

Peer Review File

Protein O-GlcNAcylation coupled to Hippo signaling drives vascular dysfunction in diabetic retinopathy



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Dr. Lei et al., investigated the roles of O-GlcNAcylation in vascular dysfunction in diabetic retinopathy (DR). They observed pronounced O-GlcNAc elevation in retina endothelial cells (ECs) of DR patients and mouse models. Endothelial-specific depletion or pharmacological inhibition of O-GlcNAc transferase efficiently mitigated vascular dysfunctions. The authors identified YAP/TAZ, key effectors of the Hippo pathway, are O-GlcNAcylated in diabetic retinopathy at T383. Additional rescue experiments revealed that YAP-T383/YAP-S397 regulatory axis attenuated, at least partially, vascular dysfunction. In short, the study was thoughtfully designed and flows adequately. However, there are some concerns need to be addressed before publication.

Major Points:

- 1, In Fig 1, the authors showed that the UDP-GlcNAc levels were significantly increased in the vitreous fluid of DR patients and mouse models. How about the key enzymes for protein O-GlcNAcylation, such as OGT and OGA protein level ? How about the rate limiting enzyme of HBP, GFPT1, in DR patients and DR models?
- 2, In Fig3, the authors used OGA inhibitor PUGNAc and the OGT inhibitor OSMI-1 to treat HRCECs and checked YAP O-GlcNAcylation/S397 phosphorylation and downstream target gene transcription. The authors should use at least two different cell lines to validate these key biochemical or functional experiments. The authors also should knockdown/overexpress OGT/OGA to examine YAP O-GlcNAcylation/S397 phosphorylation and downstream target gene transcription.
- 3, The authors should perform in vitro O-GlcNAcylation assay to confirm that OGT directly O-GlcNAcyates YAP at T383 and T358. As the 2TA mutant didn't significantly decreased YAP O-GlcNAcylation (Fig. 3G), it's essential to screen and verify the other O-GlcNAcylated sites. I strongly suggest the authors overexpressed and purified 2TA mutant from HRCECs and subjected to Mass Spectrometry analysis to identify the other potential O-GlcNAcylation sites. Does YAP T383A influence the mRNA levels of CTGF and CYR61?
4. The authors claimed that O-GlcNAcylation can directly regulate YAP phosphorylation at S397 and in turn affect YAP stability and activity. Does YAP phosphorylation at S397 (S397D or S397E mutant) affect YAP O-GlcNAcylation at T 383? Does YAP O-GlcNAcylation blocks YAP binding to LATS1/2? IF so, the authors should generate O-GlcNAcylated YAP in vitro, and then examined the O-GlcNAcylated YAP binding to LATS1/2 in vitro. Did the authors use AlfaFold2-multimer to analyze the 3D structure of YAP and determine whether O-GlcNAcylation at 383 affect its binding interface with LATS1/2?

5. The authors claimed that O-GlcNAcylation control YAP activity by inhibiting their degradation via blocking phosphorylation at YAP Ser 397. YAP Ser 397 phosphorylation event generates phosphodegron motifs that are recognized by the SCF β -TRCP E3 ligase. Does knockdown/overexpressing of OGT or YAP O-GlcNAcylation deficient mutant affects YAP recognized by bTRCP? Does YAP O-GlcNAcylation deficient mutant affect its ubiquitination level upon MG132 treatment?
6. In YAP S397A cells, does knockdown of OGT affect YAP nuclear translocation and downstream target gene (CTGF and CYR61) transcription? If so, does knockdown of OGT affect YAP S397A binding to the promoter region of CTGF and CYR61?
7. In Figure 5, does YAP T383A influence the pericyte coverage area?
8. In Figure 7, is it possible that restoring YAP/TAZ can rescue the HBP pathway and O-GlcNAc levels?

Minor Point:

1. In Figures 3B, 3C, 3F, 3H, 3G, 3I, and 3L, O-GlcNAc input should be confirmed with blotting to ascertain the efficacy of the treatments.

Reviewer #2 (Remarks to the Author):

Diabetic retinopathy (DR) is a major microvascular complication of diabetes. However, the pathogenesis of DR remains elusive. In this study, the authors illustrated that O-GlcNAcylation levels were elevated in retinal endothelial cells of DR patients and mouse models. The hyper O-GlcNAcylation level promoted pathological angiogenesis and leakage in retinal vasculature. Furthermore, the results demonstrated that YAP-T383 was a novel O-GlcNAcylation site. YAP-T383 functions in regulating the stability and activation of YAP in retinal endothelial cells. High expression of YAP contributed to vascular dysfunction. The are clear presented and convincing, and the manuscript is well written. The findings are novel and should provide new insights into pathogenesis of retinal vascular dysfunction induced by diabetes. However, the following concerns are invited to be solved.

1. Please label the nuclear layers of the retinal sections.
2. In figure 1, the western blot showed O-GlcNAcylation level, please label the exact band for O-GlcNAcylation.
3. In figure 1, different durations of diabetes were used for immunostaining. However, there is only one non-diabetic control. It is better to include age-matched non-diabetic controls for comparison.

4. In figure 2I for counting the acellular capillaries, the PAS staining sometimes was hard to identify acellular capillaries, please provide the immunofluorescence staining of EC markers. For pericyte coverage, it is more accurate to use immunostaining of both EC and pericyte markers.
5. For endothelial specific knockout of Yap/Taz mice, please provide the data verifying knockout efficiency.
6. For all the gene delivery experiments, the expression levels of the delivered genes need to be shown.
7. For STZ-diabetic model, blood glucose levels should be provided.

Reviewer #3 (Remarks to the Author):

Summary:

Lei and colleagues aimed to elucidate the role of protein O-GlcNAcylation in pathological retinal angiogenesis. The manuscript first shows elevated UDP-GlcNAc levels in the vitreous fluid of patients with proliferative diabetic retinopathy (PDR). UDP-GlcNAc is a metabolite produced in the final step of the hexosamine biosynthetic pathway (HBP), which is used for both N- and O-glycosylation of proteins. Using two models of diabetic retinopathy, the authors documented increased O-GlcNAcylation in retinal ECs. Inactivation of Ogt in ECs, the enzyme responsible for adding O-GlcNAc to proteins, attenuated pathological neovascularization in these models. In vitro approaches further suggest that O-GlcNAcylation regulates YAP activity and stability to promote pathological neovascularization. Finally, the authors postulate a feedback loop in which YAP/TAZ regulate glucose metabolism and O-GlcNAc modification in ECs, further driving pathological angiogenesis.

General comment:

In general, the rationale of the study fits into a model where high glucose signaling in diabetes may increase flux through the HBP and UDP-GlcNAc supply into protein glycosylation machinery. Unfortunately, however, the phenotypes are not convincing and the study is not mechanistically clear as to which targets may be involved in altered O-GlcNAc metabolism. Compelling evidence for a role of endothelial YAP O-GlcNAcylation in vivo is lacking. The work has several weaknesses (specified below). In some cases, there is a lack of experimental rigor and data quality to validate conclusions.

Specific comments:

1. Better quality images are required to show convincingly that GlcNAc levels are enriched in retinal ECs in STZ and OIR retinas (Fig. 1F, H). It is difficult to assess GlcNAc antibody labelling in cross-sections. Flat-mounted retinas would be better suited to appreciate GlcNAc labelling throughout the entire vascular network. In addition, neovascular tufts do not appear to form clearly in cross-sections of OIR retinas. With regard to GlcNAc signal quantification, it is not well explained how many cross-sections per retina were used with a sample size of 5/6 retinas (Fig. 1G).

2. A causal role of O-GlcNAcylation could not be established in the analysis of angiogenesis (Fig. 2). Proliferation analysis of PUGNAc-treated HRCECs lacks direct causality with increased O-GlcNAcylation. Aortic ring and choroidal sprouting assays are not exclusive to the EC component. Vascular smooth muscle and endothelial cell sprouts cannot be distinguished by phalloidin staining.

3. Postnatal retinal angiogenesis is delayed in *Ogt* Δ EC mice, but, are the levels of O-GlcNAc reduced in *Ogt* Δ EC retina? The link between *Ogt* inactivation and O-GlcNAc levels in retinal ECs has not yet been demonstrated (Fig. S2).

4. The ability of YAP to undergo O-GlcNAcylation has only been demonstrated in vitro in an artificial setting of a high glucose environment (Fig. 3). Characterization of glycosylated YAP signaling is superficial. Moreover, in vivo validation of the proposed mechanism showing upregulation of endogenous YAP O-GlcNAcylation would be needed to make this story compelling.

5. The protective effect of impaired O-GlcNAcylation in *Ogt* Δ EC mice is abolished by the expression of wild-type YAP and YAP-S897A (Fig. 5E-I). Wouldn't this result rather suggest that activating YAP through O-GlcNAcylation is not a key driver of vascular dysfunction? Alternative mechanisms of YAP activation predominate.

6. Page 7, line 223, Fig. 5A, the authors argue that the OIR model "showed high O-GlcNAcylation levels in blood vessels". Firstly, this is not properly demonstrated. Moreover, a role of ambient glucose in inducing YAP O-GlcNAcylation in the OIR model is difficult to interpret in the light of the triggering environment - hypoxia and growth factor signaling (VEGF). YAP/TAZ is expected to be induced by these conditions. It is unclear whether the OIR model depends on glucose availability and O-GlcNAcylation modifications. How the OIR model provides a means of assessing glucose-induced YAP O-GlcNAcylation, and anti-angiogenic therapies thereof, is not readily apparent.

7. It would be interesting to validate the AAV-mediated YAP-T383A and YAP-S397A tools in the physiological context of postnatal angiogenesis. This would help to consolidate the effects reported in the pathological settings.

Other comments:

1. YAP/TAZ pathway has been implicated in pathological angiogenesis. For example, inhibition of YAP alleviates retinopathies using oxygen-induced retinopathy (OIR) (Kim J, 2017 JCI) and laser-induced choroidal neovascularization (Yan Z 2018 Mol Vis). This reduces the novelty in Fig. 4.

2. Desmin is used as a pericyte marker. However, this is not accurate as Desmin is also highly expressed in smooth muscle cells SMCs (single-cell databases), especially arterial SMCs, the vessel type used throughout the manuscript to assess pericyte coverage.

3. Transcript levels of CTGF and CYR61 (Fig. 3J). Why display qPCR data when the RNA-seq experiment was performed in PUGNAC-treated HRCECs (Fig. 3A)? Methodological approach is unclear.

4. Why is YAP/TAZ deletion induced at P12 and not earlier (5A-D)? Has the effectiveness of gene deletion been verified? This variable needs to be tightly controlled to ensure a consistent baseline of OIR progression.

General response to Reviewers' comments:

We thank the Reviewers for their thorough and constructive comments which have guided us to perform multiple new experiments during the past few months. Major and fruitful new experiments include:

1. We incorporated Human Retinal Microvascular Endothelial Cells (HRMECs), a primary retinal microvascular EC type, and conducted key biochemical and functional experiments. In addition to using inhibitors, we also employed OGT/OGA knockdown or overexpression to further validate the key findings.

2. We performed comprehensive analysis to demonstrate that O-GlcNAcylation of YAP regulates its phosphorylation, stability and activation by incorporating a series of new experiments, including:

i) We conducted an *in vitro* O-GlcNAcylation assay to confirm that OGT directly O-GlcNAcyates YAP at T383.

ii) We performed immunoprecipitation analysis to demonstrate that O-GlcNAcylation of YAP affects its recognition by β TRCP and its ubiquitination level.

iii) We used CHIP-qPCR assay to prove that OGT regulates YAP/TAZ binding to the promoter of target genes, specifically requiring S397.

3. We provided the data verifying knockout efficiency for both *Ogt* ^{Δ EC} and *Yap/Taz* ^{Δ EC} mice, as well as demonstrating reduced O-GlcNAcylation levels in *Ogt* ^{Δ EC} mice.

4. For the gene delivery experiments, we have now provided the expression levels of the delivered genes in isolated brain ECs.

5. We have provided the blood glucose data in the STZ model.

6. We utilized CollIV and CD31 staining, in addition to PAS staining, to analyze vessel regression by assessing acellular capillaries in both *Ogt* ^{Δ EC} and *Yap/Taz* ^{Δ EC} mice.

7. Apart of aortic ring assay and choroid sprouting assay, we performed a bead-sprouting assay using HRCECs and demonstrated the crucial role of O-GlcNAcylation in regulating angiogenic processes.

8. We performed a proximal ligation assay (PLA) to indicate the upregulation of endogenous YAP/TAZ O-GlcNAcylation in ECs in both STZ and OIR models.

9. Using the AAV-BR1 system to specifically deliver genes to the CNS vasculature, we determined the role of YAP-T383 in retinal vessel development. Additionally, we evaluated the functionality of the YAP-T383/S397 axis in both the STZ model and OIR model using this system.

10. We analyzed the timing of VEGFA and O-GlcNAcylation elevation in the DR models. These results suggested that O-GlcNAcylation may serve as an early response factor contributing to vascular dysfunction in DR.

The outcomes of these major experiments have been incorporated into the revised manuscript. Please refer to our point-by-point reply for the detailed explanations on the many new works undertaken to improve this study.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Dr. Lei et al., investigated the roles of O-GlcNAcylation in vascular dysfunction in diabetic retinopathy (DR). They observed pronounced O-GlcNAc elevation in retina endothelial cells (ECs) of DR patients and mouse models. Endothelial-specific depletion or pharmacological inhibition of O-GlcNAc transferase efficiently mitigated vascular dysfunctions. The authors identified YAP/TAZ, key effectors of the Hippo pathway, are O-GlcNAcylated in diabetic retinopathy at T383. Additional rescue experiments revealed that YAP-T383/YAP-S397 regulatory axis attenuated, at least partially, vascular dysfunction. In short, the study was thoughtfully designed and flows adequately. However, there are some concerns need to be addressed before publication.

Response:

We thank the reviewer for the positive evaluation of our manuscript. We also greatly appreciate the very constructive review and suggestions for improving our manuscript. We addressed all of the issues in the following point-by-point response.

Major Points:

1. In Fig 1, the authors showed that the UDP-GlcNAc levels were significantly increased in the vitreous fluid of DR patients and mouse models. How about the key enzymes for protein O-GlcNAcylation, such as OGT and OGA protein level? How about the rate limiting enzyme of HBP, GFPT1, in DR patients and DR models?

Response:

We followed this suggestion and analyzed the expression levels of key enzymes at both mRNA and protein levels in patients with diabetic retinopathy (DR) and in DR models. Initially, we assessed the protein levels of OGT and OGA in the retinas of mice with streptozotocin (STZ)-induced diabetes and oxygen-induced retinopathy (OIR) using western blotting. Our findings revealed no significant changes in OGT and OGA protein levels in STZ and OIR mice compared to age-matched controls (**Figure R1A-D**). Subsequently, we analyzed mRNA levels of key enzymes, including *Gfpt1*, *Ogt*, and *Mgea5* (OGA), which are part of the HBP, using bulk RNAseq data from retinas of STZ and OIR models. Our analysis revealed no statistically significant alterations in the retinas of STZ-induced diabetic mice and OIR pups compared to age-matched controls (**Figure R1E, F**).

Since the previous data were derived from whole retinal samples, we proceeded to analyze the expression levels specifically in ECs. Specifically, in retinal microvascular ECs obtained from patients with proliferative diabetic retinopathy (PDR) and ECs from normoglycemic subjects (GSE94019, Lam et al, 2017), we observed increased expression of *GFPT1*, *OGT*, and *MGEA5*, with *OGT* exhibiting statistical significance (**Figure R1G**). Furthermore, we examined *Ogt*, *Gfpt1*, and *Mgea5* expression using single-cell RNA-seq data obtained from retinas of the OIR and STZ models (**Figure R1H, I**). Although not statistically significant, *Ogt* in the STZ model ($p=0.09$) and *Gfpt1* in the OIR model ($p=0.08$) exhibited a trend of induction. These findings suggest that the expression levels of key enzymes are altered in DR

patients and DR mouse models, primarily within retinal microvascular ECs. While we find these observations intriguing, we think that these data are preliminary and require further investigation. Hence, we opt not to include them in the current manuscript.

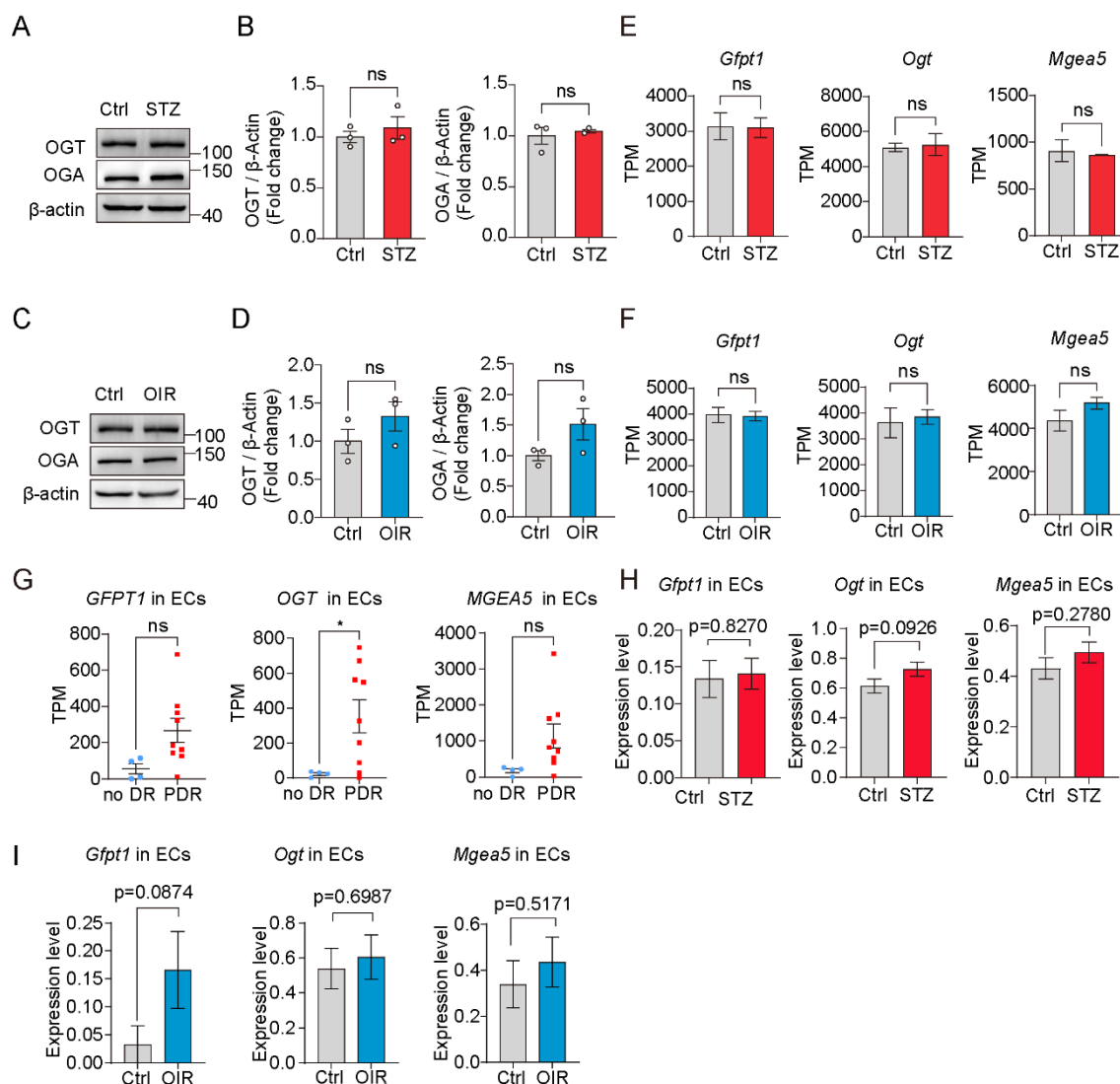


Figure R1

(A and B) Western blot analysis and (B) quantification showing the protein levels of OGT and OGA in the retina of STZ-induced diabetic mice after 4 weeks of diabetes compared to age-matched non-diabetic controls (n=3/3 mice).

(C and D) Western blot analysis and (D) quantification showing the protein levels OGT and OGA in the retina of OIR pups at P17 compared to age-matched control pups (n=3/3 mice).

(E) Relative expression levels of *Gfpt1*, *Ogt* and *Mgea5* in retinas from RNAseq data obtained from STZ-induced diabetic mice compared to age-matched control mice. Data were analyzed from Omnibus Database dataset GSE211583. TPM stands for "Transcripts per kilobase million".

(F) Relative expression levels of *Gfpt1*, *Ogt* and *Mgea5* in retinas from RNAseq data of OIR pups compared to age-matched control pups. Data were analyzed from Omnibus Database dataset GSE158799.

(G) Relative expression levels of *GFPT1*, *OGT* and *MGEA5* in retinal microvascular ECs from PDR patients compared with non-diabetic control. Data were analyzed from Omnibus Database dataset GSE94019. (n=4/9 individuals).

(H) Relative expression levels of *Gfpt1*, *Ogt* and *Mgea5* in retinal microvascular ECs from the Single-cell RNAseq of STZ-induced diabetic mice compared to age-matched non-diabetic controls. Data were analyzed from Omnibus Database dataset GSE178121.

(I) Relative expression levels of *Gfpt1*, *Ogt* and *Mgea5* in retinal microvascular ECs from the Single-cell RNAseq of normoxic and OIR pups. Data were analyzed from Omnibus Database dataset GSE150703.

*p < 0.05. Values as mean ± SEM compared by two-tailed Student's t-test in (B-I).

2. In Fig3, the authors used OGA inhibitor PUGNAc and the OGT inhibitor OSMI-1 to treat HRCECs and checked YAP O-GlcNAcylation/S397 phosphorylation and downstream target gene transcription. The authors should use at least two different cell lines to validate these key biochemical or functional experiments. The authors also should knockdown/overexpress OGT/OGA to examine YAP O-GlcNAcylation/S397 phosphorylation and downstream target gene transcription.

Response:

Following the suggestion, we incorporated Human Retinal Microvascular Endothelial Cells (HRMECs), a primary retinal microvascular EC type, and conducted key biochemical and functional experiments. In the biochemical experiments, we assessed YAP O-GlcNAcylation/S397 phosphorylation and downstream target gene transcription, as depicted in Figure 3 of our initial manuscript. We observed similar results in HRMECs as we previously observed in HRCECs, where PUGNAc increased YAP O-GlcNAcylation while decreasing S397 phosphorylation, and OSMI-1 decreased YAP O-GlcNAcylation while increasing S397 phosphorylation (**Figure R2A, B**). Additionally, PUGNAc elevated the expression of YAP/TAZ target genes *CTGF* and *CYR61*, while OSMI-1 reduced their expression in HRMECs (**Figure R2C, D**). For the functional experiments, we utilized BrdU assays, similar to Figure 2A of our initial manuscript, and found that PUGNAc also increased cell proliferation in HRMECs (**Figure R2E, F**).

In addition to utilizing inhibitors, we also employed siRNA to knock down *OGT* and *OGA* in HRCECs cells. Knocking down *OGT* decreased YAP O-GlcNAcylation and increased pYAP-S397, whereas *OGA* knockdown had the opposite effect (**Figure R2G, H**). Additionally, the expression of *CTGF* and *CYR61* decreased with si*OGT* but increased with si*OGA* (**Figure R2I, J**). To overexpress OGT/OGA, we constructed adenovirus expressing OGT and OGA, respectively (**Figure R2K, L**). OGT overexpression increased YAP O-GlcNAcylation, decreased pYAP-S397, and increased target gene expression, while OGA overexpression showed the opposite effects (**Figure R2K-N**). Collectively, these results further support the notion that O-GlcNAcylation of YAP plays a pivotal role in the regulation of YAP phosphorylation and downstream target gene expression.

The new results are now included in our revised manuscript (**revised Figure S2A, B, Figure 4F and S5E-P**)

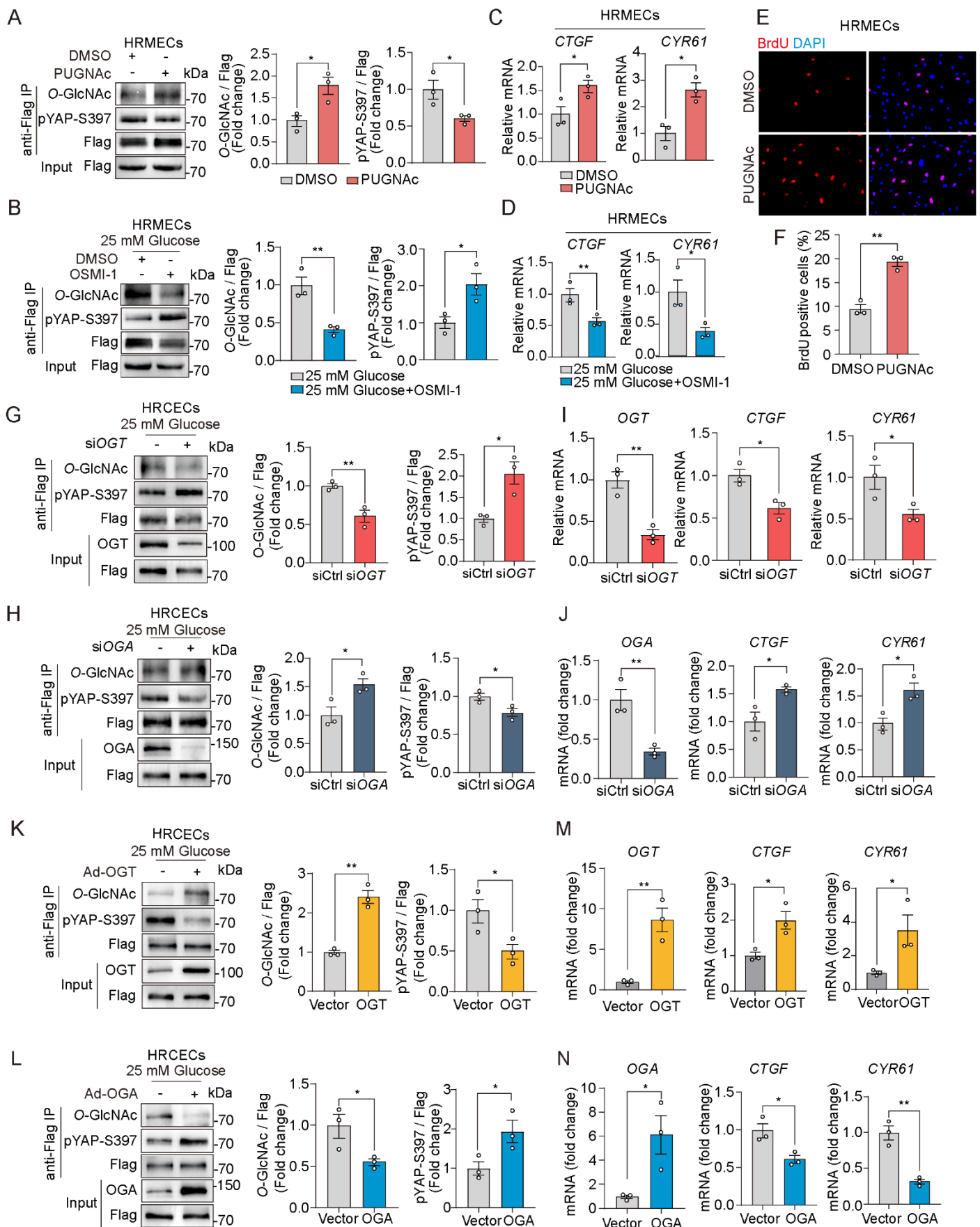


Figure R2

(A) HRMECs were infected with Ad-Flag-YAP for 24 hr followed by treatment with DMSO or 25 μ M PUGNAc for another 24 hr, YAP O-GlcNAcylation was analyzed by immunoprecipitation with anti-Flag

beads followed by western blot analysis. O-GlcNAc-modified YAP and pYAP-S397 in HRMECs were quantified (n=3 independent experiments).

(B) HRMECs were infected with Ad-Flag-YAP for 24 hr followed by treatment with DMSO or 15 μ M OSML-1 for another 24 hr under high glucose (25 mM) culture conditions, YAP O-GlcNAcylation was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis. O-GlcNAc-modified YAP and pYAP-S397 in HRMECs were quantified (n=3 independent experiments).

(C) HRMECs were treated with DMSO or 25 μ M PUGNAc for 24 hr, relative mRNA levels of *CTGF* and *CYR61* were analyzed by qPCR (n=3 independent experiments).

(D) HRMECs were treated with DMSO or 15 μ M OSML-1 for 24 hr under high glucose (25 mM) culture conditions, relative mRNA levels of *CTGF* and *CYR61* were analyzed by qPCR (n=3 independent experiments).

(E) BrdU (red) incorporation in HRMECs treated with DMSO or 25 μ M PUGNAc for 24 hr.

(F) Quantification of BrdU⁺ cells as a percentage of the total cell number in (E) (n = 3/3 independent experiments).

(G) HRCECs were infected with Ad-Flag-YAP for 24 hr followed by transfection with siCtrl or siOGT and cultured in 25 mM glucose medium, YAP O-GlcNAcylation was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis. O-GlcNAc-modified YAP and pYAP-S397 in HRCECs were quantified (n=3 independent experiments).

(H) HRCECs were infected with Ad-Flag-YAP for 24 hr followed by transfection with siCtrl or siOGA and cultured in 25 mM glucose medium, YAP O-GlcNAcylation was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis. O-GlcNAc-modified YAP and pYAP-S397 in HRCECs were quantified (n=3 independent experiments).

(I and J) HRCECs were transfected with siCtrl or siOGT/siOGA and cultured in 25 mM glucose medium. Relative mRNA level of *OGT/OGA*, *CTGF* and *CYR61* were analyzed by qPCR (n=3 independent experiments).

(K and L) HRCECs were infected with Ad-Flag-YAP for 24 hr followed by infection with Ad-Vector or Ad-OGT/Ad-OGA for another 24 hr, YAP O-GlcNAcylation was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis. O-GlcNAc-modified YAP and pYAP-S397 in HRCECs were quantified (n=3 independent experiments).

(M and N) HRCECs were infected with Ad-Vector or Ad-OGT/OGA for 24 hr, relative mRNA level of *OGT/OGA*, *CTGF* and *CYR61* in HRCECs were analyzed by qPCR (n=3 independent experiments).

*p < 0.05, **p < 0.01. Values as mean $\pm$ SEM compared by two-tailed Student's t-test in (A-N).

3. The authors should perform in vitro O-GlcNAcylation assay to confirm that OGT directly O-GlcNAcyates YAP at T383 and T358. As the 2TA mutant didn't significantly decreased YAP O-GlcNAcylation (Fig. 3G), it's essential to screen and verify the other O-GlcNAcyated sites. I strongly suggest the authors overexpressed and purified 2TA mutant from HRCEs and subjected to Mass Spectrometry analysis to identify the other potential O-GlcNAcylation sites. Does YAP T383A influence the mRNA levels of *CTGF* and *CYR61*?

Response:

We appreciate these excellent suggestions. To confirm that OGT directly O-GlcNAcyates YAP at T383 and T358, we conducted an *in vitro* O-GlcNAcylation assay. Recombinant proteins were generated for YAP-WT, YAP-T358A, and YAP-T383A for the *in vitro* O-GlcNAcylation assay. The results showed a significant reduction in the O-GlcNAcylation level mediated by OGT in the YAP-T383A mutant compared to YAP-WT *in vitro*, while marginal changes were observed in T358A mutants. This finding suggests that T383 is the principal O-GlcNAcylation site on YAP (**Figure R3A**).

In response to the reviewer's suggestion, we overexpressed and purified the 2TA mutant from HRCECs and subjected it to Mass Spectrometry analysis. We conducted two biological repeats of the Mass Spectrometry analysis. While YAP was successfully enriched, no additional O-GlcNAcylation sites on YAP-2TA were identified. As shown in the revised Figure 3F, YAP-2TA still exhibited O-GlcNAcylation. This suggests the possibility of other potential O-GlcNAcylation sites on YAP. However, based on our results, we believe that T383 is a crucial O-GlcNAcylation site on YAP in retinal ECs.

To explore whether YAP T383A influence the mRNA levels of *CTGF* and *CYR61*, we knocked down endogenous YAP using siRNA targeting the 3'UTR of YAP (**Figure R3B, C**) and infected HRCECs with adenovirus expressing YAP-WT and YAP-T383A. Cells were collected for analysis after 48 hr. *CTGF* and *CYR61* expression were significantly reduced in YAP-T383A expressing cells, further indicating that T383A expression reduced YAP activity.

The new results are now included in our revised manuscript (**revised Figure 3G, S5Q, R**).

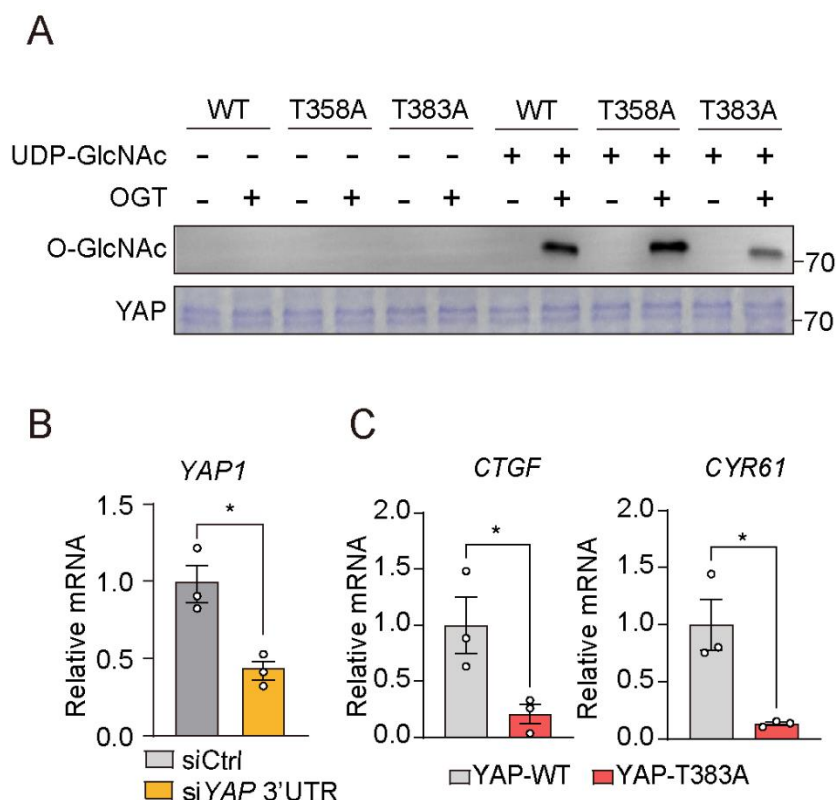


Figure R3

(A) Identification of the O-GlcNAcylation modification sites of YAP by *in vitro* O-GlcNAcylation assay. Purified wild-type or mutant YAP was used as substrates. Coomassie blue staining shows the YAP protein

used in the assay. Three independent experiments were performed.

(B) HRCECs were transfected with control siRNA or YAP 3'UTR siRNA in HRCECs, relative mRNA level of *YAP1* were analyzed.

(C) HRCECs were transfected with YAP 3'UTR siRNA, followed by infection with Ad-Flag-YAP-WT or Ad-Flag-YAP-T383A for 48hr. The expression of *CTGF* and *CYR61* were analyzed (n=3 independent experiments).

*p < 0.05, **p < 0.01. Values as mean ± SEM compared by two-tailed Student's t-test in (B), (C).

4. The authors claimed that O-GlcNAcylation can directly regulate YAP phosphorylation at S397 and in turn affect YAP stability and activity. Does YAP phosphorylation at S397 (S397D or S397E mutant) affect YAP O-GlcNAcylation at T383? Does YAP O-GlcNAcylation blocks YAP binding to LATS1/2? IF so, the authors should generate O-GlcNAcylated YAP in vitro, and then examined the O-GlcNAcylated YAP binding to LATS1/2 in vitro. Did the authors use AlphaFold2-multimer to analyze the 3D structure of YAP and determine whether O-GlcNAcylation at 383 affect its binding interface with LATS1/2?

Response:

To investigate whether YAP phosphorylation at S397 (S397D or S397E mutant) affects YAP O-GlcNAcylation at T383, we constructed Flag-YAP-S397D and Flag-YAP-S397E mutants and transfected them into HEK293 cells to analyze the O-GlcNAcylation level of YAP. The results revealed that neither the YAP S397D nor S397E mutant exerted an effect on YAP O-GlcNAcylation (**Figure R4A**). To explain this result, we referred to a previous study that described the interplay between phosphorylation and O-GlcNAcylation. Using a kinetic-based high-resolution mass spectrometry assay, this study highlighted specific residues (pSp/T) P(V/A/T) (gS/gT) that, when phosphorylated, hamper O-GlcNAcylation at nearby sites (Leney *et al*, 2017). As YAP S397 site does not exhibit correlation with the (pSp/T) P(V/A/T) (gS/gT) motif, the phosphorylation of YAP at S397 does not influence YAP O-GlcNAcylation at T383.

We acknowledge the importance of understanding whether YAP O-GlcNAcylation blocks YAP binding to LATS1/2. A previous study assessed the interaction between O-GlcNAcylated YAP and LATS1 (Peng *et al*, 2017). They generated YAP with or without O-GlcNAcylation by *in vitro* glycosylation assay and evaluated the direct interaction between YAP (with or without O-GlcNAcylation) and LATS1 by *in vitro* pull-down assay. As showed in the figure below, they demonstrated that YAP O-GlcNAcylation significantly blocked YAP-LATS1 interaction in a cell free system (**Figure R4B**).

Following the reviewer's suggestion, we employed AlphaFold2-multimer to analyze the potential impact of O-GlcNAcylation at T383 on its binding interface with LATS1/2 (**Figure R4C, 4D**). The predicted structure showed a binding interface of Lats1/2 and YAP at S397. Furthermore, both S397 and T383 are closely located and are situated in the loop region of YAP. Therefore, based on the predicted structure, it is possible that O-GlcNAcylation at T383 affects its binding interface with LATS1/2 at S397. A series of experiments further demonstrated that O-GlcNAcylation of T383 indeed influences phosphorylation at S397. The new results are now included in our revised manuscript (**revised Figure S5B, C**).

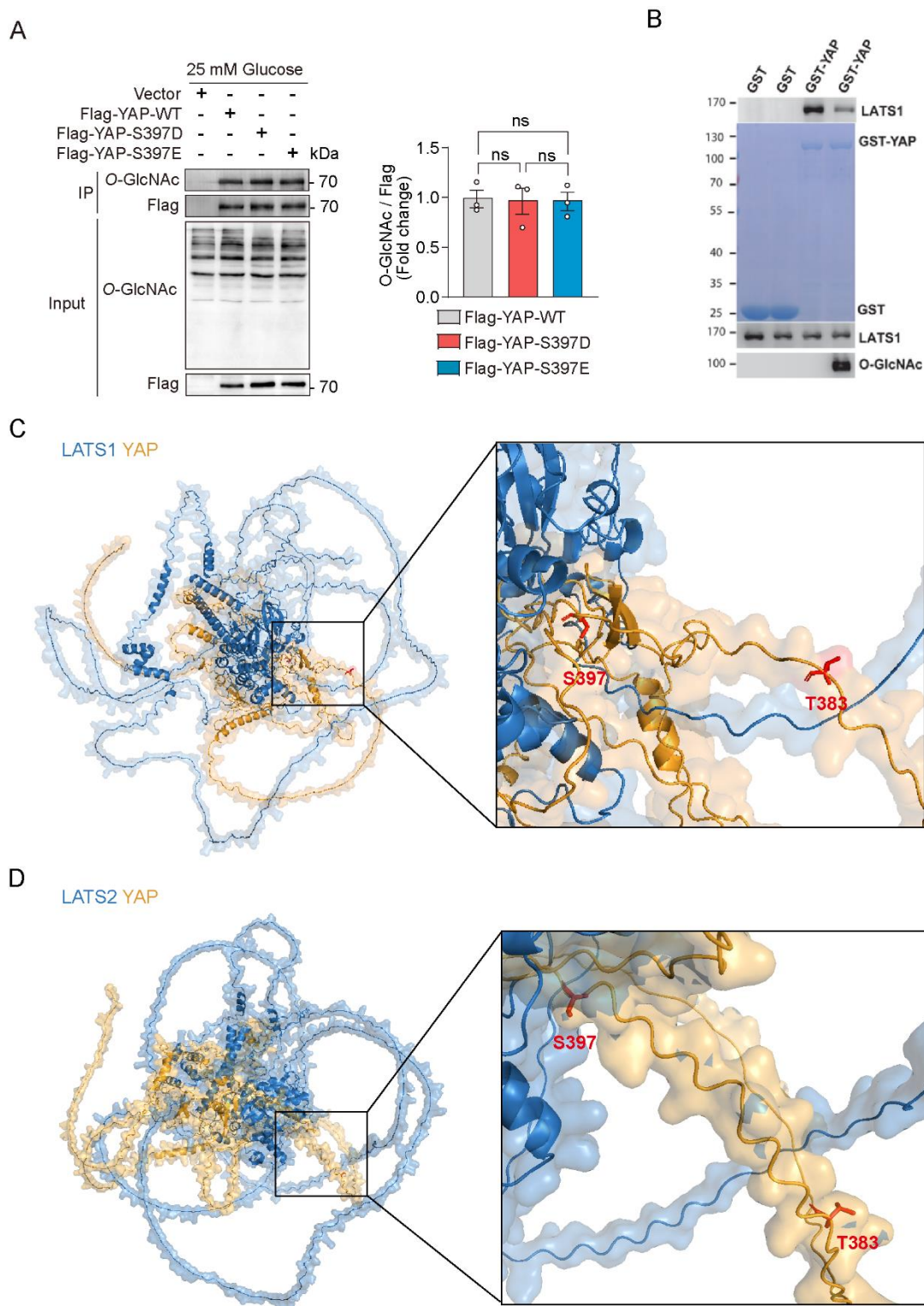


Figure R4

(A) HEK 293T cells were transfected with Flag-YAP-WT, Flag-YAP- S397D or Flag-YAP- S397E plasmids, YAP O-GlcNAcylation was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis (n=3 independent experiments).

(B) Blots from Peng et al. *Mol Cell* 2017: GST-YAP with or without O-GlcNAcylation was generated by *in vitro* glycosylation assay, LATS1 was purified from *E. coli*, and the direct interaction between YAP (with or without O-GlcNAcylation) and LATS1 was detected by *in vitro* pull-down assay (Peng et al., 2017).

(C and D) The structure details of the interaction between YAP and LATS1/2, LATS1 or LATS2 is shown in blue and YAP is shown in yellow. YAP T383 site and YAP S397 site are marked in red. All structure predictions were generated with AlphaFold2-multimer and PyMol.

Values as mean $\pm$ SEM compared by one-way ANOVA followed by Tukey's multiple comparisons test in (A).

5. The authors claimed that O-GlcNAcylation control YAP activity by inhibiting their degradation via blocking phosphorylation at YAP Ser 397. YAP Ser 397 phosphorylation event generates phosphodegron motifs that are recognized by the SCF β -TRCP E3 ligase. Does knockdown/overexpressing of OGT or YAP O-GlcNAcylation deficient mutant affects YAP recognized by β TRCP? Does YAP O-GlcNAcylation deficient mutant affect its ubiquitination level upon MG132 treatment?

Response:

We thank the reviewer for the excellent suggestion. Following this suggestion, we determined whether the binding of YAP and SCF β -TRCP is affected by knockdown/overexpressing of OGT or YAP O-GlcNAcylation deficient mutant through Co-IP experiments. The results indicated that OGT knockdown increased the association of YAP and β -TRCP (**Figure R5A**), while overexpression of OGT reduced this association (**Figure R5B**). Additionally, the YAP O-GlcNAcylation-deficient mutant (YAP-T383A) showed an increased level of ubiquitination, which could be inhibited by MG132 treatment (**Figure R5C**). These results further supporting our hypothesis that O-GlcNAcylation controls YAP activity by inhibiting its proteasome-dependent degradation. These results have been incorporated into our revised manuscript (**Figure 4I, S6G, H**).

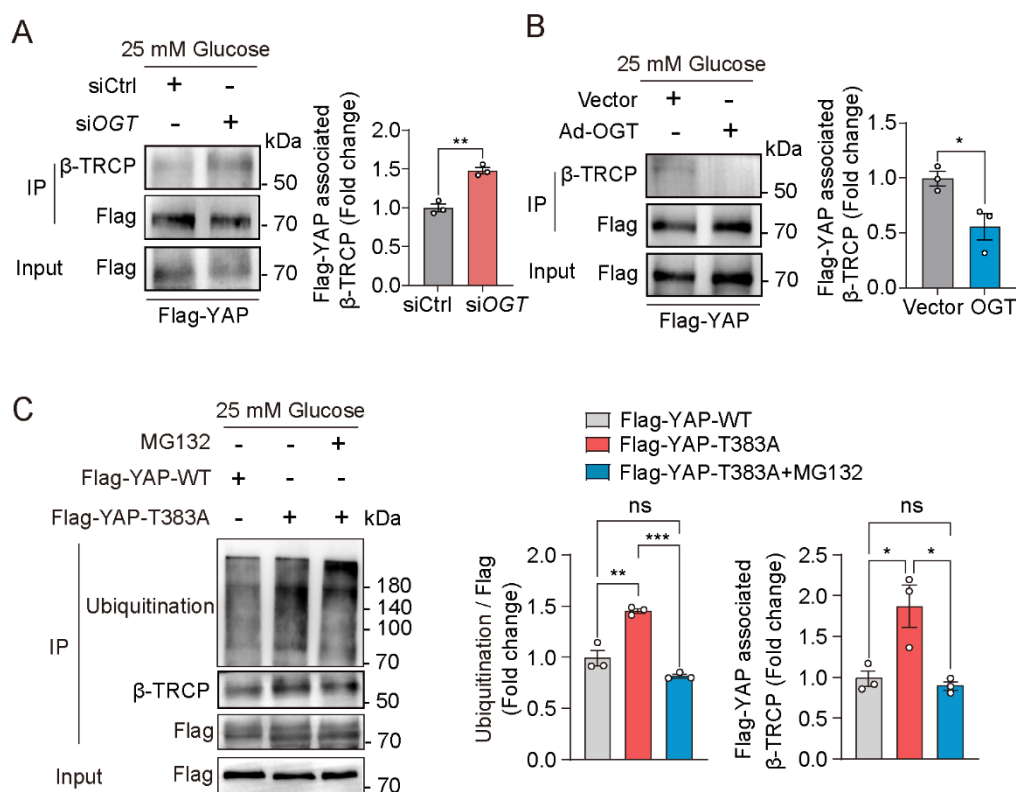


Figure R5

(A) HRCECs were infected with Ad-Flag-YAP for 24 hr followed by transfection with siCtrl and siOGT and

cultured in 25 mM glucose medium, the association of β -TRCP and Flag-YAP was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis (n=3 independent experiments). (B) HRCECs were infected with Ad-Flag-YAP for 24 hr followed by infection with Ad-Vector or Ad-OGT and cultured in 25 mM glucose medium, β -TRCP was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis (n=3 independent experiments).

(C) HRCECs were infected with Ad-Flag-YAP-WT or Ad-Flag-YAP-S397A and cultured in 25 mM glucose medium for 40 hr, followed by administration with or without MG132 for another 8 hr, ubiquitination and β -TRCP was analyzed by immunoprecipitation with anti-Flag beads followed by western blot analysis (n=3 independent experiments).

*p < 0.05, **p < 0.01, ***p < 0.001. Values as mean $\pm$ SEM compared by two-tailed Student's t-test in (A, B) and one-way ANOVA followed by Tukey's multiple comparisons test in (C).

6. In YAP S397A cells, does knockdown of OGT affect YAP nuclear translocation and downstream target gene (*CTGF* and *CYR61*) transcription? If so, does knockdown of OGT affect YAP S397A binding to the promoter region of *CTGF* and *CYR61*?

Response:

To address this question, we expressed Flag-YAP-WT or Flag-YAP-S397A by adenovirus and transfected siCtrl or siOGT in HRCECs. YAP 3'UTR siRNA was also transfected to knockdown endogenous YAP. The YAP-S397A mutant exhibited increased nuclear translocation, consistent with previous findings (Hu *et al*, 2017b). Furthermore, knockdown of OGT resulted in reduced nuclear localization and target gene expression in YAP-WT expressing cells but not in YAP-S397A, suggesting that nuclear translocation by O-GlcNAcylation depends on the S397 site of YAP (**Figure R6A-C**).

We further conducted ChIP-qPCR analyses to determine whether knockdown of OGT affect YAP-S397A binding to the promoter region of *CTGF* and *CYR61*. Our results indicated that the YAP-S397A mutant showed increased binding to the promoter regions of *CTGF* and *CYR61*. OGT knockdown reduced the binding in YAP-WT expressing cells but not in YAP-S397A expressing cells (**Figure R6D**).

We have now included these results in our revised manuscript (**revised Figure S6I-L**).

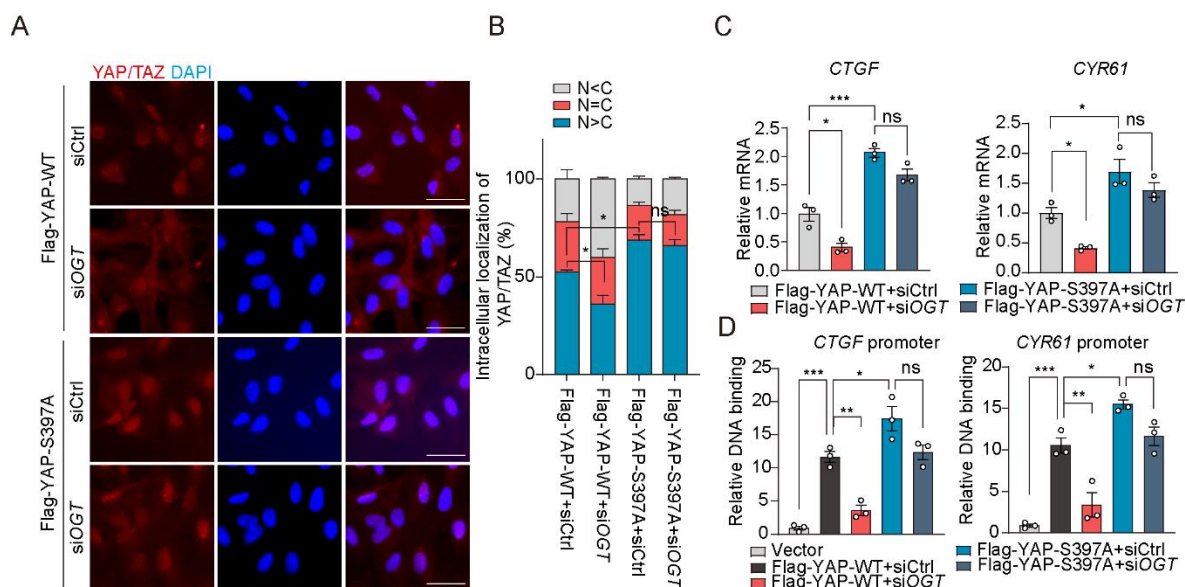


Figure R6

(A) HRCECs were infected with Ad-Flag-YAP-WT or Ad-Flag-YAP-S397A and then transfected with siCtrl or siOGT. YAP 3'UTR siRNA was also transfected to knockdown endogenous YAP. Immunofluorescence of YAP (red) and DAPI (blue) were shown.

(B) Quantification of YAP subcellular localization of (A). (n=more than 25 cells from 3 independent experiments)

(C) Relative mRNA levels of *CTGF* and *CYR61* in HRCECs treated as in (A).

(D) HRCECs were transfected with siCtrl and siOGT for 24 hr, followed by infection with Ad-Vector, Ad-Flag-YAP-WT or Ad-Flag-YAP-S397A for another 24 hr. ChIP were performed using anti-Flag beads and ChIP-qPCR showing YAP binding to the promoters associated with *CTGF* and *CYR61*. The relative DNA binding was calculated as fraction of input and normalized to Ad-Vector; data from 3 biological replicates from one representative experiment are shown.

*p < 0.05, **p < 0.01, ***p < 0.001. Values as mean ± SEM compared by one-way ANOVA followed by Tukey's multiple comparisons test in (B-D).

7. In Figure 5, does YAP T383A influence the pericyte coverage area?

Response:

Following the reviewers' suggestion, we investigated the necessity of YAP-T383 in the regulation of the pericyte coverage in the STZ model. We expressed wild-type YAP (AAV-YAP-WT) or a mutant YAP-T383A (AAV-YAP-T383A) in *Yap/Taz*^{ΔEC} mice, AAV-EGFP served as the control. Similar to the findings in the OIR model, our results demonstrated that the expression of wild-type YAP significantly decreased Desmin positive cell coverage, while the AAV-YAP-T383A group exhibited levels comparable to the control group (**Figure R7A-C**). Furthermore, we also took into consideration the suggestion from Reviewer 3 that Desmin is highly expressed in vascular smooth muscle cells. To ensure greater accuracy, we revised our statement to describe Desmin-labeled cells and changed the term "pericytes" to "vascular mural cells". We have now included these results in our revised manuscript (**revised Figure 7D-F**).

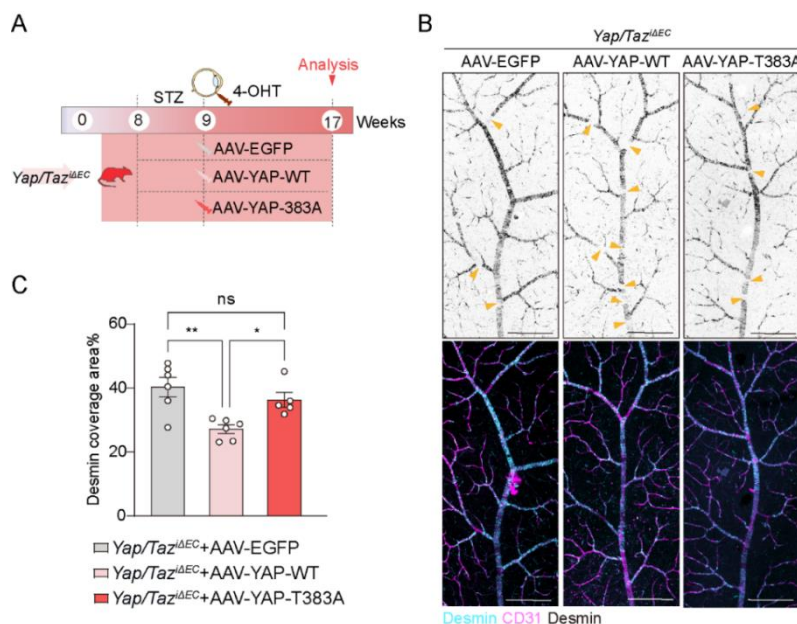


Figure R7

(A) Scheme of AAV-BR1 injection in STZ model. 10 μ g 4-OHT in 1 μ l DMSO was intravitreally injected into *Yap/Taz*^{*iΔEC*} mice one week after the onset of STZ. At the same time, AAV- EGFP, AAV- YAP-WT, or AAV- YAP-T383A were retro-orbitally injected into *Yap/Taz*^{*iΔEC*} mice as indicated. After 8 weeks, the retinas were harvested for analysis.

(B) Desmin and CD31 staining of retinas from AAV-BR1 injected *Yap/Taz*^{*iΔEC*} mice. Arrowheads indicate vessels without mural cell coverage.

(C) Quantification of vascular mural cell coverage area (%) within the CD31⁺ vessels area of (B) (n = 6/6/5 mice).

*p < 0.05, **p < 0.01. Values as mean $\pm$ SEM compared by one-way ANOVA followed by Tukey's multiple comparisons test in (C). Scale bars, 100 μ m in (B).

8. In Figure 7, is it possible that restoring YAP/TAZ can rescue the HBP pathway and O-GlcNAc levels?

Response:

To address this question, we knocked down YAP/TAZ by transfecting siRNA targeting the 3'-UTR, followed by infecting Ad-Flag-YAP and Ad-Flag-TAZ to restore the expression of YAP/TAZ in HRCECs. The results showed that the overall levels of O-GlcNAc-modified protein were significantly reduced in YAP/TAZ knockdown cells, while the restoration of YAP/TAZ rescued O-GlcNAcylation levels in HRCECs (**Figure R8A, B**). We have now included these results in our revised manuscript (**revised Figure 9F**).

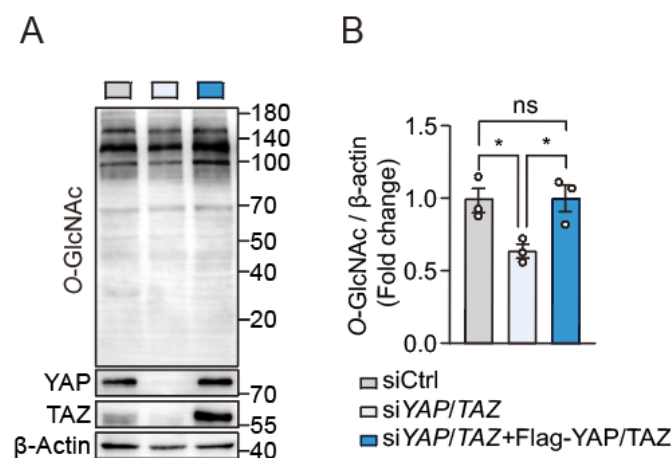


Figure R8

(A and B) Western blot and quantification (B) of overall levels of O-GlcNAc-modified proteins in HRCECs transfected with siCtrl and siYAP/TAZ followed by infection with Ad-Flag-YAP and Ad-Flag-TAZ and cultured in 25 mM glucose medium (n=3/3 independent experiments).

*p < 0.05. Values as mean $\pm$ SEM compared by one-way ANOVA followed by Tukey's multiple comparisons test in (B).

Minor Point:

9. In Figures 3B, 3C, 3F, 3H, 3G, 3I, and 3L, O-GlcNAc input should be confirmed with blotting to ascertain the efficacy of the treatments.

Response:

O-GlcNAc input blots were added to confirm the efficacy of these treatments, and all O-GlcNAc input blots were added in (revised Figure 3B, C, F, 4A, B, C, H).

Reviewer #2 (Remarks to the Author):

Diabetic retinopathy (DR) is a major microvascular complication of diabetes. However, the pathogenesis of DR remains elusive. In this study, the authors illustrated that O-GlcNAcylation levels were elevated in retinal endothelial cells of DR patients and mouse models. The hyper O-GlcNAcylation level promoted pathological angiogenesis and leakage in retinal vasculature. Furthermore, the results demonstrated that YAP-T383 was a novel O-GlcNAcylation site. YAP-T383 functions in regulating the stability and activation of YAP in retinal endothelial cells. High expression of YAP contributed to vascular dysfunction. The are clear presented and convincing, and the manuscript is well written. The findings are novel and should provide new insights into pathogenesis of retinal vascular dysfunction induced by diabetes. However, the following concerns are invited to be solved.

Response:

We thank you for endorsement. To address these questions, please refer to our point-by-point response.

1. Please label the nuclear layers of the retinal sections

Response:

We have now labeled the layers of the retinal sections.

2. In figure 1, the western blot showed O-GlcNAcylation level, please label the exact band for O-GlcNAcylation.

Response:

We have labeled the molecular weights for the whole blots of O-GlcNAcylation.

3. In figure 1, different durations of diabetes were used for immunostaining. However, there is only one non-diabetic control. It is better to include age-matched non-diabetic controls for comparison.

Response:

We appreciate this suggestion, and we agree that it is more proper to use age-matched non-diabetic controls. In the revised manuscript, we have now included age-matched non-diabetic control groups at various stages of the STZ model. The new results align with previous findings, showing that O-GlcNAc intensity in blood vessels increased with the duration of diabetes (**Figure R9A-C**). We have now revised these data as (**revised Figure 1H, I, 5H, I**).

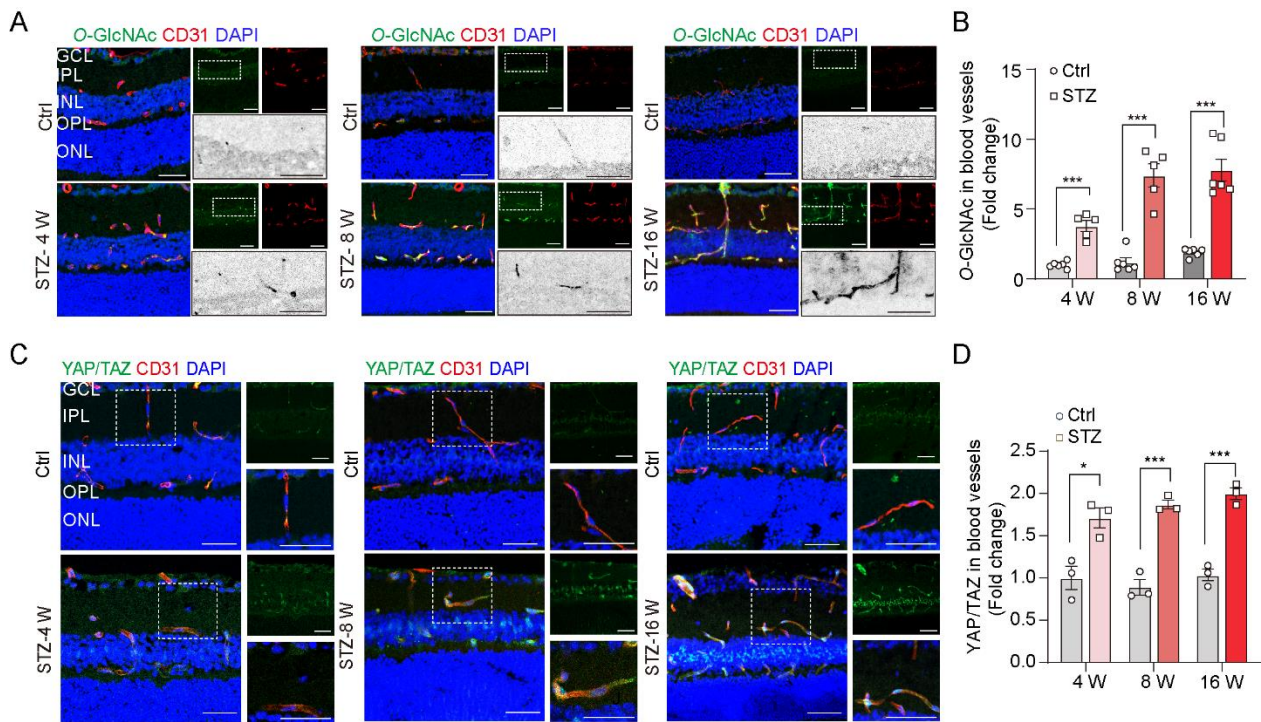


Figure R9

(A) Representative immunostaining images of O-GlcNAc (green or black), CD31 (red), and DAPI (blue) in frozen sections of eyeballs from age-matched control and diabetic mice at 4, 8 and 16 weeks of diabetes.

(B) Quantification of O-GlcNAc-modified protein levels within retinal vessels (CD31⁺ area) of (A).

(C) Representative images of mouse retina sagittal sections immunostained for YAP/TAZ (green) and CD31 (red) in different stages of STZ model and age-matched controls.

(D) Quantification of YAP/TAZ fluorescence intensity in CD31⁺ area of (C) (n = 3/3 mice each time point).

*p < 0.05, ***p < 0.001; Values as mean ± SEM compared by two-tailed Student's t-test in (B) and (D). Scale bars: 30 μm in (A) and (C).

4. In figure 2I for counting the acellular capillaries, the PAS staining sometimes was hard to identify acellular capillaries, please provide the immunofluorescence staining of EC markers. For pericyte coverage, it is more accurate to use immunostaining of both EC and pericyte markers.

Response:

We appreciate this suggestion and we share the concern that PAS staining sometimes was hard to identify acellular capillaries. The regressing vessel branches leave collagen type IV (CollIV⁺) empty sleeves behind (Baffert *et al*, 2006). Therefore, we employ CollIV and CD31 staining to analyze vessel regression by quantifying the number of CollIV⁺CD31⁻ sleeves. Consistent with the PAS staining results, quantification of CollIV⁺CD31⁻ sleeves showed decreased vessel regression in *Ogt*^{ΔEC} mice and *Yap/Taz*^{ΔEC} mice (**Figure R10A-D**). We have now revised these data as **revised Figure 2K, L, 6G, H**.

For pericyte coverage, we agree with the reviewer that it is more accurate to use both EC and pericyte markers. To analyze pericyte coverage in the STZ model, we stained CD31 as EC marker and Desmin as pericyte marker (**revised Figures 2G, 6C, 7E, 7H, 8C and S10I**). Furthermore, we also took into consideration the suggestion from Reviewer 3 that Desmin is also highly expressed in vascular smooth

muscle cells. To ensure greater accuracy, we revised our statement to describe Desmin-labeled cells and changed the term "pericytes" to "vascular mural cells".

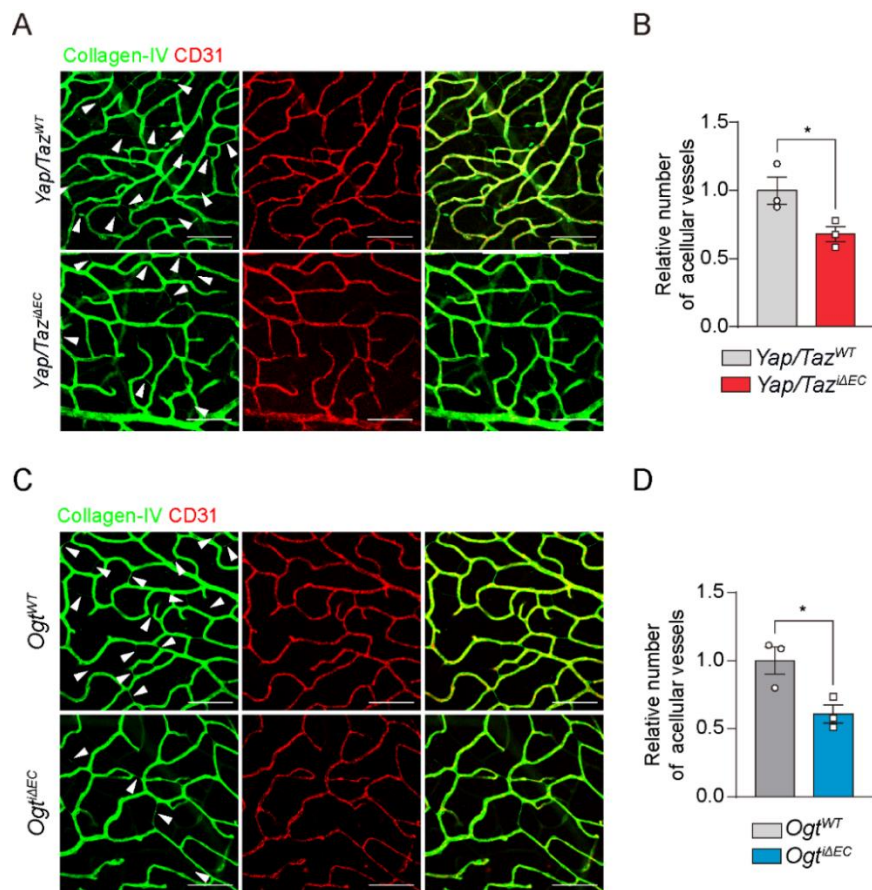


Figure R10

(A) CollIV and CD31 staining of retinas from *Yap/Taz*^{WT} and *Yap/Taz*^{ΔEC} 9 weeks after the onset of STZ-induced diabetes. Arrowheads indicate acellular capillaries.

(B) Quantification of the number of CollIV⁺CD31⁻ sleeves in (A). Number of CollIV⁺CD31⁻ sleeves were quantified per field of retina and normalized to *Yap/Taz*^{WT} average number. 15 random field were involved per retina (n=3/3 mice).

(C) CollIV and CD31 staining of retinas from *Ogt*^{WT} and *Ogt*^{ΔEC} 9 weeks after the onset of STZ-induced diabetes. Arrowheads indicate acellular capillaries.

(D) Quantification of the number of CollIV⁺CD31⁻ sleeves in (C). Number of CollIV⁺CD31⁻ sleeves were quantified per field of retina and normalized to *Ogt*^{WT} average number. 15 random field were involved per retina (n=3/3 mice).

*p < 0.05, Values as mean ± SEM compared by two-tailed Student's t-test in (B) and (D). Scale bars: 100 μm in (A) and (C).

5. For endothelial specific knockout of Yap/Taz mice, please provide the data verifying knockout efficiency.

Response:

The *Yap/Taz*^{ΔEC} (*Cdh5*-(PAC)-*CreERT2*; *Yap/Taz*^{fl/fl}) mice were utilized in two disease models: the OIR model and the STZ-induced diabetes model. In the OIR model, Cre recombination was induced by intraperitoneal administration of 70 μl tamoxifen (1mg/ml) at postnatal days P12, P13, P14, and P16. Due

to challenges in isolating a sufficient number of ECs from the retina, we chose to isolate ECs from the brains of OIR mice (**Figure R11A**). The mRNA levels of *Yap* and *Taz* was significantly reduced in the isolated brain ECs from *Yap/Taz*^{iΔEC} mice, validating the efficacy of the knockout approach (**Figure R11B**). In the STZ model, Cre recombination was induced via intravitreal injection of 10 μg 4-OHT one week after the onset of STZ-induced diabetes. We performed immunostaining to assess the expression of YAP/TAZ within ECs of retinal sections (**Figure R11C, D**). YAP/TAZ expression were significantly reduced in blood vessels in *Yap/Taz*^{iΔEC} mice confirmed the successful knockout efficiency in this model. We have included the new results in our revised manuscript (**revised Figure S8A, B, F, G**).

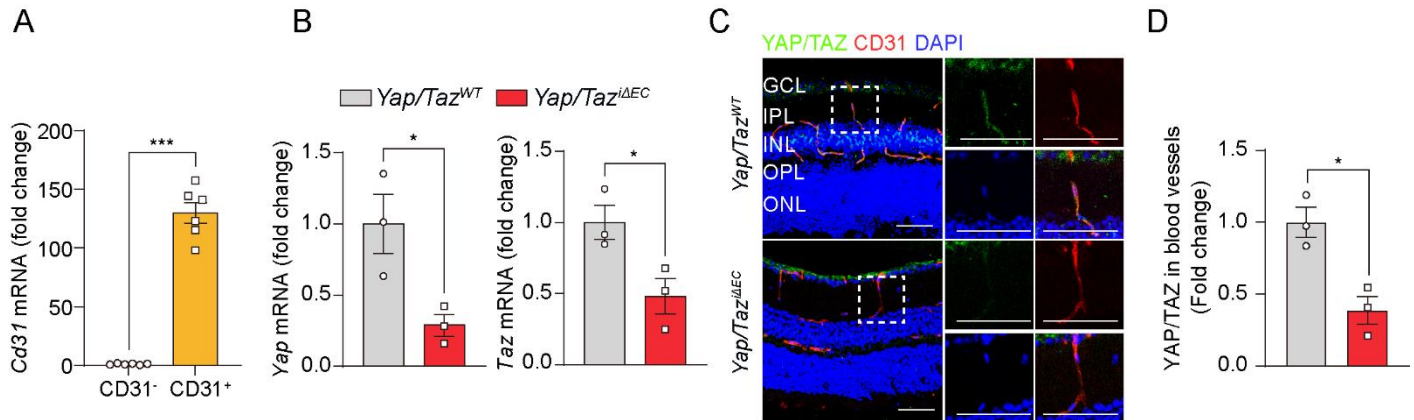


Figure R11

(A) qRT-PCR showing the expression of *Cd31* mRNA in the isolated EC fraction (CD31⁺) compared with CD31⁻ cells from brains of *Yap/Taz*^{WT} and *Yap/Taz*^{iΔEC} OIR pups (n = 6 mice).

(B) qRT-PCR showing the expression of *Yap* and *Taz* mRNA in the isolated EC fraction (CD31⁺) from *Yap/Taz*^{WT} and *Yap/Taz*^{iΔEC} OIR pups (n = 3/3 mice).

(C) Representative images of YAP/TAZ (green), CD31 (red) and DAPI (blue) in frozen sections of eyeball from *Yap/Taz*^{WT} and *Yap/Taz*^{iΔEC} STZ mice.

(D) Quantification of YAP/TAZ expression within retinal vessels (CD31⁺ area) in *Yap/Taz*^{WT} and *Yap/Taz*^{iΔEC} STZ mice (n=3/3 mice).

*p < 0.05, ***p < 0.001, Values as mean ± SEM compared by two-tailed Student's t-test in (A), (B), (D). Scale bars: 30 μm in (C).

6. For all the gene delivery experiments, the expression levels of the delivered genes need to be shown.

Response:

We appreciate this suggestion to show the expression levels of the delivered genes. We utilized the AAV-BR1 system, which can specifically target CNS vasculature (including the brain and the retina) (Korbelin *et al*, 2016; Marchio *et al*, 2016). Firstly, to confirm the delivery specificity of this system in our model, we injected AAV-EGFP into P3 neonatal mice. GFP expression was detected in both retinal and brain vasculature, indicating efficient and specific gene delivery (**Figure R12A**). Subsequently, we assessed the expression levels of the delivered genes. Due to challenges in isolating a sufficient number of retina ECs, we isolated the ECs from the brain for qPCR analysis. The injection of AAV constructs encoding YAP-WT, YAP-T383A, and YAP-S397A significantly elevated *Yap* mRNA expression levels in isolated brain ECs,

indicating successful gene delivery (**Figure R12B**). The new results are included in our revised manuscript (**revised Figure S9A, B**).

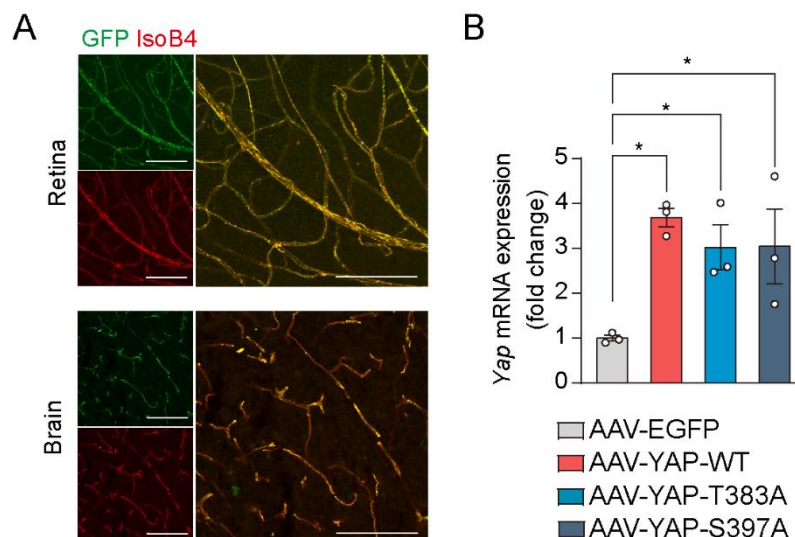


Figure R12

(A) Representative image from the retina and the brain showing that 10 days after AAV-EGFP retro-orbital injection, GFP expression could be observed in the blood vessels (IsoB4⁺ areas).

(B) qRT-PCR showing the expression of *Yap* mRNA in the isolated EC fraction from the brain treated as in (A).

* $p < 0.05$; Values as mean $\pm$ SEM compared by one-way ANOVA followed by Dunnett's multiple comparisons test in (B). Scale bars: 30 μ m in (A).

7. For STZ-diabetic model, blood glucose levels should be provided.

Response:

The blood glucose levels were monitored throughout the entire experimental procedure. Mice subjected to STZ treatment exhibited pathologically elevated blood glucose levels (>16.8 mM) at 4, 8, and 16 weeks (**Figure R13A**). In the case of *Yap/Taz* ^{Δ EC} and *Ogt* ^{Δ EC} mice, Cre recombination was induced by intravitreal injection of 4-OHT in the STZ model. We compared blood glucose levels between *Yap/Taz*^{WT} and *Yap/Taz* ^{Δ EC} mice, as well as between *Ogt*^{WT} and *Ogt* ^{Δ EC} mice after 8 weeks of diabetes. No differences in blood glucose levels were observed between genotypes (**Figure R13B, C**). We have now revised these data as **revised Figure S1A, S4C, S8C**.

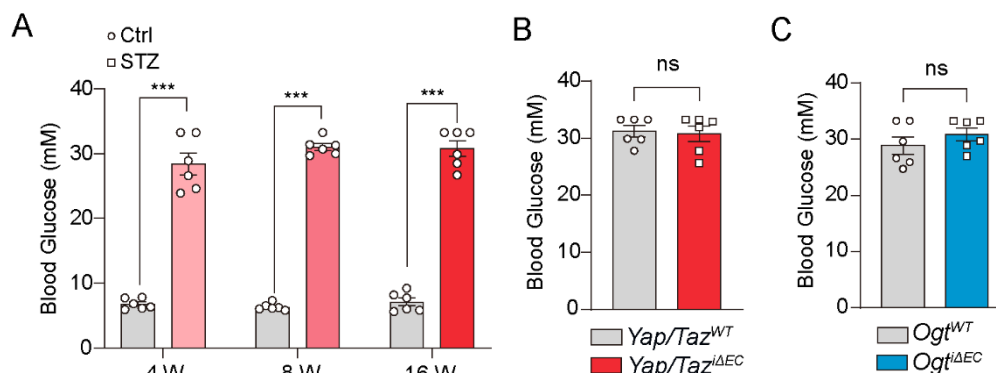


Figure R13

(A) Blood glucose of control mice and mice with diabetes for 4 weeks, 8 weeks, and 16 weeks (n=6/6 mice).

(B) Blood glucose of *Yap/Taz*^{WT} and *Yap/Taz*^{*iΔEC*} mice after 8 weeks of diabetes (n=6/6/6/6 mice).

(C) Blood glucose of *Ogt*^{WT} and *Ogt*^{*iΔEC*} mice after 8 weeks of diabetes (n=6/6/6/6 mice).

***p < 0.001; Values as mean ± SEM compare by two-tailed Student's t-test in (A-C).

Reviewer #3 (Remarks to the Author):

Summary:

Lei and colleagues aimed to elucidate the role of protein O-GlcNAcylation in pathological retinal angiogenesis. The manuscript first shows elevated UDP-GlcNAc levels in the vitreous fluid of patients with proliferative diabetic retinopathy (PDR). UDP-GlcNAc is a metabolite produced in the final step of the hexosamine biosynthetic pathway (HBP), which is used for both N- and O-glycosylation of proteins. Using two models of diabetic retinopathy, the authors documented increased O-GlcNAcylation in retinal ECs. Inactivation of Ogt in ECs, the enzyme responsible for adding O-GlcNAc to proteins, attenuated pathological neovascularization in these models. In vitro approaches further suggest that O-GlcNAcylation regulates YAP activity and stability to promote pathological neovascularization. Finally, the authors postulate a feedback loop in which YAP/TAZ regulate glucose metabolism and O-GlcNAc modification in ECs, further driving pathological angiogenesis.

General comment:

In general, the rationale of the study fits into a model where high glucose signaling in diabetes may increase flux through the HBP and UDP-GlcNAc supply into protein glycosylation machinery. Unfortunately, however, the phenotypes are not convincing and the study is not mechanistically clear as to which targets may be involved in altered O-GlcNAc metabolism. Compelling evidence for a role of endothelial YAP O-GlcNAcylation in vivo is lacking. The work has several weaknesses (specified below). In some cases, there is a lack of experimental rigor and data quality to validate conclusions.

Response:

We appreciate the critical appraisal and suggestions. By addressing all the comments of the reviewer, we were able to substantially improve our work and manuscript.

Specific comments:

1. Better quality images are required to show convincingly that GlcNAc levels are enriched in retinal ECs in STZ and OIR retinas (Fig. 1F, H). It is difficult to assess GlcNAc antibody labelling in cross-sections. Flat-mounted retinas would be better suited to appreciate GlcNAc labelling throughout the entire vascular network. In addition, neovascular tufts do not appear to form clearly in cross-sections of OIR retinas. With regard to GlcNAc signal quantification, it is not well explained how many cross-sections per retina were used with a sample size of 5/6 retinas (Fig. 1G).

Response:

We agree with the reviewer that flat-mounted retinas would be better suited to appreciate GlcNAc labelling throughout the entire vascular network. We performed O-GlcNAc staining on flat-mounted retinas from both the OIR and STZ models, and the results revealed higher O-GlcNAc levels in the vessel areas compared to age-matched controls (**Figure R14A, B**). For the results from the cryosections, to ensure comprehensive representation of the entire vascular network (**Figure R14C**), we analyzed the staining using 3-5 sections per retina, and for each group, 5/6 retinas were used for quantification. We have now included this information in our revised manuscript (**Page 34, Line 1096-1097, 1100**).

We thank the reviewer for pointing that the neovascular tufts do not appear to form clearly in cross-sections of OIR retinas in our initial manuscript. Therefore, we replace the images, which shows clear neovascular tufts and high O-GlcNAc level in IsoB4 positive blood vessels (**Figure R14D**).

The new results are included in our revised manuscript (**revised Figure 1F, G, J**).

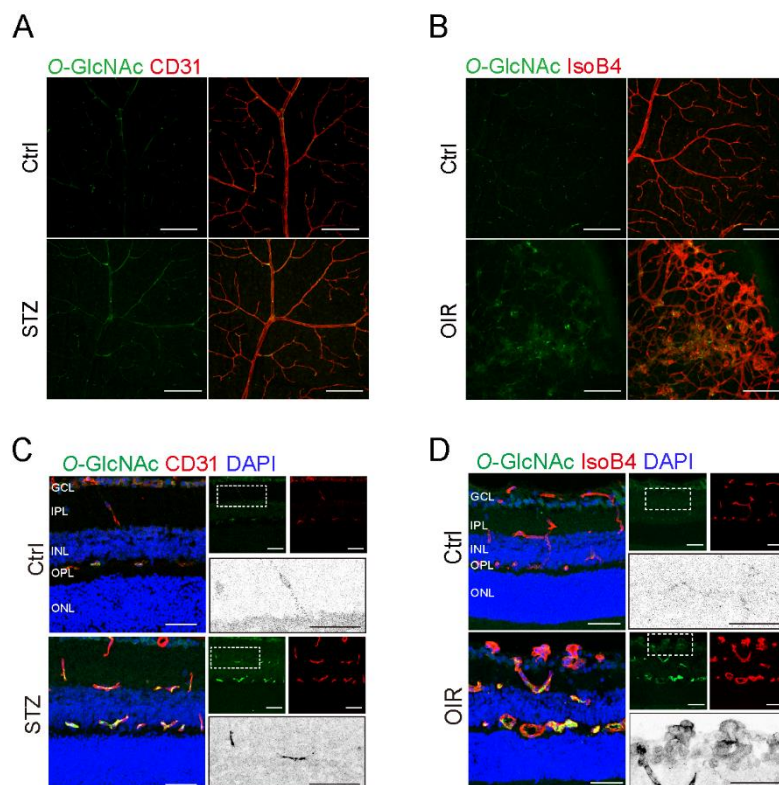


Figure R14

(A and B) Flat-mounted retinas from control and 8 weeks of diabetic mice or OIR pups stained for O-GlcNAc (green) and CD31/IsoB4 (red) showing the overall O-GlcNAc-modified protein levels.

(C and D) Representative images of O-GlcNAc (green or black), CD31/IsoB4 (red) and DAPI (blue) in frozen sections of eyeball from STZ and OIR pups and age-matched control pups.

Scale bars: 100 μ m in (A) and (B), 30 μ m in (C) and (D).

2. A causal role of O-GlcNAcylation could not be established in the analysis of angiogenesis (Fig. 2). Proliferation analysis of PUGNAc-treated HRCECs lacks direct causality with increased O-GlcNAcylation. Aortic ring and choroidal sprouting assays are not exclusive to the EC component. Vascular smooth muscle and endothelial cell sprouts cannot be distinguished by phalloidin staining.

Response:

We are grateful to the reviewer for highlighting this concern. We acknowledge that relying solely on PUGNAc for *in vitro* proliferation assays may not robustly establish O-GlcNAcylation as causative for angiogenesis. Furthermore, we agree with the reviewer's point regarding the limitations of the aortic ring assay in distinguishing vascular smooth muscle and endothelial cell sprouts. To address these concerns comprehensively, we took the following approaches:

1) In addition to utilizing PUGNAc as a gain-of-function approach, we incorporated a loss-of-function assay to complement our *in vitro* assays. Specifically, we performed angiogenesis assays (including

proliferation assay and sprouting assay) after knocking down *OGT*, the key enzyme responsible for catalyzing O-GlcNAc addition on proteins.

2) Apart from the aortic ring assay, we conducted the bead-sprouting assay, a well-established model for studying EC sprouting (Wang *et al*, 2017a).

Specifically, to evaluate the impact of inhibiting O-GlcNAcylation on proliferation, we transfected *OGT* siRNA into HRCECs and conducted a BrdU assay. Our findings revealed reduced EC proliferation in HRCECs transfected with si*OGT* (**Figure R15A, B**). Additionally, we assessed EC sprouting in HRCECs treated with PUGNAc or subjected to *OGT* knockdown. The results indicated a significant increase in sprout length with PUGNAc treatment, whereas si*OGT* led to decreased sprouting. These observations provide further *in vitro* evidence supporting the crucial role of O-GlcNAcylation in regulating angiogenic processes (**Figure R15C-F**). The new results have been incorporated into the revised manuscript (**revised Figure S2C, D, G-J**).

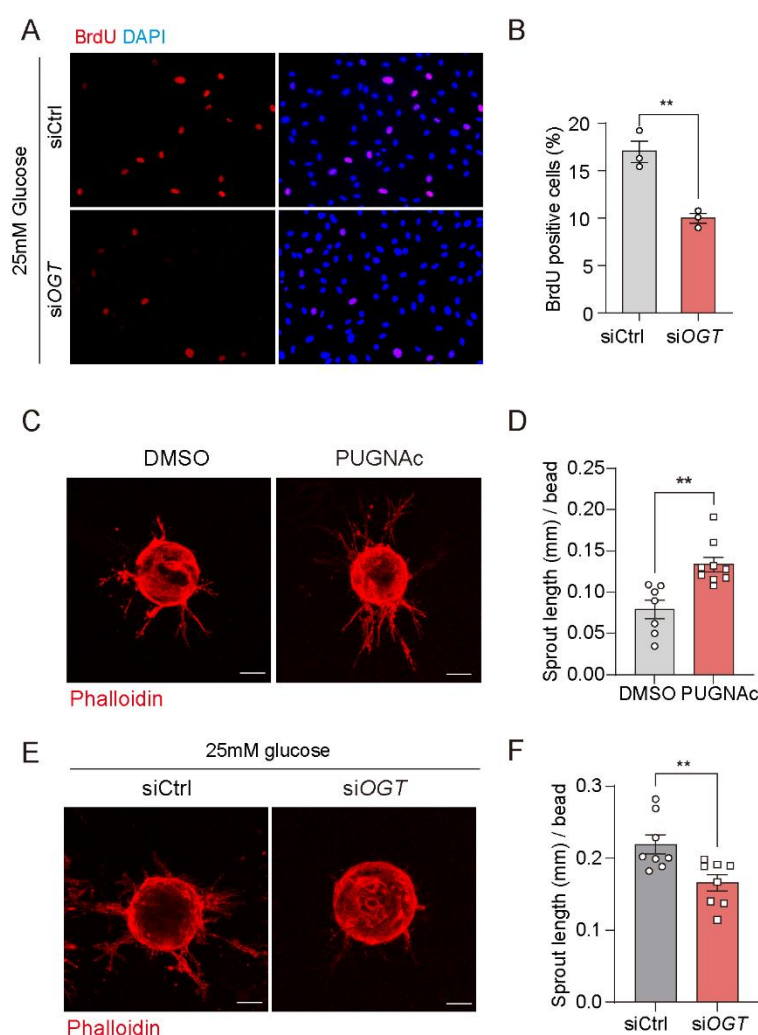


Figure R15

(A) BrdU (red) incorporation in HRCECs treated with siCtrl or si*OGT* for 24 hr.

(B) Quantification of BrdU⁺ cells as a percentage of the total cell number in (A) (n = 3/3 independent experiments).

(C) Representative images of bead-sprouting assay using HRCECs treated with DMSO or 25 μ M PUGNAc for 48 hr and stained with Phalloidin.

(D) Quantification of bead-sprouting length of (C) (n = 7/9 beads).

(E) Representative images of bead-sprouting assay using HRCECs transfected with siCtrl or siOGT for 48 hr and stained with Phalloidin.

(F) Quantification of bead-sprouting length of (E) (n = 8/8 beads).

*p < 0.05; Values as mean ± SEM compared by two-tailed Student's t-test in (B), (D) and (F) Scale bars: 30 µm in (A), (C) and (E).

3. Postnatal retinal angiogenesis is delayed in *Ogt* Δ EC mice, but, are the levels of O-GlcNAc reduced in *Ogt* Δ EC retina? The link between *Ogt* inactivation and O-GlcNAc levels in retinal ECs has not yet been demonstrated (Fig. S2).

Response:

We thank the reviewer for pointing this out. To demonstrate the reduction of O-GlcNAc in *Ogt* Δ EC mice, we are providing two evidences:

1) We isolated brain ECs and conducted qPCR analysis. The results showed a significant reduction in *Ogt* expression in ECs from *Ogt* Δ EC mice compared with *Ogt*^{WT} littermate controls (**Figure R16 A, B**).

2) We conducted O-GlcNAc staining on retinas from *Ogt*^{WT} and *Ogt* Δ EC mice across three models used in this study: the developmental model (P6 pups retina), the STZ model, and the OIR model. Remarkably, O-GlcNAcylation levels were consistently and significantly decreased in CD31/IsoB4-labeled blood vessels across all three models (**Figure R16 C-H**).

Based on these data, we provide evidence that inactivating *Ogt* leads to reduced O-GlcNAc levels in retinal vasculature. These results have been incorporated into the revised manuscript (**revised Figure S3N, O, S4A, B, F-I**).

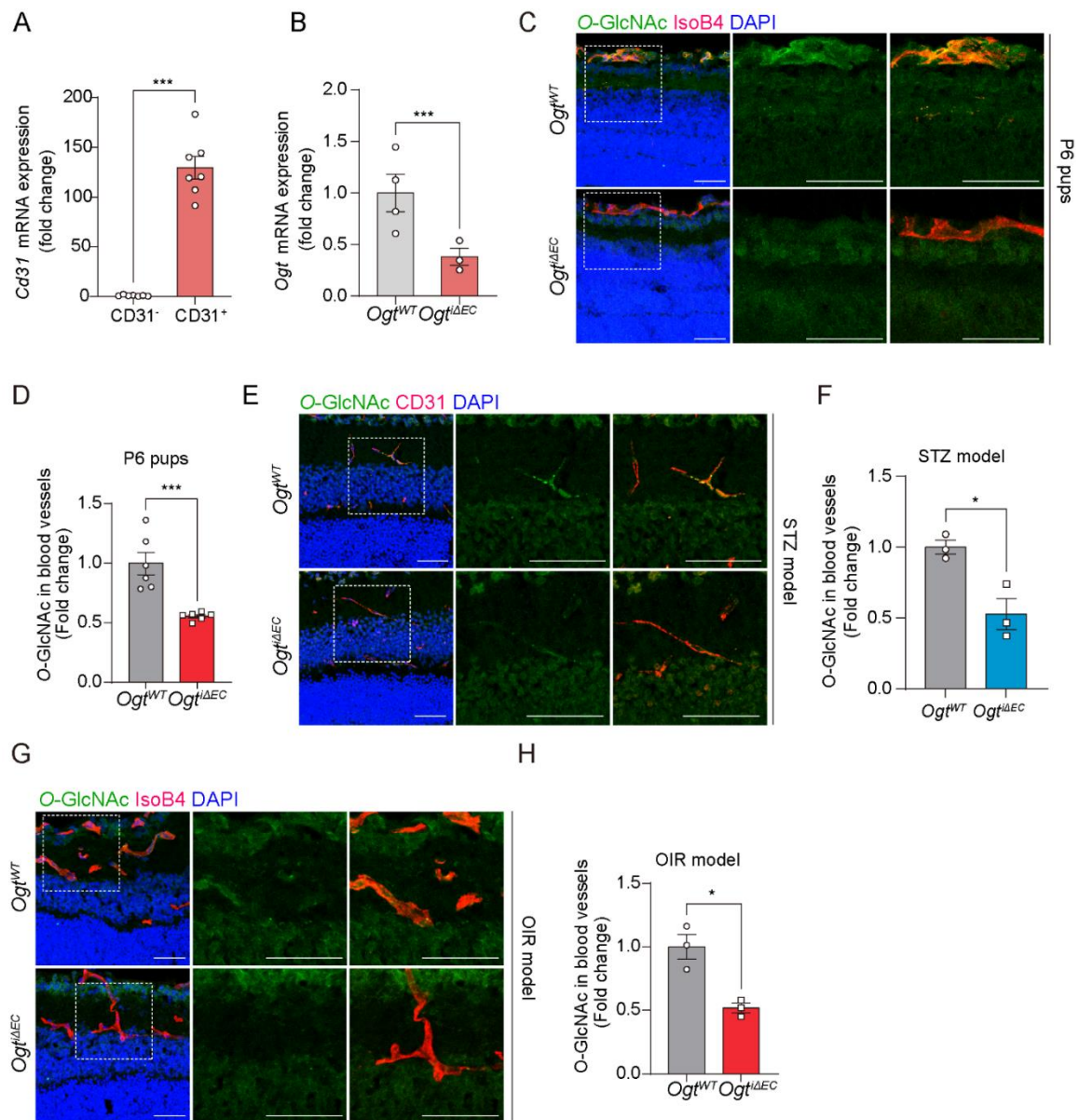


Figure R16

(A) qRT-PCR showing the expression of *Cd31* mRNA in the isolated EC fraction (CD31⁺) compared with CD31⁻ cells from brains of *Ogt*^{WT} and *Ogt*^{ΔEC} OIR pups (n = 7/7 mice).

(B) qRT-PCR showing the expression of *Ogt* mRNA in the isolated EC fraction (CD31⁺) from *Ogt*^{WT} and *Ogt*^{ΔEC} OIR pups (n = 4/3 mice).

(C) Representative immunostaining images of O-GlcNAc (green), IsoB4 (red), and DAPI (blue) in frozen sections of eyeballs from the *Ogt*^{WT} and *Ogt*^{ΔEC} P6 pups.

(D) Quantification of O-GlcNAc-modified protein levels within retinal vessels (IsoB4⁺ area) in P6 *Ogt*^{WT} and *Ogt*^{ΔEC} pups (n=6/6 mice).

(E) Representative images of O-GlcNAc (green), CD31 (red) and DAPI (blue) in frozen sections of eyeball from the *Ogt*^{WT} and *Ogt*^{ΔEC} STZ mice.

(F) Quantification of O-GlcNAc-modified protein levels within retinal vessels (CD31⁺ area) in the *Ogt*^{WT} and *Ogt*^{ΔEC} STZ mice (n=3/3 mice).

(G) Representative images of O-GlcNAc (green), IsoB4 (red) and DAPI (blue) in frozen sections of eyeball from the *Ogt*^{WT} and *Ogt*^{ΔEC} OIR pups.

(H) Quantification of O-GlcNAc-modified protein levels within retinal vessels (IsoB4⁺ area) in the *Ogt*^{WT} and *Ogt* ^{Δ EC} OIR pups (n=3/3 mice).

*p < 0.05; **p < 0.01; ***p < 0.001; Values as mean $\pm$ SEM compared by two-tailed Student's t-test in (A), (B), (D), (F), (H), Scale bars: 30 μ m in (C), (E), (G).

4. The ability of YAP to undergo O-GlcNAcylation has only been demonstrated *in vitro* in an artificial setting of a high glucose environment (Fig. 3). Characterization of glycosylated YAP signaling is superficial. Moreover, *in vivo* validation of the proposed mechanism showing upregulation of endogenous YAP O-GlcNAcylation would be needed to make this story compelling.

Response:

We appreciate this suggestion. Conducting extensive *in vivo* biochemical experiments similar to those in revised Figures 3 and 4 is challenging due to the limited quantity of retinal tissue available. Therefore, we opted to utilize the proximity ligation assay (PLA), a technique for detecting protein–protein interactions *in situ* at endogenous protein levels. This method has been previously used to assess the regulation of O-GlcNAcylation by targeting a substrate protein to OGT (Zhu & Hart, 2023). Using this approach, we examined the association between OGT and YAP/TAZ to assess O-GlcNAcylation of YAP/TAZ in both the STZ and OIR models. The results revealed a significant increase in interactions between OGT and YAP/TAZ in ECs in both models compared to age-matched controls (**Figure R17A-D**). These findings provide compelling evidence supporting the upregulation of endogenous YAP/TAZ O-GlcNAcylation in DR ECs. These results are now included in the revised manuscript (**revised Figure 5A-D**).

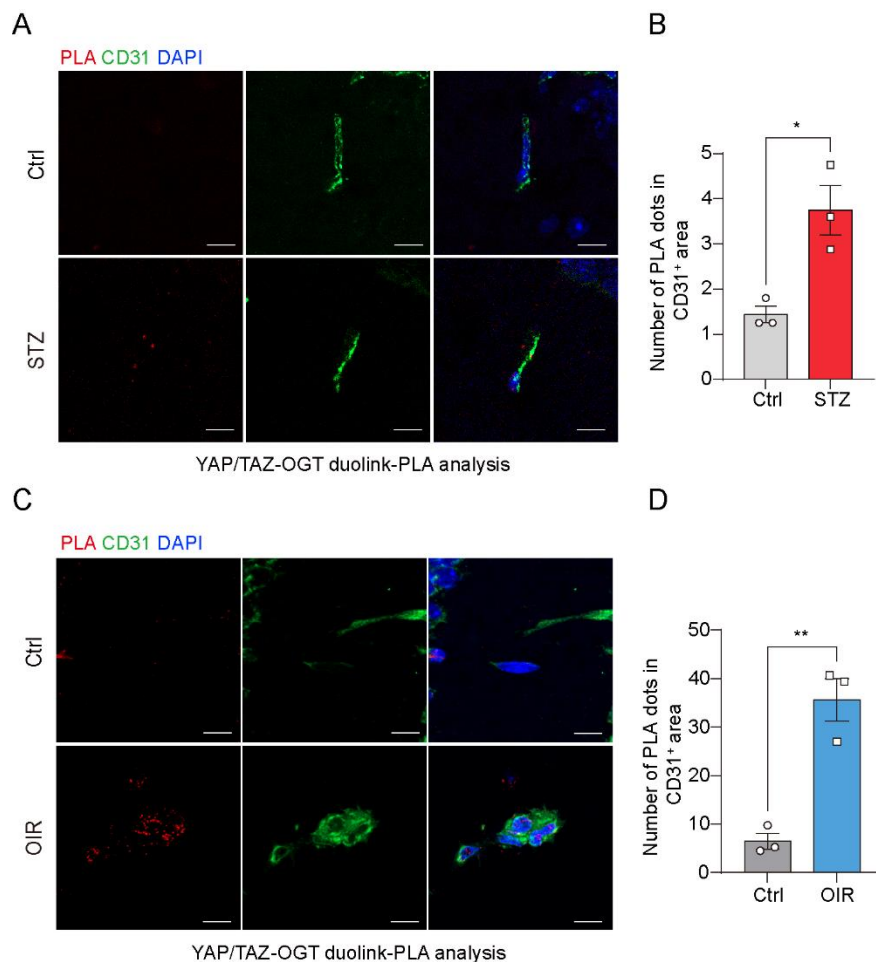


Figure R17

(A) PLA analysis showing the association between YAP/TAZ and OGT in frozen sections of eyeballs from control and STZ mice. CD31 was stained for blood vessels.

(B) Quantification of counts of PLA in CD31⁺ area of (A) (n=3/3 mice, 4-8 sections per mouse).

(C) PLA analysis showing the association between YAP/TAZ and OGT in frozen sections of eyeballs from control and OIR pups. CD31 was stained for blood vessels.

(D) Quantification of counts of PLA in CD31⁺ area of (C) (n=3/3 mice, 4-5 sections per mouse).

*p < 0.05; **p < 0.01; Values as mean ± SEM compared by two-tailed Student's t-test in (B), (D), Scale bars: 10 μm in (A), (C).

5. The protective effect of impaired O-GlcNAcylation in *Ogt*^{ΔEC} mice is abolished by the expression of wild-type YAP and YAP-S897A (Fig. 5E-I). Wouldn't this result rather suggest that activating YAP through O-GlcNAcylation is not a key driver of vascular dysfunction? Alternative mechanisms of YAP activation predominate.

Response:

We thank the reviewer for highlighting this point. In order to more clearly demonstrate that activating YAP through O-GlcNAcylation is a key driver of vascular dysfunction, we introduced a new group into our study: *Ogt*^{WT} mice injected with AAV-YAP-WT and further compared all the five groups in the STZ model. In *Ogt*^{WT} mice, the overexpression of YAP-WT significantly decreased the mural cell coverage compared with EGFP

group. Conversely, in *Ogt* ^{Δ EC} mice, overexpression of YAP-WT resulted in only a slight reduction of mural cell coverage. These results suggest that in the STZ model, the effect of YAP in promoting mural cell loss depends on OGT. However, the expression of YAP-S397A could nullify the protective effect of *Ogt* ^{Δ EC}, suggesting that the effect of S397A is not dependent on OGT (**Figure R18A, B**). Similar results were also observed in the OIR model (**Figure R18C, D**).

In a separate *in vivo* experiment from our initial manuscript, we introduced AAV-YAP-WT or AAV-YAP-T383A into *Yap/Taz* ^{Δ EC} mice and subjected them to the OIR model. The results demonstrated that AAV-YAP-WT injection significantly increased neovascularization, whereas the AAV-YAP-T383A group exhibited comparable levels to the control group, indicating that non-O-GlcNAc-modified YAP remains inactive in the OIR model (**revised Figure 7B, C**). To further prove the necessity of YAP-T383 in the regulation of the pericyte coverage in the STZ model, we performed a similar set of experiment by expressing AAV-YAP-WT or AAV- YAP-T383A in *Yap/Taz* ^{Δ EC} mice, AAV-EGFP served as the control. Similar to the findings in the OIR model, our results demonstrated that the expression of YAP-WT significantly decreased Desmin-positive cell coverage, while the YAP-T383A group exhibited levels comparable to the control group confirm this regulation is conserved in STZ-induced diabetes model (**Figure R18E, F**). The collective findings strongly suggest that OGT plays a pivotal role in YAP activation, contributing to neovascularization in the OIR model and mural cell loss in the STZ model.

These results are now included in the revised manuscript (**revised Figure 7**).

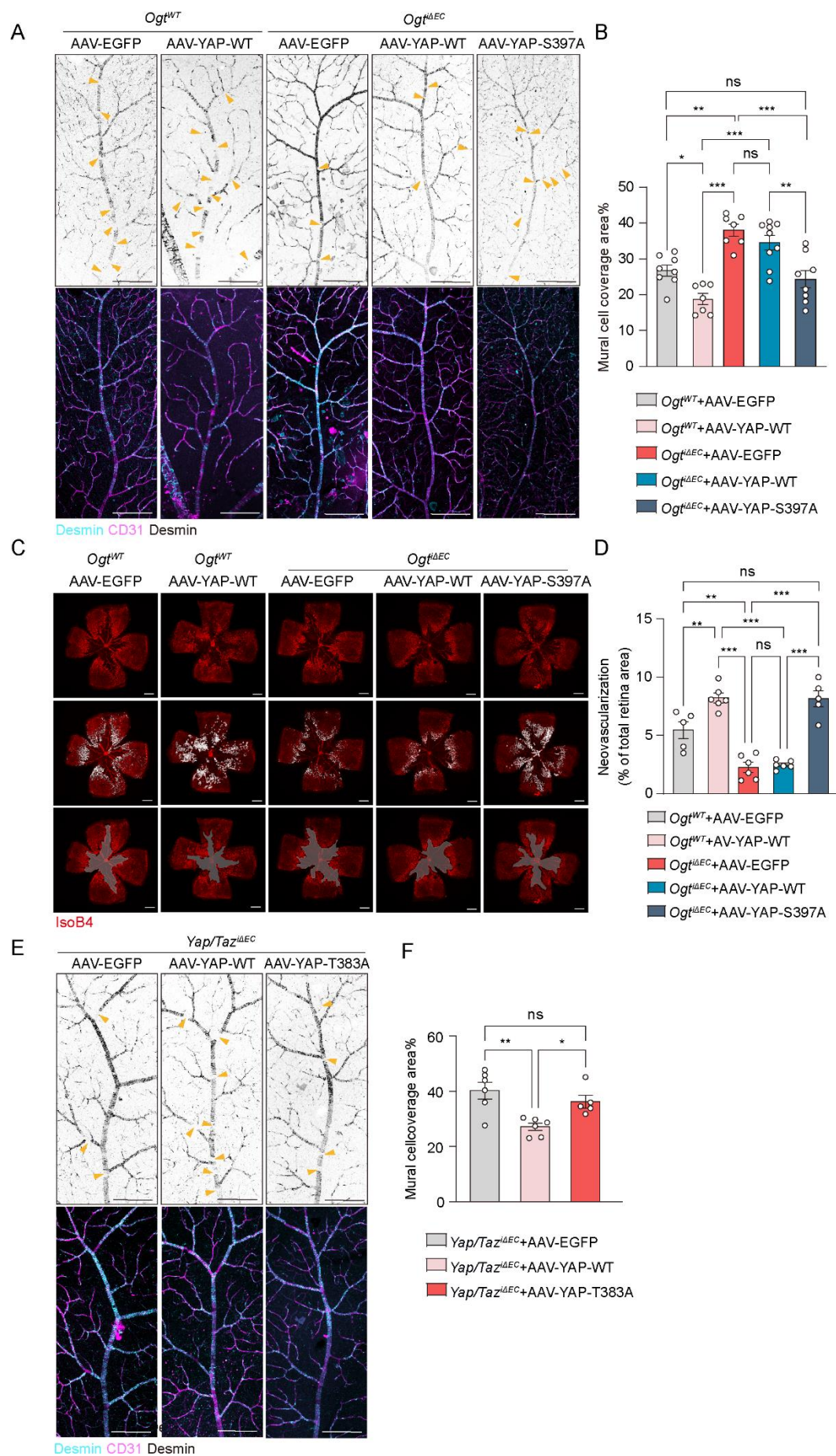


Figure R18

(A) Desmin and CD31 staining of retinas from AAV-BR1 injected *Ogt*^{ΔEC} mice. Arrowheads indicate vessels

without mural cell coverage.

(B) Quantification of mural cell coverage area (%) within the CD31⁺ vessels area of (A) (n = 8/7/7/9/8 mice).

(C) Representative images of the retina vasculature stained with IsoB4 in OIR retinas of AAV-BR1 injected *Ogt*^{WT} and *Ogt*^{ΔEC} pups.

(D) Quantitation of oxygen-induced retinal neovascularization in AAV-BR1 injected *Ogt*^{WT} and *Ogt*^{ΔEC} pups (n = 5/6/6/6/5 pups).

(E) Desmin and CD31 staining of retinas from AAV-BR1 injected *Yap/Taz*^{ΔEC} mice. Arrowheads indicate vessels without mural cell coverage.

(F) Quantification of mural cell coverage area (%) within the CD31⁺ vessels area of (C) (n = 6/6/5 mice).

*p < 0.05; **p < 0.01; ***p < 0.001; Values as mean ± SEM compared by one-way ANOVA followed by Tukey's multiple comparisons test in (B), (D), (F). Scale bars: 100 μm in (A), (E), 500 μm in (C).

6. Page 7, line 223, Fig.5A, the authors argue that the OIR model “showed high O-GlcNAcylation levels in blood vessels”. Firstly, this is not properly demonstrated. Moreover, a role of ambient glucose in inducing YAP O-GlcNAcylation in the OIR model is difficult to interpret in the light of the triggering environment - hypoxia and growth factor signaling (VEGF). YAP/TAZ is expected to be induced by these conditions. It is unclear whether the OIR model depends on glucose availability and O-GlcNAcylation modifications. How the OIR model provides a means of assessing glucose-induced YAP O-GlcNAcylation, and anti-angiogenic therapies thereof, is not readily apparent.

Response:

We thank the reviewer for highlighting this important concern. O-GlcNAcylation, as a posttranslational modification of proteins, acts as a nutrient sensor, responding to changes in the cellular microenvironment and regulating cell metabolism. Previous research has suggested that hypoxia-induced metabolic changes can also lead to increased O-GlcNAcylation levels (Ferrer *et al*, 2014; Yi *et al*, 2012). In our initial manuscript, we demonstrated that hypoxia induces a rise in O-GlcNAcylation in ECs (**revised Figure 1J, K, M**). We have now provided further clarification on this aspect in our revised manuscript (**page 5, line 112-114**).

However, we acknowledge the reviewer's point regarding the role of VEGF, which is indeed important. Previous studies conducted by our team and other researchers have highlighted VEGF as a potent activator of YAP/TAZ (Kim *et al*, 2017; Wang *et al.*, 2017a). Therefore, we analyzed the timing of VEGFA elevation and O-GlcNAcylation increase in the OIR model. Our observations revealed that retinal *Vegfa* levels increased on P15, whereas the elevation in O-GlcNAcylation occurred earlier, on P12 (**Figure R19A, B**). This discrepancy suggests that O-GlcNAcylation is a rapid response process and may contribute to vascular dysfunction at an earlier stage. This relationship was observed not only in the OIR model but also in the early stages of diabetes. In STZ-treated mice, *Vegfa* levels remained comparable to those in vehicle-treated littermates until week 14, as previously reported (Cerani *et al*, 2013). However, O-GlcNAcylation exhibited a notable increase at 4 weeks post-diabetes onset. These findings suggest that O-GlcNAcylation may serve as an early response factor contributing to vascular dysfunction in DR.

We have included these data in our revised manuscript (**revised Figure S7C, D**).

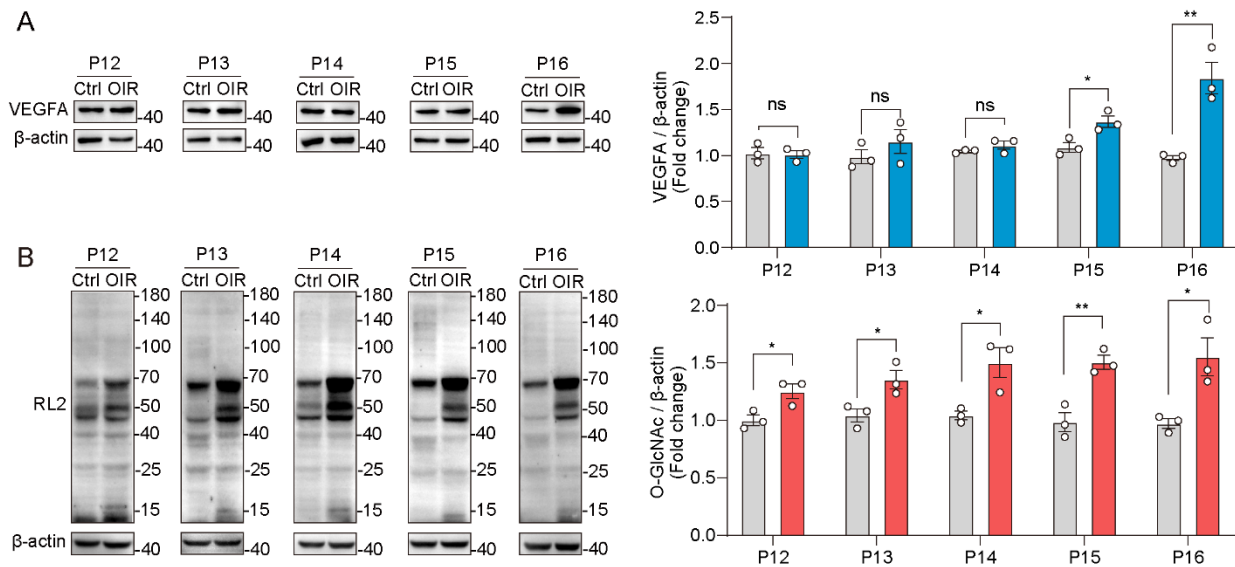


Figure R19

(A) Western blot analysis showing the overall levels of VEGFA in the retinas of OIR pups at indicated time points compared to age-matched control pups (n = 3/3/3 mice).

(B) Western blot analysis showing the expression levels of O-GlcNAc-modified proteins in the retina of OIR pups at indicated time points compared to age-matched control pups (n = 3/3/3 mice).

*p < 0.05; **p < 0.01; Values as mean $\pm$ SEM compared by two-tailed Student's t-test in (A) and (B).

7. It would be interesting to validate the AAV-mediated YAP-T383A and YAP-S397A tools in the physiological context of postnatal angiogenesis. This would help to consolidate the effects reported in the pathological settings.

Response:

Following the reviewer's suggestion, we evaluated the impact of AAV-mediated YAP-T383A and YAP-S397A mutations on physiological angiogenesis using the developing retina model. As AAV mediated gene expression requires relative long period, and we have observed expression of EGFP 10 days after injection in the vasculature of the retina and brain (**Figure R20A**), we retro-orbitally injected AAV-EGFP, AAV-YAP-WT, AAV-YAP-T383A, and AAV-YAP-S397A in P3 pups and analyzed the outcomes at P13. The injection of AAV constructs encoding YAP-WT, YAP-T383A, and YAP-S397A significantly elevated *Yap* mRNA expression levels in isolated brain ECs, indicating successful gene delivery (**Figure R20B**).

In mice, the retinal vasculature develops as ECs migrate from the optic nerve to the retinal surface at birth, progressing radially to form the superficial plexus. By postnatal day 7 (P7), sprouting vessels extend into the outer plexus layer (OPL), establishing the deep plexus. Between P11 and P12, vessels from the deep plexus ascend into the inner plexus layer (IPL), forming the intermediate plexus, which completes vascularization around P15 (Chen *et al*, 2021). We separately assessed the vascular coverage in each layer. The results revealed no statistical difference between the four groups in the superficial layer and the deep layer. Interestingly, when analyzing the intermediate layer, we found that the AAV-YAP-WT group increased vascular density compared to the AAV-EGFP group, while the AAV-YAP-T383A group showed a similar density to that in the AAV-EGFP group. The AAV-YAP-S397A group exhibited the highest vascular

coverage in the intermediate layer (**Figure R20C-E**). These results suggest that while YAP-WT and YAP-S397A promote developmental angiogenesis, YAP-T383A fails to do so. These results are now included in the revised manuscript (**revised Figure S9A-G**).

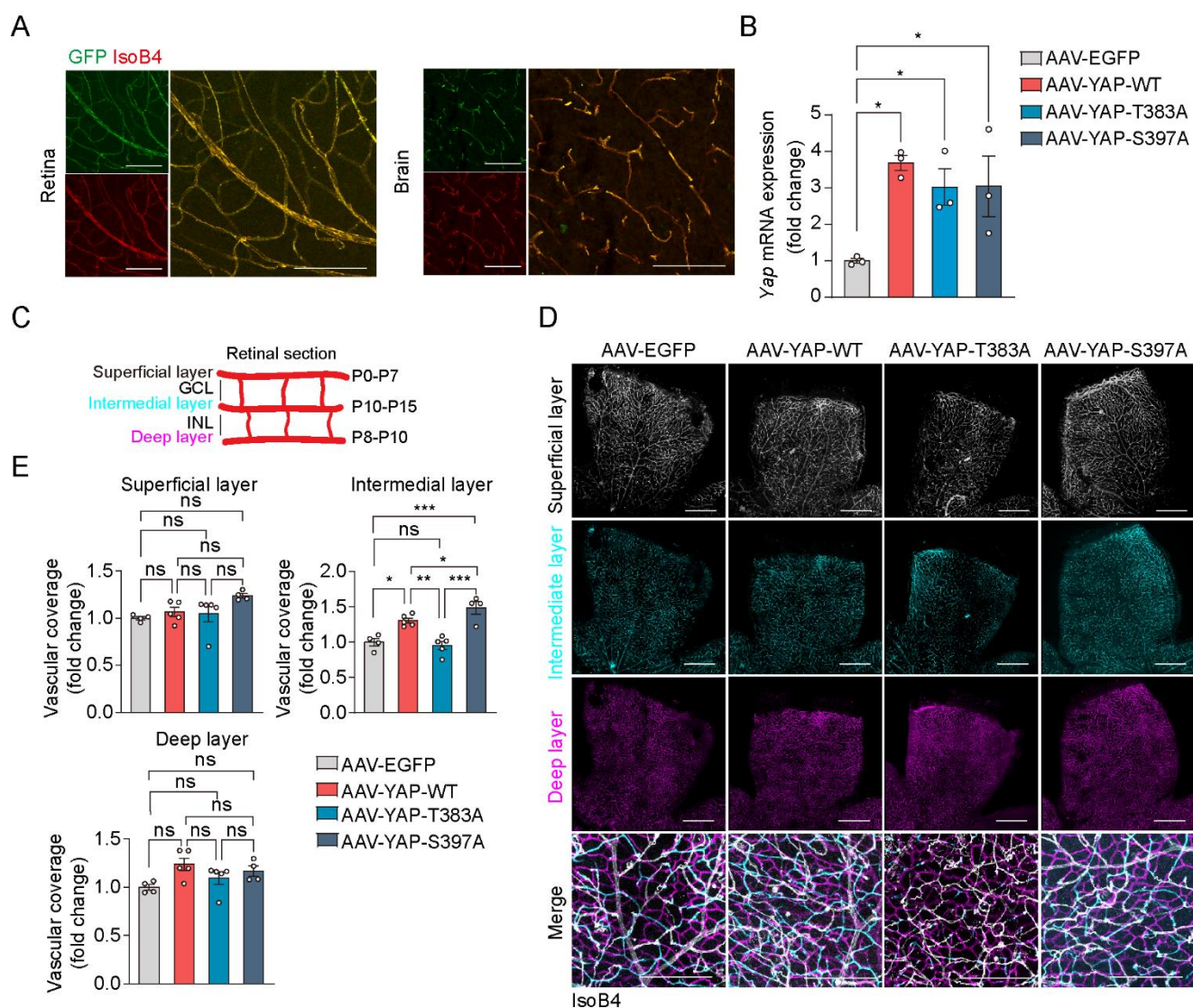


Figure R20

(A) Representative image from the retina and the brain showing that 10 days after AAV- EGFP retro-orbital injection, GFP expression could be observed in the blood vessels (IsoB4⁺ areas).

(B) qRT-PCR showing the expression of *Yap* mRNA in the isolated EC fraction from the brain treated as in (A).

(C) Schematic diagram of the development of mouse retinal blood vessel. Superficial layer develops during P0-P7, deep layer develops during P8-P10, and the intermedia layer develops during P10-P15.

(D) AAV- EGFP, AAV- YAP-WT, AAV- YAP-T383A, and AAV- YAP-S397A were retro-orbitally injected at P3 and the retina were harvest at P13. Representative images of the retina vasculature stained with IsoB4 were shown.

(E) Quantitation of vascular coverage in the three layers of retinas of (D) (n =4/5/5/4 pups).

*p < 0.05; **p < 0.01; ***p < 0.001; Values as mean ± SEM compared by one-way ANOVA followed by Dunnett's multiple comparisons test in (B) and one-way ANOVA followed by Tukey's multiple comparisons

test in (E). Scale bars: 30 μ m in (A), 500 μ m in (D).

Other comments:

8. YAP/TAZ pathway has been implicated in pathological angiogenesis. For example, inhibition of YAP alleviates retinopathies using oxygen-induced retinopathy (OIR) (Kim J, 2017 JCI) and laser-induced choroidal neovascularization (Yan Z 2018 Mol Vis). This reduces the novelty in Fig. 4.

Response:

Previous studies, including ours (Kim *et al.*, 2017; Wang *et al.*, 2017b), have established YAP/TAZ pathway as a crucial regulator in pathological angiogenesis. While some studies have shown that YAP/TAZ contribute to pathological retinal angiogenesis and that inhibiting YAP/TAZ alleviates angiogenesis (Kim *et al.*, 2017; Yan *et al.*, 2018), to the best of our knowledge, our study is the first to utilize endothelial-specific *Yap/Taz* knockout mice to demonstrate the critical role of YAP/TAZ in regulating pathological retinal angiogenesis in DR models (STZ model and OIR model). Furthermore, we elucidate the regulatory mechanism in this study. Given the crucial role of YAP/TAZ in the regulation of pathological angiogenesis, we believe that this study contributes new insights and mechanisms to this field.

9. Desmin is used as a pericyte marker. However, this is not accurate as Desmin is also highly expressed in smooth muscle cells SMCs (single-cell databases), especially arterial SMCs, the vessel type used throughout the manuscript to assess pericyte coverage.

Response:

We share the concern that it's not accurate to use Desmin as pericyte marker. It is true that there is no perfect marker for identifying pericytes, as markers like platelet-derived growth factor receptor β (PDGFR β), neural/glial antigen 2 (NG2), and Desmin are also expressed in other cell types in certain tissues (Figueiredo *et al.*, 2020).

Desmin has been widely utilized to label retinal pericytes in numerous studies (Chen *et al.*, 2021; Figueiredo *et al.*, 2020; Hu *et al.*, 2017a), making it a commonly used marker in this context. However, we acknowledge that Desmin is also highly expressed in smooth muscle cells. Vascular mural cells encompass both pericytes and smooth muscle cells and are essential components of the retinal vasculature. They play crucial roles in maintaining the inner blood-retinal barrier, regulating vascular tone, and supporting ECs. Histopathologic studies on donor eyes have highlighted widespread loss of both pericytes and smooth muscle cells in DR, indicating their significant involvement in the pathogenesis of DR (Huang *et al.*, 2023). To improve accuracy, we have updated our statement to refer to Desmin-labeled cells as "vascular mural cells" instead of specifically mentioning "pericytes" in our revised manuscript.

10. Transcript levels of CTGF and CYR61 (Fig. 3J). Why display qPCR data when the RNA-seq experiment was performed in PUGNAc-treated HRCECs (Fig. 3A)? Methodological approach is unclear.

Response:

Our initial goal was to validate the RNA-seq experiment; however, we acknowledge the reviewer's point that this data may be redundant. To complement the inhibitor use, we conducted OGT/OGA siRNA

knockdown experiments and overexpression experiments for validation purposes. Knockdown of *OGT* led to a decrease in the expression of YAP/TAZ target genes *CTGF* and *CYR61*, whereas knockdown of *OGA* resulted in increased expression in HRCECs (**Figure R21A, B**). Furthermore, we employed adenovirus-mediated overexpression of *OGT* and *OGA* to strengthen our findings. *OGT* overexpression increased the expression of YAP/TAZ target genes *CTGF* and *CYR61*, while *OGA* overexpression reduced their expression in HRCECs (**Figure R21C, D**). These results support the hypothesis that O-GlcNAcylation of YAP plays a crucial role in regulating YAP downstream genes in retinal ECs. The new results are included in our revised manuscript (**revised Figure 4F, G, Figure S5I, J, K, L, O, P**).

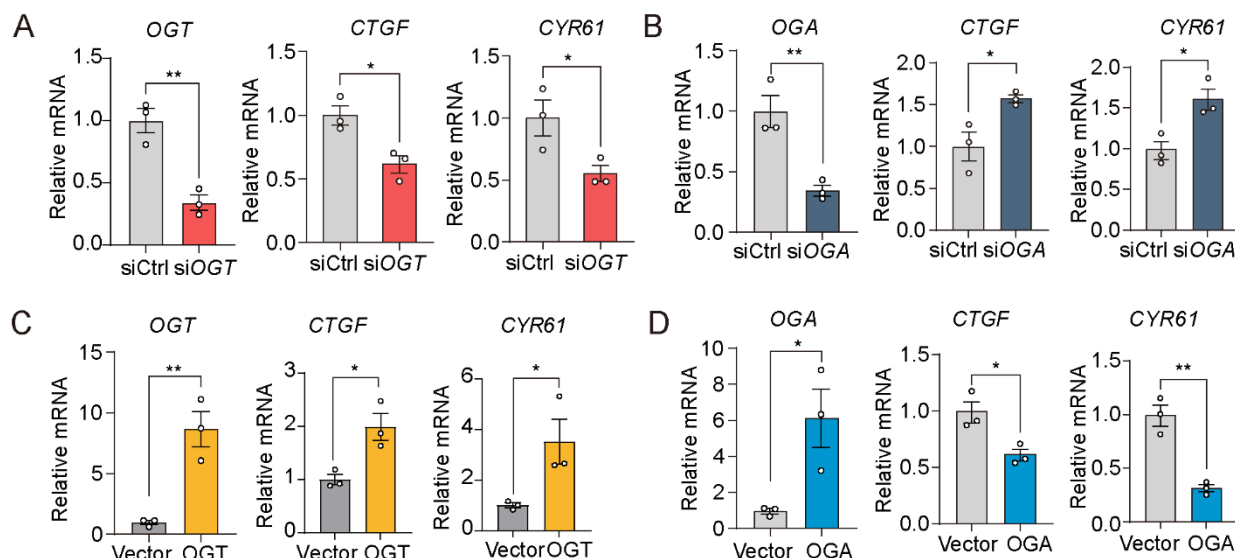


Figure R21

(A and B) HRCECs were transfected with siCtrl or siOGT/siOGA and cultured in 25 mM glucose medium. Relative mRNA level of *CTGF* and *CYR61* were analyzed by qPCR (n=3 independent experiments).

(C and D) HRCECs were infected with Ad-Vector or Ad-OGT/OGA for 24 hr, relative mRNA level of *CTGF* and *CYR61* in HRCECs were analyzed by qPCR (n=3 independent experiments).

*p < 0.05; **p < 0.01; ***p < 0.001; Values as mean ± SEM compared by two-tailed Student's t-test in (A-D).

11. Why is YAP/TAZ deletion induced at P12 and not earlier (5A-D)? Has the effectiveness of gene deletion been verified? This variable needs to be tightly controlled to ensure a consistent baseline of OIR progression.

Response:

In the OIR model, P7 mouse pups, accompanied by nursing mothers, are exposed to hyperoxia (75% oxygen) for 5 days, leading to inhibited retinal vessel growth and significant vessel loss. On P12, the mice are returned to room air, initiating a phase of relative hypoxia. This hypoxic phase stimulates pathological neovascularization characterized by the formation of abnormal blood vessels as the retina attempts to compensate for prior oxygen-induced damage (Connor *et al*, 2009). In our study, we followed the previously reported Tamoxifen induction strategy, which starts right after the pups were returned to room air (at P12) (Tisch *et al*, 2019), focusing specifically on the process of pathological neovascularization.

We agree with the reviewer that the effectiveness of gene deletion should be verified. In the OIR model, we induced Cre recombination through intraperitoneal administration of Tamoxifen. Due to challenges in isolating a sufficient number of ECs from the retina, we isolated ECs from the brains of OIR mice (**Figure R22A**). The mRNA levels of *Yap* and *Taz* was significantly reduced in the isolated brain ECs from *Yap/Taz^{ΔEC}* mice, validating the efficacy of the knockout approach (**Figure R22B**). In the STZ model, Cre recombination was induced via intravitreal injection of 10 μg 4-OHT one week after the onset of STZ-induced diabetes. We performed immunostaining to assess the expression of YAP/TAZ within ECs of retinal sections (**Figure R22C, D**). The observed decrease in YAP/TAZ expression in *Yap/Taz^{ΔEC}* mice confirmed the successful knockout efficiency in this model. We have included the new results in our revised manuscript (**revised Figure S8F-I**).

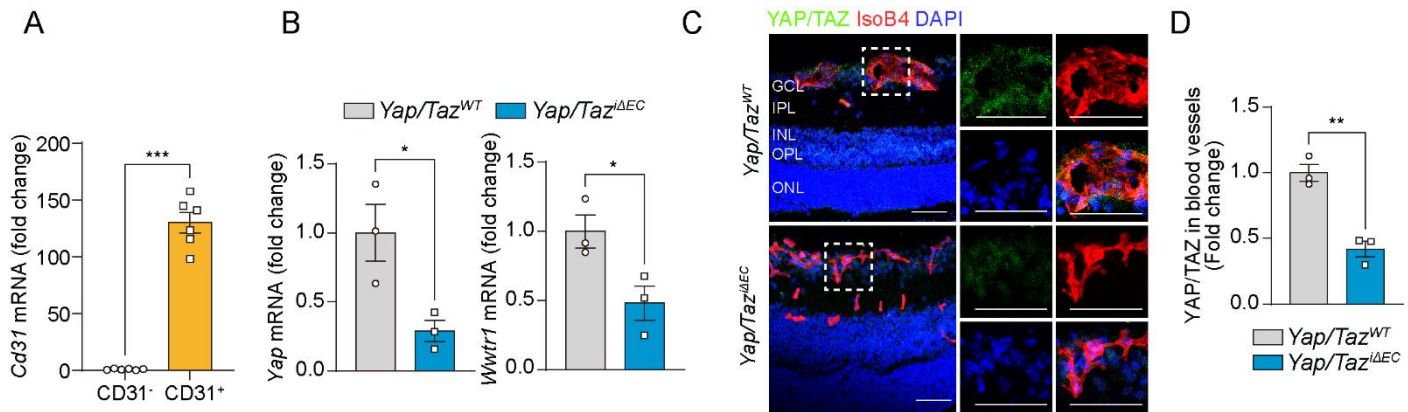


Figure R22

(A) qRT-PCR showing the expression of *Cd31* mRNA in the isolated EC fraction (CD31⁺) compared with CD31⁻ cells from brains of *Yap/Taz^{WT}* and *Yap/Taz^{ΔEC}* OIR pups (n = 6 mice).

(B) qRT-PCR showing the expression of *Yap* and *Taz* mRNA in the isolated EC fraction (CD31⁺) from *Yap/Taz^{WT}* and *Yap/Taz^{ΔEC}* OIR pups (n = 3/3 mice).

(C) Representative images of YAP/TAZ (green), IsoB4 (red) and DAPI (blue) in frozen sections of eyeball from *Yap/Taz^{WT}* and *Yap/Taz^{ΔEC}* STZ mice.

(D) Quantification of YAP/TAZ expression within retinal vessels (IsoB4⁺ area) in *Yap/Taz^{WT}* and *Yap/Taz^{ΔEC}* STZ mice (n=3/3 mice).

*p < 0.05, **p < 0.01, ***p < 0.001; Values as mean ± SEM compared by two-tailed Student's t-test in (A), (B), (D). Scale bars: 30 μm in (C).

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REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

Lei et al. have submitted a revised version of their manuscript, in which they describe a role for O-GlcNAcylation in regulating YAP signaling in diabetic vascular disease in the retina. The revised manuscript contains additional data and clarifications where requested. Some of the data, particularly the mechanistic biochemical data—such as the sites of O-GlcNAcylation—are still not clear yet, but overall the work is improved and consistent with previous studies describing YAP GlcNAcylation in cancer cells (Zhang et al., Nature Communications, 2017). Although I am not entirely enthusiastic, I consider the manuscript to be acceptable.

Reviewer #4 (Remarks to the Author):

The authors have been VERY responsive to the previous critiques of this manuscript and added key experimental data that clarifies and further supports their findings (multiple orthogonal approaches all coming to same significant conclusions) that were already met with enthusiasm. This manuscript is of significant moment as it illustrates how pathological (diabetic retinopathy) shifts in the nutrient sensing O-GlcNAc pathway impact the Hippo pathway leading to vascular dysfunction and pro-pathology transcriptional profiles. This important work not only provides a better mechanistic insight into how O-GlcNAc and the Hippo pathways work together in regulation of transcription but also provides potential insight into therapeutic approaches for an unfortunately too common pathology (diabetic retinopathy). Thus, enthusiasm is extremely high for this revised manuscript.