 6B, 6D), despite reduced polyubiquitination of this variant (Fig. 5G). Again, a similar effect was observed with T82A and S86A variants (Fig. 6D). Thus, integrity of the PEST domain was not necessary for interaction with the KLHL25-CUL3 E3 ubiquitin ligase complex. One possible explanation is that reduced eIF4E interaction permits increased association with KLHL25-CUL3. However, both 4E-BP1 and CUL3 coimmunoprecipitated with eIF4E, suggesting that the interactions of CUL3 and eIF4E are not mutually exclusive (Fig. 6E).
same site as elf4E.\textsuperscript{29} In support of the report, coimmunoprecipitation of elf4E with N-terminally HA-tagged 4E-BP1 was not elevated as compared to C-terminally tagged 4E-BP1 (Fig. 6A). Overall, these findings support that the 4E-BP1 interaction with CUL3 is independent of elf4E binding. To evaluate the effect of hyperglycemia on CUL3 binding, cells were exposed to hyperglycemic conditions prior to immunoprecipitation. Exposure to hyperglycemic conditions increased the coimmunoprecipitation of CUL3 with wild-type 4E-BP1 to a level similar to that observed with the T82A/S86A variant (Fig. 6F). Overall, the data support a model where modification of the PEST motif via 4E-BP1 O-GlcNAcylation or alanine substitution at Thr82/ Ser86 promotes CUL3 retention by blocking polyubiquitination and release of the E3 ubiquitin ligase complex (Supplementary Fig. S2).

**DISCUSSION**

The failure of key regulatory proteins to be expressed appropriately leads to an array of human pathologies. The findings of the present study provide new insight into the mechanism whereby diabetes-induced hyperglycemia enhances expression of the translational repressor 4E-BP1 in the retina. While elevated 4E-BP1 expression has been previously linked to hyperglycemia-mediated eIF4E expression in the retina of diabetic rodents,\textsuperscript{3} we demonstrated here that 4E-BP1/2 ablation protects against early visual dysfunction in diabetic mice. Moreover, we demonstrated that elevated 4E-BP1 expression was mediated by hyperglycemia per se, as phlorizin administration to normalize blood glucose levels prevented the effect. This observation is supported by previous studies utilizing retinal Müller cells in culture, where hyperglycemic conditions enhanced 4E-BP1 abundance.\textsuperscript{5} Retinal Müller cells are a likely target of the pathologic effects of hyperglycemia, as they are the only cell type in the retina to express GLUT2 glucose transporters,\textsuperscript{31} which exhibit a high capacity (but low affinity) for glucose. In cells lacking GLUT2, glucose uptake is quickly saturated under hyperglycemic conditions; however, GLUT2-mediated glucose transport will continue to rise under such conditions. Herein we extended the previous studies by demonstrating that O-GlcNAcylation elevated 4E-BP1 expression in the retina.

Since its discovery in the 1980s,\textsuperscript{32} protein O-GlcNAcylation has emerged as a critical mediator in a range of cellular functions,\textsuperscript{33–35} including protein translation.\textsuperscript{15} The addition of O-GlcNAc (O-GlcNAcylation) to the hydroxyl side chain of serine or threonine residues is driven by glucose flux through the hexosamine biosynthetic pathway, which converts the sugar to UDP-N-acetylglucosamine (UDP-GlcNAc), the donor substrate for addition of N-acetyl glucosamine.\textsuperscript{32} The O-GlcNAcylation cycling enzymes OGT and OGA both strongly associate with ribosomes,\textsuperscript{36} suggesting an important role in regulating mRNA translation. Although only a small percentage of glucose enters the hexosamine biosynthetic pathway under normal physiological conditions, diabetes and hyperglycemic conditions dramatically elevate flux through the pathway and promote production of UDP-GlcNAc.\textsuperscript{37} Protein O-GlcNAcylation and phosphorylation site-specifically modify hydroxy side chains of serine/threonine residues in a competitive manner that allows for extensive crosstalk.\textsuperscript{38} While there is not a strict consensus sequence for O-GlcNac modification, most sites occur in a serine/threonine-enriched region near a proline and valine residue (i.e., PVS/T).\textsuperscript{34} Thus, it is not surprising that many known O-GlcNAcylation sites are coregulated by proline-directed kinases, such as GSK3.\textsuperscript{39} In the present study, alanine substitution to disrupt a consensus GSK3 phosphorylation site in 4E-BP1 at Thr82 was sufficient to promote polyubiquitination and degradation of the protein.
Phosphorylation of 4E-BP1 on Thr82 and Ser86 has been previously detected in large-scale phosphoproteomic studies. Moreover, GSK3β was recently shown to phosphorylate 4E-BP1. Intriguingly, a number of kinases that inactivate GSK3, including PKC, are activated by hyperglycemia in the retina. 

In the present study, we observed a small but significant increase in 4E-BP1 expression in the retina of diabetic as compared to control rodents. It is important to consider that this moderate elevation in 4E-BP1 expression (less than 2-fold) is potentially key to the shift in mRNA translation that occurs in response to hyperglycemic conditions. A similar moderate increase in 4E-BP1 expression mediates a shift from cap-dependent to cap-independent mRNA translation in breast cancer tumors. However, at much higher expression levels, 4E-BP1 is inhibitory to cell growth and proapoptotic. Thus, moderately elevated 4E-BP1 expression in retinal cells is likely critical in promoting the shift in mRNA translation that facilitates hyperglycemia-induced VEGF expression.

4E-BP1 was initially reported to undergo proteasome-mediated degradation in a manner that could be repressed with either the mTOR inhibitor rapamycin or the proteasome inhibitor MG132. Similarly, hyperphosphorylation of 4E-BP1 in response to phosphatase inhibition also promotes polyubiquitination and degradation of the protein. Conversely, exposure of cells to DNA damage or ionizing radiation results in dephosphorylation of 4E-BP1 and an increase in its stability. Together these findings support a model in which phosphorylation of 4E-BP1 promotes its degradation by the proteasome. However, more recently Yanagiya et al. provide evidence for a model in which subpopulations of hypophosphorylated non-eIF4E-bound 4E-BP1 are degraded by the proteasome following KLHL25-CUL3-dependent polyubiquitination. In their model, eIF4E binding to hypophosphorylated 4E-BP1 at the central Y helical Y(X) 4L motif (X, variable amino acids; Φ, hydrophobic amino acids) obstructs polyubiquitination at Lys57. In the absence of eIF4E, 4E-BP1 can be polyubiquitinated by the KLHL25-CUL3 complex; however, hyperphosphorylated 4E-BP1 is resistant to the effect.

In support of this model, the results here demonstrate that eIF4E-bound 4E-BP1 also interacts with dormant KLHL25-CUL3 E3 ligase complex in the presence of eIF4E. The 4E-BP1 T82A/S86A variant exhibited reduced eIF4E binding as compared to wild-type 4E-BP1, yet polyubiquitination and degradation of this protein was attenuated as compared to wild-type 4E-BP1. Thus, in addition to the phosphorylation events that regulate eIF4E binding, other regulatory mechanisms must exist for modulating the stability of 4E-BP1.

Herein, we extend the previous studies by exploring how crosstalk between 4E-BP1 phosphorylation and O-GlcNacylation regulates its stability in response to diabetes-induced hyperglycemia. The findings support a model wherein hyperglycemia-induced O-GlcNacylation represses Lys57 ubiquitination by KLHL25-CUL3 (Supplementary Fig. S2). One potential mechanism whereby O-GlcNacylation of 4E-BP1 blocks ubiquitination is by preventing accelerated protein instability via PEST motif phosphorylation. Importantly, of the 13 phosphorylation sites observed on 4E-BP1, only some are responsible for modulating eIF4E binding and electrophoretic mobility. Thus, if phosphorylation events distinct from those that regulate eIF4E binding promote 4E-BP1 turnover, the model of Yanagiya et al. could be resolved with the previous reports suggesting that 4E-BP1 phosphorylation promotes its polyubiquitination and degradation. Of the previously characterized phosphorylation sites, Ser85 is of particular interest with regard to the present study, since it is located within the PEST motif. Phosphorylation of 4E-BP1 at Ser85 is rapamycin insensitive, is unresponsive to either insulin or amino acids, and does not appear to influence eIF4E binding. Importantly, the stoichiometry of 4E-BP1 phosphorylation at Ser83 is notably lower than that of other phosphorylation sites, consistent with the idea that the modification may lead to a less stable isoform. Thus, it is tempting to speculate that 4E-BP1 phosphorylation at Ser85 serves to promote recognition of the motif and binding by the KLHL25-CUL3 E3 ligase complex. In this scenario, hyperglycemia-induced O-GlcNacylation at Thr82 would serve as a checkpoint prior to targeting 4E-BP1 for degradation via polyubiquitination.

The results presented herein establish the role of residues within a C-terminal PEST motif in regulating 4E-BP1 expression. In the retina of diabetic rodents, both 4E-BP1 expression and its sequestration of the cap-binding protein eIF4E are elevated. Remarkably, 4E-BP1/2-deficient mice were protected from early diabetes-induced visual dysfunction. Thus, attenuation of 4E-BP1 degradation potentially drives the pathologic expression of genes linked to the development of diabetic retinopathy. Notably, early vision protection in diabetic 4E-BP1/2-deficient mice was later followed by manifestation of visual dysfunction similar to that of wild-type mice. Thus, 4E-BP1/2 ablation protects from early events that contribute to visual dysfunction, yet other mechanism(s) (e.g., transcriptional changes in VEGF expression) must contribute to later stages of this disease. Whereas, SF was attenuated in diabetic wild-type mice as compared to nondiabetic wild-type mice, diabetic and nondiabetic 4E-BP1/2-deficient mice exhibited similar SF. Although no statistical difference among strain means was observed 6 weeks post STZ, there was a trend toward lower SF in 4E-BP1/2-deficient mice (P = 0.11). Thus, there is tempered confidence in concluding a protective effect of 4E-BP1/2 ablation on SF, as unknown genetic variances in mouse strains with the same genetic background could also potentially contribute subtle phenotypic differences, such as the rate of SF decline in response to diabetes. The protective effect of 4E-BP1/2 ablation was principally observed in C57Bl/6J, which is largely associated with inner retinal information processing, as compared to the outer retina and photoreceptors. Thus, the findings herein support a role for elevated 4E-BP1 expression in the development of inner retinal dysfunction in diabetic mice. Overall, the findings suggest that the molecular mechanisms whereby altered mRNA translation in the retina contributes to visual dysfunction represent a promising target for novel therapeutics that address the development and progression of diabetic retinopathy.

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