Supplemental data

Supplemental materials and methods

Cloning and production of cZNF532 shRNA adeno-associated virus

Three different shRNA vectors were designed for cZNF532 silencing. In brief, 200 pmol of the forward and reverse primers containing the shRNA sequence were dissolved in 2 × annealing buffer (20 mM Tris, pH 7.8, 100 mM NaCl and 0.2 mM EDTA). The solution was boiled for 5 minutes and then cooled to room temperature. Annealed oligos were ligated to the Bgl2/Hind3-cut backbone fragment of pSUPER-hSyn-EGFP-CytB-AS.

This first cloning step resulted in the vectors, pSUPER-hSyn-EGFP-H1-cZNF532 shRNA. From these vectors, the insert containing the H1-promoter and the shRNA were cut out. Then, shRNA or scrambled sequences were cloned into the AAV vector (pAAV-U6-GFP-shRNA). For AAV production, the vectors were co-transfected with the pAAV-RC1 and pHelper vector in HEK293T packaging cell line. Forty-eight hours after transfection, these cells were harvested and purified by ultracentrifugation.

cZNF532 overexpression vector construction

To overexpress cZNF532, the genomic sequence for cZNF532 together with the 300 bp upstream and 300 bp downstream sequence was chemically synthesized. These sequences were sufficient to allow the intervening exons of ZNF532 to circularize in vivo. These sequences were finally cloned into the recombinant adeno-associated virus (rAAV). rAAV were packaged in HEK293T cell cotransfected

with the pAAV-RC1 and pHelper vector. The vector preparations were purified by dialysis and titered by qRT-PCRs.

MTT assay

Cell viability was detected by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay (Biosharp Company, BS030). After the required treatment, pericytes were cultured on a 96-well plate (3×10^4 cells per well), and then the plate was returned to the standard tissue incubator condition for another 6 hours at 37℃. After that, cells were incubated with MTT (5 mg/mL in PBS) for 3 hours at 37°C. DMSO solution was added to dissolve the purple-blue MTT formazan precipitate. The absorbance was detected at 570 nm wavelength by a microplate reader (Molecular Devices, FilterMax F5).

RNase R treatment

For RNase R treatment, about 2 μg of total RNAs from pericytes were incubated with or without 3U μg^{-1} of RNase R (Sigma-Aldrich, R4642) for 30 minutes at 37°C. The resulting RNAs were subsequently purified using an RNeasy MinElute cleaning Kit (Qiagen, 74204).

RNA fluorescent in situ hybridization (RNA-FISH)

RNA in situ hybridization was conducted using FITC- or Cy3-labelled RNA probes to miRNA-29a-3p or cZNF532 sequence. Pericytes were grown to the exponential phase and fixed at 80-90% confluence. After the prehybridization (1×PBS/0.5% Triton X-100), they were incubated with the RNA probes in the hybridization buffer (40% formamide, 10% Dextran sulfate, 1×Denhardt's solution,

4×SSC, 10 mM DDT, 1 mg ml^{-1} yeast transfer RNA, 1 mg ml^{-1} sheared salmon sperm DNA) at 60℃ overnight. The nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, Molecular Probe, D1306).

Cell proliferation detected by Ki67 staining

Ki67 staining was used to detect the proliferation of pericytes or HRVECs. The cells were fixed with 4% paraformaldehyde solution for 15 minutes at room temperature and washed three times with PBS buffer solution. These cells were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, T9284) at 4℃ for 5 minutes, and then blocked with 0.1% Triton X-100 mixed with 5% BSA for 30 minutes at 37℃. These cells were incubated with Ki67 antibody (1:100, Abcam, ab16667) overnight at 4°C, and then incubated with the Alexa 594 conjugated goat anti-rabbit IgG (1:1000, Invitrogen, A11012) for 3 hours at room temperature in the dark. They were washed with PBS twice and stained with DAPI to show nuclei. The images were captured by the Olympus IX73P1F fluorescence microscopy (Olympus Corporation) and analyzed by Image J. The ratio of $Ki67^+$ cell number to total cell number was used for comparing proliferation ability of pericytes.

Matrigel co-culture assay

About 2×10^5 HRVECs were seeded onto the 24-well plate coated with the Matrigel layer in a maximum volume of 500 μl and incubated for 4 hours. Afterwards, the medium was removed and about 3×10^4 pericytes were added in a maximum volume of 500 μl for 4 hours. Pericyte culture medium and ECM (1:1) were added to reach a final volume of 600 μl. The plate was incubated for 8 hours or 12 hours. Next,

these cells were fixed for 15 minutes, stained with CD31 (1:100, BD Pharmingen, 550300) to label endothelial cells, and then stained with NG2 to label pericytes (1:100, Abcam, ab50009). When the two different colors merged together, it was considered that pericytes were recruited to endothelial cells.

Cell cycle evaluation by flow cytometry

Pericytes were fixed with 70% ethanol after the transfection of scramble siRNAs or cZNF532 siRNAs. These cells were washed with FACS buffer (phosphate-buffered saline containing 0.1% bovine serum albumin) and incubated with FACS buffer containing 50 μg/ml RNase A (Sigma-Aldrich, R4642) for 30 minutes at 37°C. After incubation, these cells were stained with propidium iodide (25 μg/ml, Molecular Probes, P3566) to show the nuclei and analyzed using FACSCalibur and Cell Quest Pro software (BD Biosciences). The synchronization of pericytes was conducted through serum deprivation. The cells were re-stimulated with serum for the indicated time.

Measurement of barrier permeability

The Transwell filters (0.4-μm pore size, 12-well, Corning) were coated with fibronectin (30 μg/mL) and used for the co-culture of pericytes and HRVECs. After the required treatment, pericyte monolayer was seeded onto the basolateral chamber of the filter at a density of 3×10^4 cells. HRVECs were grown to confluence were seeded onto the apical chamber of the filter at a density of 5×10^4 cells. They were co-cultured for 8 hours or 12 hours. The measurement of barrier permeability was conducted by adding Fluorescein Isothiocyanate-dextran (0.25 mg/ml, average

molecular weight 70,000, Sigma-Aldrich, FD70S-100MG) to the apical chamber. After 30, 60, 90, and 120 minutes, 100 µl samples were collected from the basolateral chamber and placed in a 96 well dish. The sample was collected from the apical chamber at the last time point. The fluorescence of the aliquots was detected by a microplate reader using 485 nm and 538 nm as the excitation and emission wave-lengths, respectively. The diffusive rate of flux, *P*o, was determined for the 2-hour period in which samples were taken and calculated by the following formula:

$$Po = [(F_A/\Delta t)\ V_A\ ] / (F_L A)$$

where, *Po* is diffusive flux; F_A is basolateral fluorescence; F_L is apical fluorescence; Δt is change in time; A is the surface area of the filter (in square centimeters); and V_A is the volume of the basolateral chamber (in cubic centimeters).

Propidium iodide (PI) staining

The apoptosis percentage of pericytes was measured by PI staining. The cells were washed twice in cold 1 × PBS, stained with PI for 15 minutes at room temperature, and then stained with DAPI (0.5 µg/ml, Molecular Probe, D1306) for 5 minutes to show cell nuclei.

Caspase-3/7 activity assay

Caspases 3/7 activity was measured by the Apo-ONE™ Homogeneous Caspase 3/7 assay kit (Promega Corp, G8090) according to the manufacturer's protocol. Human pericytes (1×10^4 per well) or HRVECs (1×10^4 per well) were seeded in a 96-well plate. After the required treatment, equal volumes of DMEM and Apo-ONE™ caspase reagent (1:100 pro-fluorescent substrate and lysis buffer) were

added to cells. The mixture was incubated for 3 hours. The fluorescence was detected by a microplate reader at the wavelengths of 480 nm and 535 nm for excitation and emission. The background fluorescence was detected by the fluorescence from DMEM alone and subtracted from all experimental values.

Luciferase reporter assay

The entire cZNF532 sequence and mutant cZNF532 sequence without miRNA binding site were chemically synthesized. They were inserted into the KpnI and Hind III sites of pGL3-basic to generate expression vectors. For overexpression of miR-29a-3p and miR-21, a fragment of ~200 nt encompassing the miRNA gene was amplified from genomic DNA and cloned into the pJEBB vector. HEK293T cells were seeded in 96-well plates at a density of 6×10^3 cells per well 24 hours before transfection. HEK293T cells were co-transfected as shown in figure legend. 48 hours after transfection, the cells were collected and luciferase activity was measured by the Dual-Luciferase Reporter Assay kit (Promega). The vector pRL-TK expressing *Renilla luciferase* was used as the internal control for transfection. The empty vector pGL3-basic was used as the negative control. For comparison, the *firefly luciferase* activity of pGL3 vector was normalized with *Renilla luciferase* activity.

Luciferase reporter assay was also used to investigate the regulation of miR-29a-3p on the expression of its target genes. The 3'-untranslated regions (3'-UTR) of *CSPG4*, *LOXL2*, and *CDK2* were amplified by PCR assays and inserted into the downstream of the luciferase gene of pGL3-control vector (Promega). Two hundred nanograms of pGL3-vector containing corresponding gene sequence were

transfected in combination with miR-29a-3p mimic and Vector pRL-TK. The luciferase activity was detected 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega).

RNA isolation and qRT-PCR analysis

Total RNAs were isolated using the TRIzol reagent (Life Technologies, 15596-026) according to manufacturer's protocol. The concentration of RNA was detected by a spectrophotometer at 260 nm. Reverse transcription was conducted with 1 μg of total RNA using the superscript first-strand synthesis system. qRT-PCRs were conducted using SYBR Green PCR kit. Each sample was detected in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression. The formation of a single product was confirmed by observing the melting curve graph generated by the sequence-detection system. All data were normalized to *GAPDH* expression and shown as the fold increase compared with control group.

Antagomir and agomir sequence

The sequences of miRNA agomirs and antagomirs were shown as below: miR-29a-3p agomir: 2′-O-methylated-sulfo-5′-Cy3-UAGCACCAUCUGAAAUCG GUUACUGU-cholesterol-3′ (sense), 2′-O-methylated-sulfo-5′-Cy3-AGUAACCGAUUUCAGAUGGUGCUAUU-cholesterol-3′ (antisense); miR-29a-3p antagomir: 2′-O-methylated-sulfo-5′-Cy3-UAACCGAUUUCAGAUGGUGCUA -cholesterol-3′. The universal negative controls for both agomir and antagomir are based on the sequences of *cel*-miR-67 in *Caenorhabditis elegans*, which has been confirmed to have minimal sequence identity with miRNAs in human, mouse, and rat.

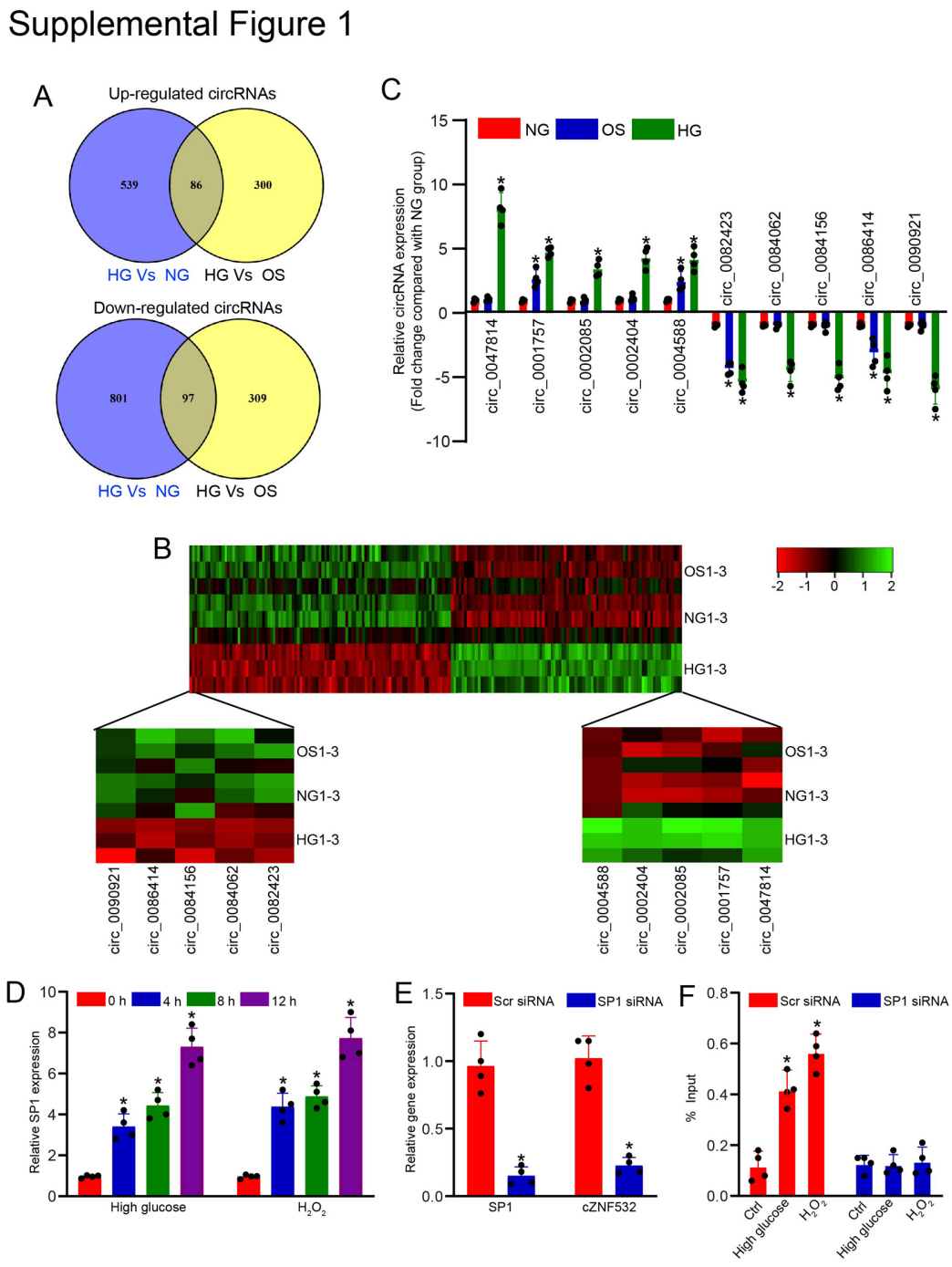
Vitreous sample preparation

This study was conducted according to the tenets of the Declaration of Helsinki and received approval from the institutional review committee of the authors' institute. Vitreous specimens were obtained from 36 subjects undergoing pars plana vitrectomy for the treatment of DR (n=28 eyes) and other retinal disorders (n=8 eyes). All subjects were informed of the purpose and the procedures of the study. Inclusion criteria for patients with DME included the diagnosis of diabetes and the presence of ME or PDR. Exclusion criteria for control patients included non-diabetes or any known ischemic retinal diseases. Vitreous samples from patients with ME or PDR were used as experimental group. Vitreous samples from non-diabetic patients for the treatment of macular hole were used as control group. The harvested vitreous samples were collected in the tubes, placed immediately on ice, centrifuged for 15 minutes to remove insoluble material, and stored at 80°C until use.

PCR primer sequence, siRNA sequence, and shRNA target sequence

Primer sequence		
cZNF532	Forward	5’- TCTTCCAGGACGAGACATCAC-3’
	Reverse	5’- AATGCTGCCAGGAGGTCATC -3’
cZnf532	Forward	5’- GTTATCTCAGCTCCGGCAAG-3’
	Reverse	5’- TATCCCCCATGGTCATGAAT-3’
ZNF532	Forward	5’- CTTCATGGCAGCCAAAAGCA-3’
	Reverse	5’- AACCAAGTGTCCCCACCCTA-3’
Znf532	Forward	5’- TCCCTTTGATTTGGGCTTGGT-3’
	Reverse	5’-CGACGTCTGAGGATGATGGG-3’
GAPDH	Forward	5’-AAGACGGGCGGAGAGAAACC-3’
	Reverse	5’-CGTTGACTCCGACCTTCACC-3’
Gapdh	Forward	5’-GTCAAGGCTGAGAACGGGAA-3’
	Reverse	5’-AAATGAGCCCCAGCCTTCTC-3’
CSPG4	Forward	5’- GAGCCCAGGCACGAAAAATG-3’
	Reverse	5’- GTATGTTTGGCCCCTCCGAA-3’
LOXL2	Forward	5’- AGGGACCCTCCCATCATTCA-3’
	Reverse	5’- GGAGGAGAAATGGGGTTCGG-3’
CDK2	Forward	5’-TCTTTGCTGAGATGGTGACTCG-3’
	Reverse	5’- TGTTAGGGTCGTAGTGCAGC-3’
siRNA sequence		
cZNF532 siRNA1		5’- GACAAAACATCTGCTCAAATT-3′
cZNF532 siRNA2		5’- AGATACGAGTGGACAAAACAT-3′
cZNF532 siRNA3		5’- GGACAAAACATCTGCTCAAAT-3′
shRNA target sequence		
circZNF532 shRNA1		5’- GTGGACAAAACTGATCAAATTCA-3′
circZNF532 shRNA2		5’- GACAAAACTGATCAAATTCATGA-3′
circZNF532 shRNA3		5’- CGAGTGGACAAAACATCTGCT-3′

Supplemental Figure 1

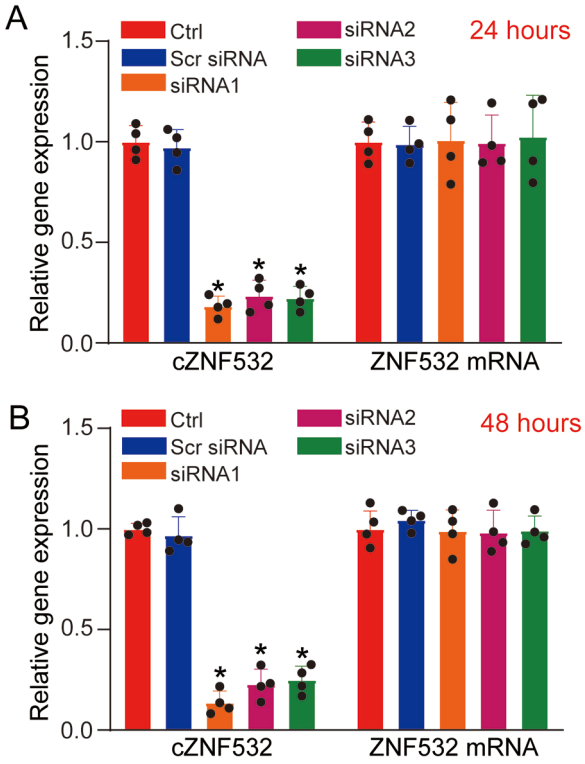


Supplemental Figure 1: Identification of cZNF532 as a high glucose-regulated circRNA and its transcriptional regulatory mechanism

(A)Venn diagrams showed the number of differentially expressed circRNAs in the pairwise comparison (HG vs. NG and HG vs. OS, Fold change >2; $P< 0.05$) by the SBC Human (4×180K) Circular RNA Microarray (Shanghai Biotechnology Co., Ltd.)

and the common circRNAs shared by the two comparisons. (B) Hierarchical clustering of differentially expressed circRNAs among HG, NG, and OS group based on their expression profiles. Colored bar indicates the range of normalized log2-based signal: red, transcript level below the median; green, greater than median. Each row represents a sample; each column represents a single circRNA. (C) Total RNA (80 μg per sample) were isolated from pericytes treated with 5.55 mM glucose (Normal glucose, NG), 5.55 mM glucose plus 24.45 mM pyruvate (Osmotic control, OS) or 30 mM glucose (High glucose, HG) for 24 hours. The expression of indicated circRNAs were detected by qRT-PCRs (n=4, **P*<0.05 versus NG group). (D) Pericytes were incubated with high glucose (30 mM) or H_2O_2 (100 μm) for 0 hour (Ctrl), 4 hours, 8 hours and 12 hours. qRT-PCRs were conducted to detect SP1 expression (n=4, **P*<0.05 versus Ctrl group). (E) Pericytes were transfected with SP1 siRNA or scrambled (Scr) siRNA for 12 hours. qRT-PCRs were conducted to detect SP1 and cZNF532 expression (n=4, **P*<0.05 versus Scr group). (F) Pericytes were transfected with SP1 siRNA or Scr siRNA for 12 hours. The sonicated chromatin of cell lysates was subjected to ChIP using anti-SP1 followed by qPCR analysis using the primer for cZNF532 promoter. Data were presented as % input for each IP sample relative to the input chromatin (1%) for each ChIP sample as indicated (n=4, **P*<0.05 versus Ctrl group). For Fig. 1C-F: The significant difference was evaluated by one-way ANOVA followed by Bonferroni's post-hoc test. Error bar = ± SD.

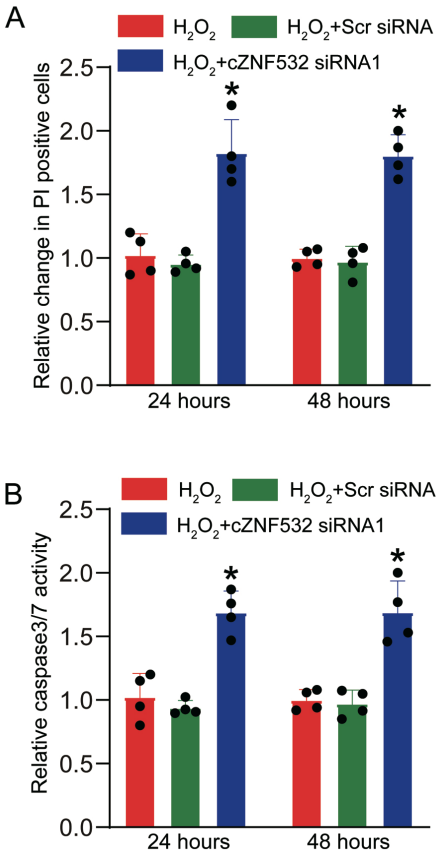
Supplemental Figure 2



Supplemental Figure 2: cZNF532 siRNA transfection down-regulates cZNF532 expression but does not alter ZNF532 mRNA expression

Pericytes were transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of cZNF532 (siRNA 1-3), or left untreated (Ctrl) for 24 hours or 48 hours. qRT-PCR assays were conducted to detect cZNF532 and ZNF532 mRNA expression (n=4, **P*<0.05, one-way ANOVA followed by post-hoc Bonferroni's comparison test). Error bar = ± SD.

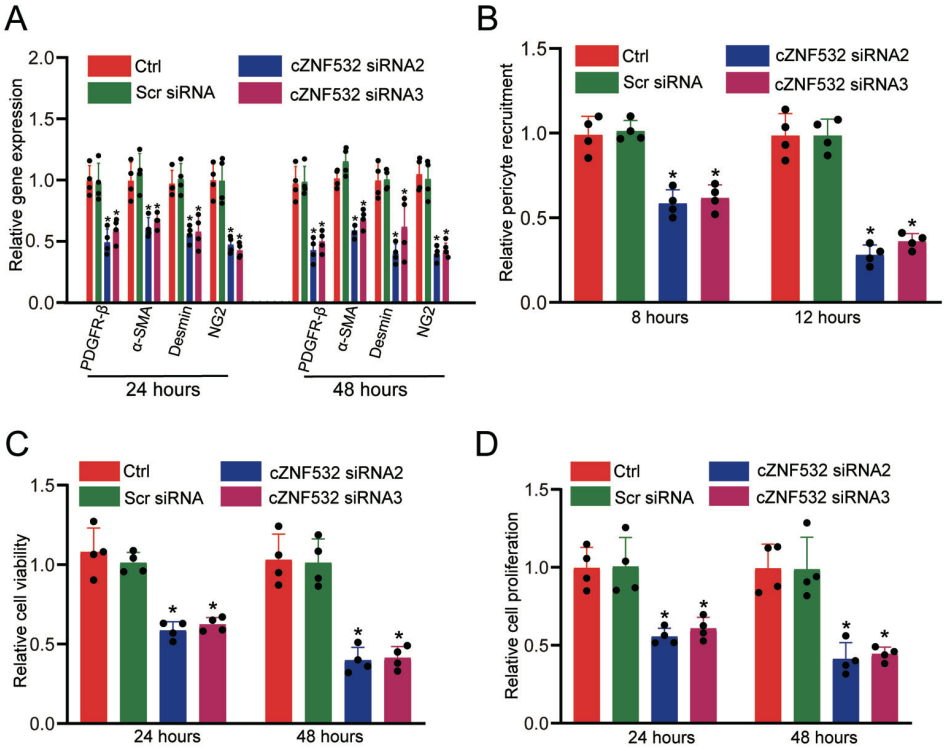
Supplemental Figure 3



Supplemental Figure 3: cZNF532 silencing by cZNF532 siRNA1 aggravates oxidative stress-induced pericyte apoptosis

(A and B) Pericytes were transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of cZNF532 (siRNA1), or left untreated (Ctrl), and then exposed to H_2O_2 (100 μm) for 24 hours or 48 hours. Apoptotic cells were detected by PI staining (A) or caspase 3/7 activity (B). The significant difference was evaluated by one-way ANOVA followed by Bonferroni's post-hoc test (n=4, *P<0.05). Error bar = ± SD.

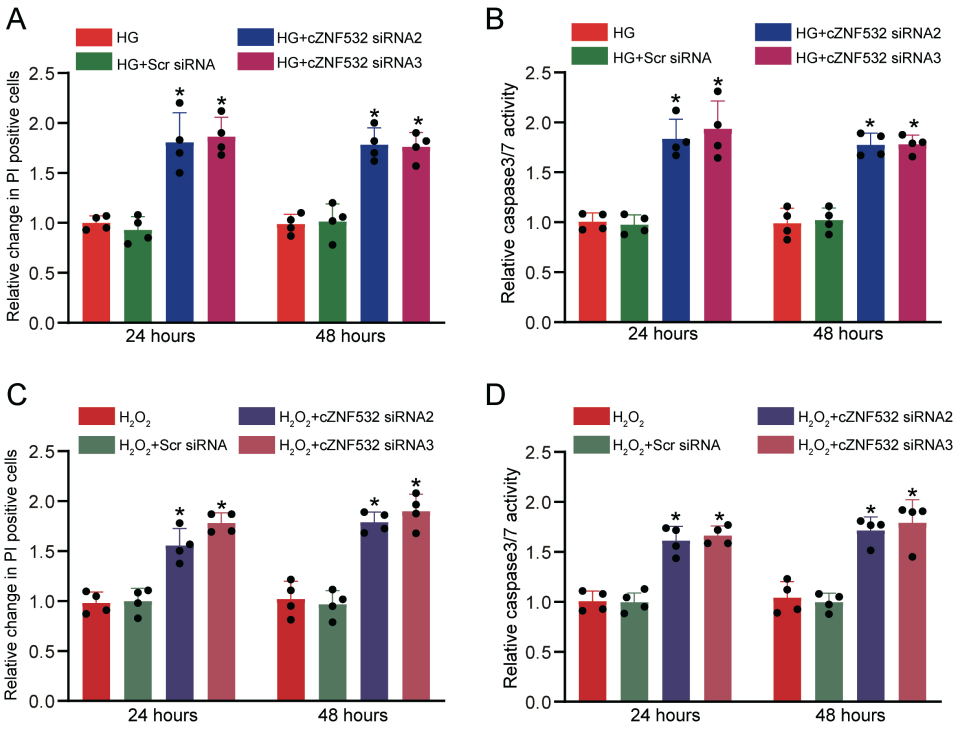
Supplemental Figure 4



Supplemental Figure 4: cZNF532 silencing by cZNF532 siRNA2 or siRNA3 affects the differentiation, recruitment, viability, and proliferation of pericytes

(A) Pericytes were transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of cZNF532 (siRNA2 or siRNA3), or left untreated (Ctrl) for 24 hours or 48 hours. qRT-PCRs were conducted to detect the expression of pericyte markers, including PDGFR-β, α-SMA, desmin and NG2 (n=4). (B) Wild-type (Ctrl), cZNF532 siRNA2, cZNF532 siRNA3, or Scr siRNA-transfected pericytes were co-cultured with HRVECs for 8 hours or 12 hours, and then stained with NG2 and CD31 to detect the recruitment of pericytes towards HRVECs (n=4). (C and D) Pericytes were transfected with Scr siRNA, cZNF532 siRNA2, cZNF532 siRNA3, or left untreated (Ctrl) for 24 hours or 48 hours. Cell viability was detected by MTT method (C, n=4). Cell proliferation was detected by Ki67 staining (D, n=4). The significant difference was evaluated by one-way ANOVA followed by Bonferroni's post-hoc test. Error bar = ± SD, *P<0.05.

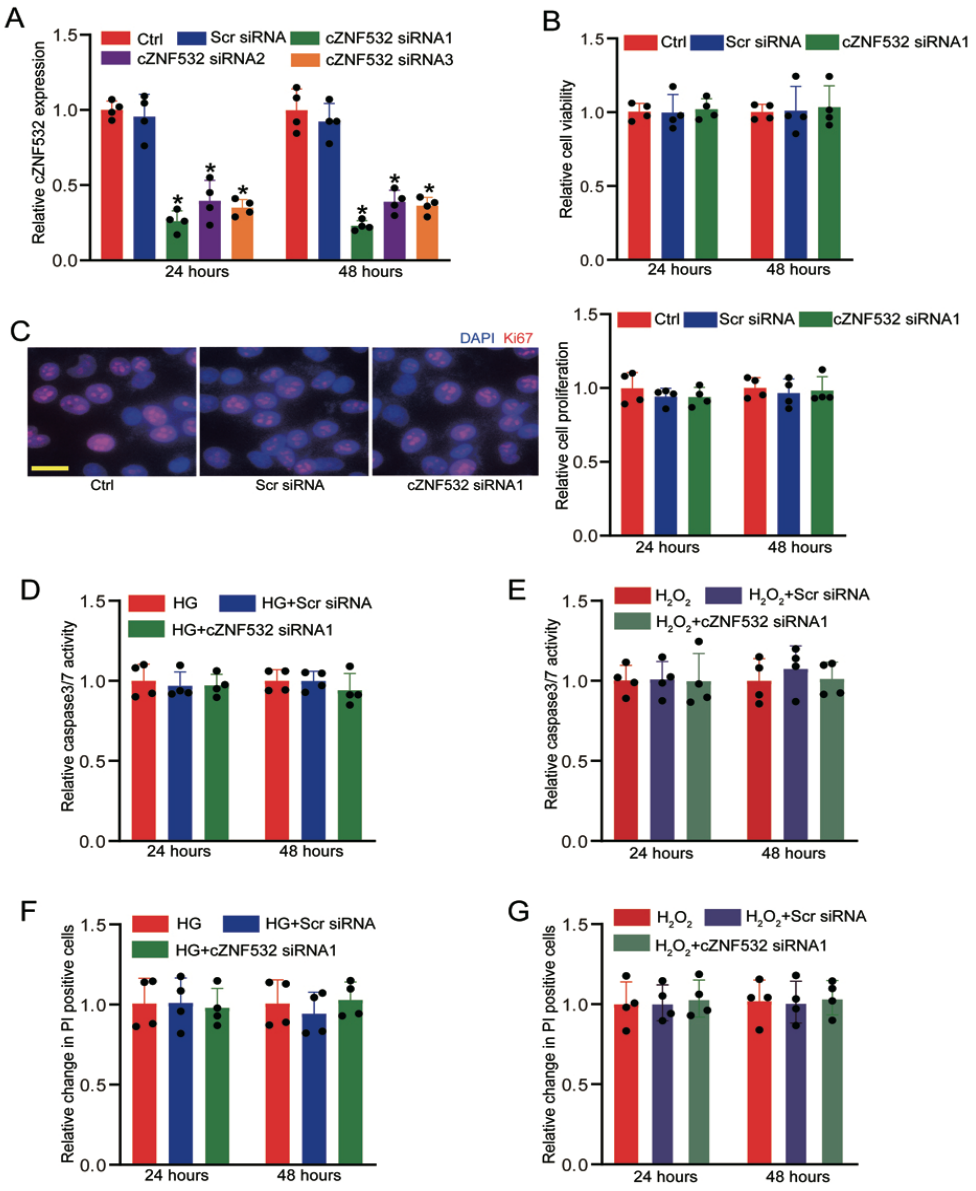
Supplemental Figure 5



Supplemental Figure 5: cZNF532 silencing by cZNF532 siRNA2 or siRNA3 affects the development of pericyte apoptosis

Pericytes were transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of cZNF532 (siRNA2 or siRNA3), or left untreated (Ctrl), and then exposed to 30 mM glucose (A and B) or H_2O_2 (100 μm, C and D) for 24 hours or 48 hours. Cell apoptosis was analyzed by PI staining (A and C) or caspase 3/7 activity (B and D). The significant difference was evaluated by one-way ANOVA followed by Bonferroni's post-hoc test (n=4). Error bar= ± SD, *P<0.05.

Supplemental Figure 6

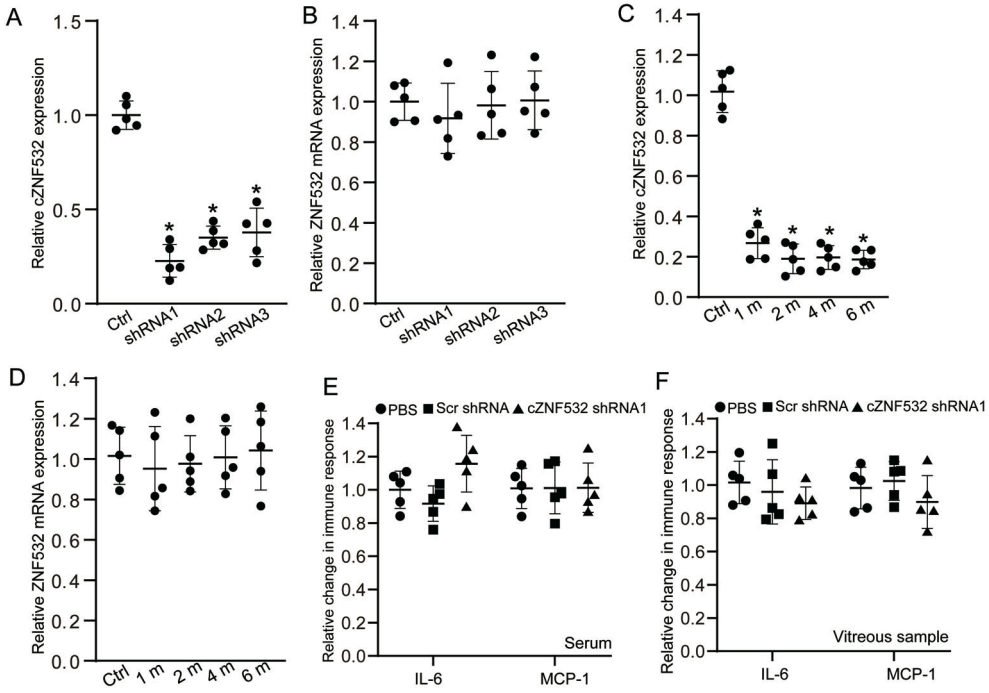


Supplemental Figure 6: cZNF532 silencing has no effect on the viability, proliferation, and apoptosis of retinal endothelial cells

(A and B) HRVECs were transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of cZNF532 (siRNA1, siRNA2 or siRNA3), or left untreated (Ctrl) for 24 hours or 48 hours. qRT-PCRs were conducted to detect cZNF532 expression (n=4). (B and C) Pericytes were transfected with Scr siRNA, cZNF532 siRNA1, or left untreated (Ctrl) for 24 hours or 48 hours. Cell viability was detected by MTT assay (B, n=4). (C) Cell proliferation was detected by Ki67 staining (C, n=4). The representative images at 48 hours were shown (Scale bar: 20 μm). (D-G)

Pericytes were transfected with scrambled (Scr) siRNA, cZNF532 siRNA1, or left untreated (Ctrl), and then exposed to high glucose (30 mM) or H_2O_2 (100 μm) for 24 hours or 48 hours. Cell apoptosis was detected by caspase 3/7 activity (D and E, n=4) and PI staining (F and G, n=4). The significant difference was evaluated by one-way ANOVA followed by Bonferroni's post-hoc test. Error bar = ± SD, *P<0.05.

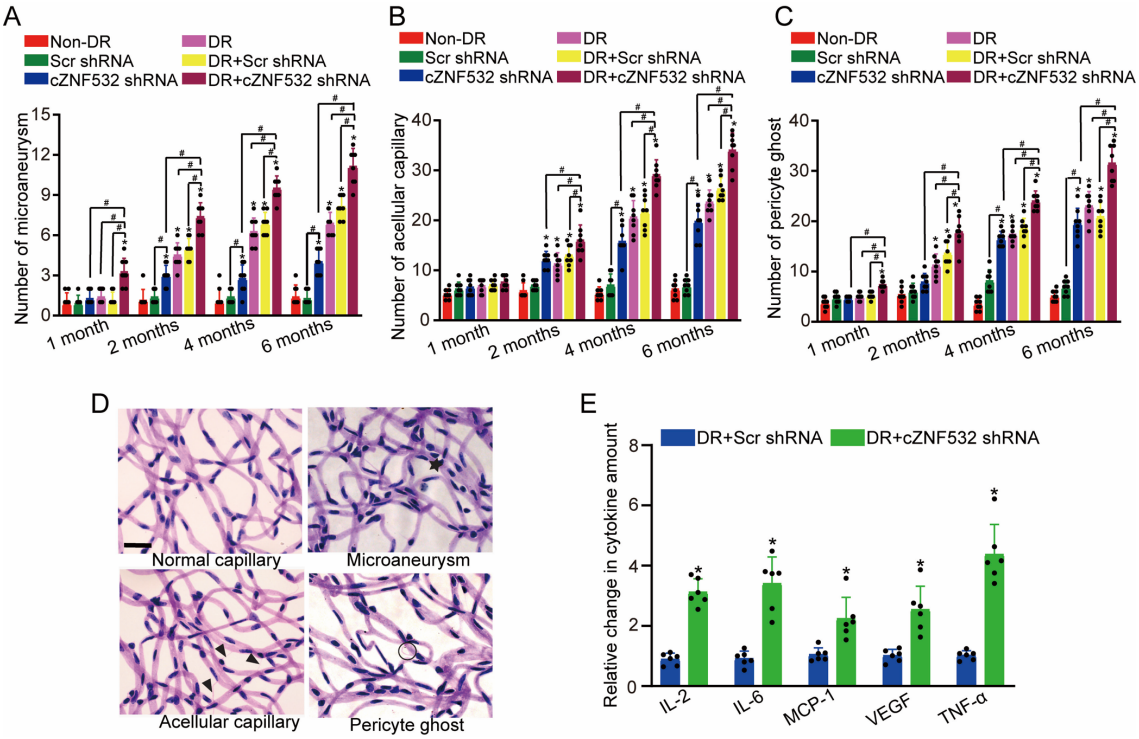
Supplemental Figure 7



Supplemental Figure 7: cZNF532 shRNA injection significantly down-regulates cZNF532 expression

(A and B) Diabetic C57BL/6 mice (2-month old, male) received an intravitreous injection of cZNF532 shRNA targeting the backsplice sequence of cZNF532 (shRNA1-3) or left untreated (Ctrl). qRT-PCRs were conducted to detect the expression of cZNF532 (A) and ZNF532 mRNA (B) at 1 month after cZNF532 shRNA injection (n=5, **P*<0.05). (C and D) Diabetic C57BL/6 mice (2-month old, male) received an intravitreous injection of cZNF532 shRNA1, or left untreated (Ctrl) for the indicated time points. qRT-PCRs were conducted to detect cZNF532 (C) and ZNF532 mRNA (D) expression at the indicated time periods (n=5, **P*<0.05). (E and F) Diabetic C57BL/6 mice (2-month old, male) received an intravitreous injection of cZNF532 shRNA1, scrambled (Scr) shRNA, or PBS. One month after injection, ELISAs were conducted to detect MCP-1 and IL-6 levels in the serum and vitreous sample (n=5, **P*<0.05). The significant difference was evaluated by Kruskal-Wallis test followed by post-hoc Bonferroni's test. Error bar = ± SD.

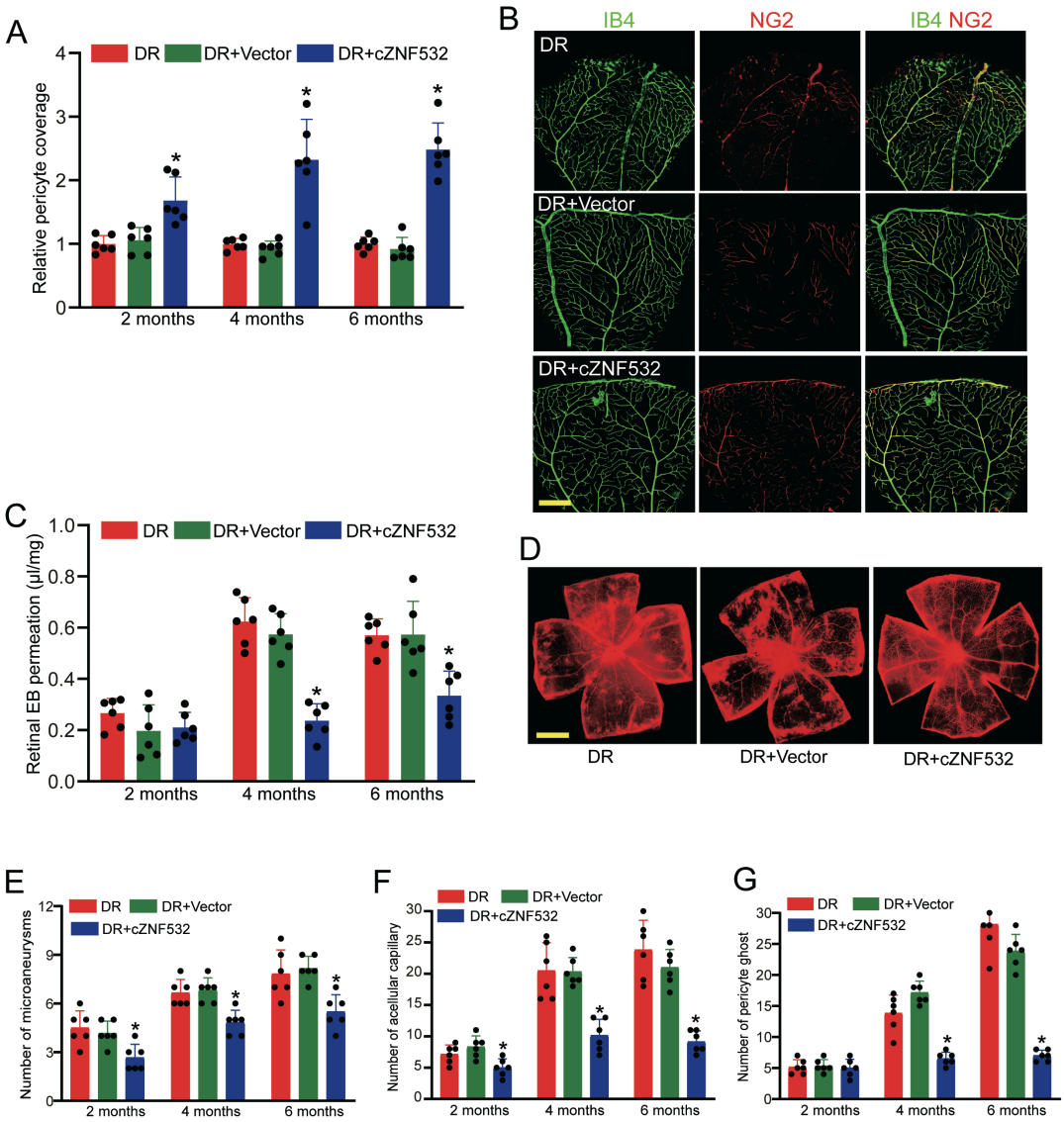
Supplemental Figure 8



Supplemental Figure 8: cZNF532 regulates diabetes-induced retinal vascular dysfunction in vivo

Retinal trypsin digestion and PAS staining was conducted to detect the number of microaneurysm (A, n=8, per mm^2 retina), acellular capillary (B, n=8, per mm^2 retina), and pericyte ghost (C, n=8, per mm^2 retina). The star indicates microaneurysm; arrow indicates acellular capillary; circle indicates pericyte ghost (D, Scale bar, 100 μm) (E) ELISA assays were conducted to detect the amount of IL-2, IL-6, MCP-1, VEGF, and TNF-α in retinal lysates after 6-month treatment (n=6). Blood glucose levels of diabetic mice were above 300 mg/dl. All significant difference was evaluated by Mann-Whitney's *U* test or Kruskal-Wallis's test followed by post hoc Bonferroni's test. Error bar = ± SD. $^{*}P < 0.05$ compared with non-DR group (A-C) or DR+Scr shRNA (E). $^{\#}P < 0.05$ between the marked groups.

Supplemental Figure 9

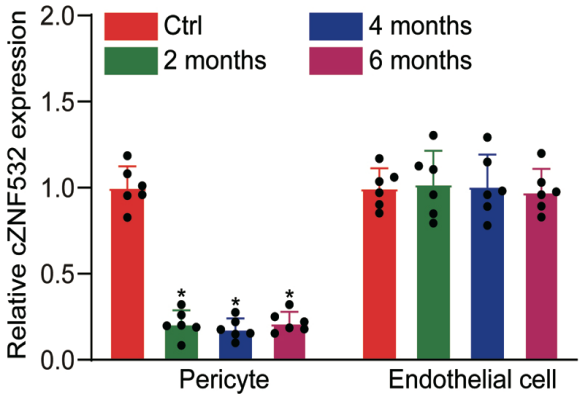


Supplemental Figure 9: cZNF532 overexpression alleviates retinal vascular dysfunction in vivo

Diabetic C57BL/6 mice (2-month old, male) received an intravitreous injection of null vector (Vector) and cZNF532 overexpression vector, or left untreated (DR). Two months, four months, or six months after diabetes induction, pericyte coverage was quantified by staining the whole-mount retinas with IB4 and NG2. The statistical result (A, n=6) and representative composite images after six-month treatment are shown (B, Scale bar: 100 μm). (C and D) The mice were infused with Evans blue (EB) dye for 2 hours. The tile-scanning images of whole retinal vessels were taken using a 4×lens with identical gain settings. The statistical result of EB extravasation (C, n=8)

and representative images of flat-mounted retinas after four-month treatment (D; Scale bar, 500 μm). The red fluorescent indicates EB signaling. (E-G) Retinal trypsin digestion and PAS staining was conducted to detect the number of microaneurysm (E, n=5, per mm^2 retina), acellular capillary (F, n=5, per mm^2 retina), and pericyte ghost (G, n=5, per mm^2 retina). The blood glucose levels of diabetic mice were above 300 mg/dl. All significant difference was evaluated by Kruskal-Wallis's test followed by post hoc Bonferroni's test, Error bar = ±SD. *P<0.05.

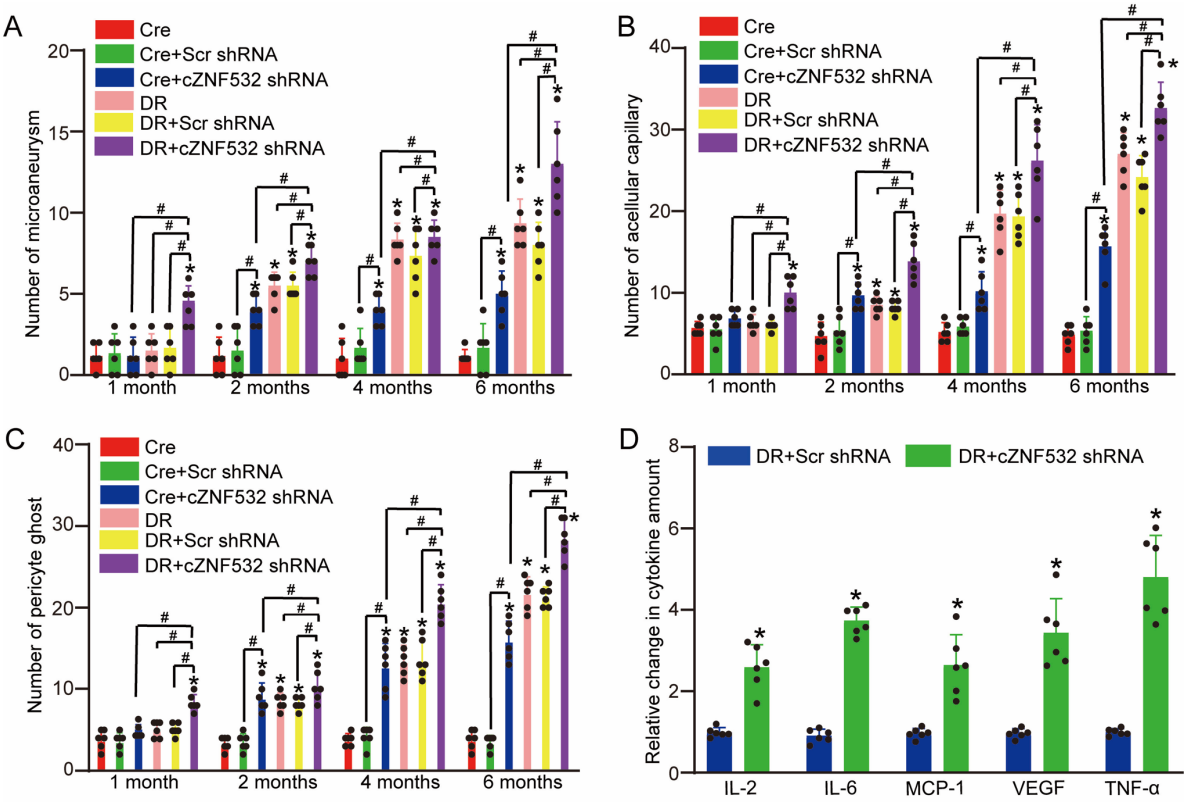
Supplemental Figure 10



Supplemental Figure 10: Cre-dependent cZNF532 shRNA reduces cZNF532 expression in the pericytes of PDGFR-β-Cre mice

cZNF532 expression was detected by qRT-PCRs in the primarily isolated pericytes and endothelial cells from the retinas of PDGFR-β-Cre mice at 2-month, 4-month, and 6-month after Cre-dependent cZNF532 shRNA administration. The pericytes or endothelial cells from PDGFR-β-Cre mice without Cre-dependent cZNF532 shRNA administration were used as the control group (n=6). The significant difference was evaluated by Kruskal-Wallis's test followed by post hoc Bonferroni's test. Error bar = ± SD, *P<0.05.

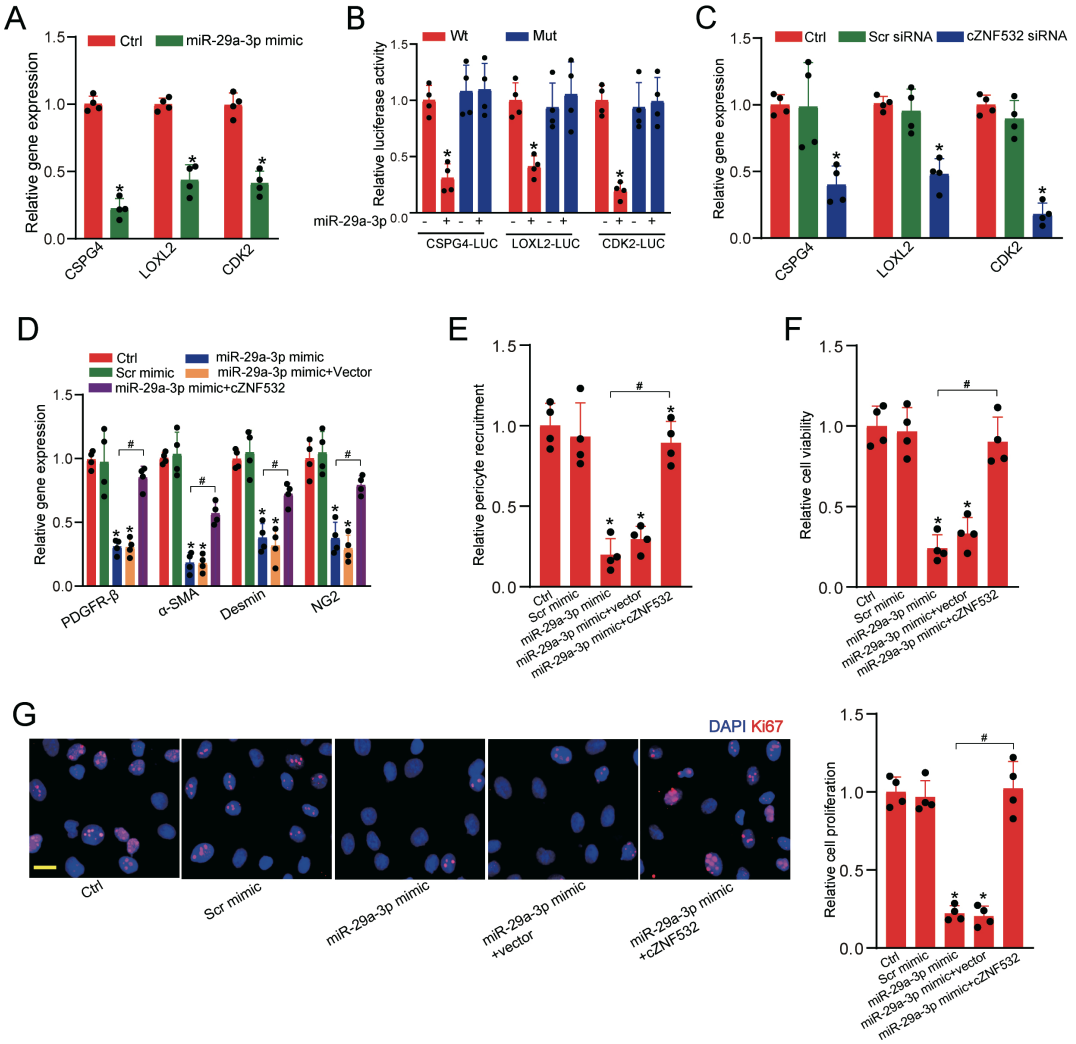
Supplemental Figure 11



Supplemental Figure 11: Conditional knockdown of cZNF532 in pericyte affects retinal vascular dysfunction in vivo

Retinal trypsin digestion and PAS staining was conducted to detect the number of the number of microaneurysm (A, n=6, per mm^2 retina), acellular capillary (B, n=6, per mm^2 retina), and pericyte ghost (C, n=6, per mm^2 retina). (D) ELISA assays were conducted to detect the amount of IL-2, IL-6, MCP-1, VEGF, and TNF-α in retinal lysates after 6-month treatment (n=6). The blood glucose levels of diabetic mice were above 300 mg/dl. All significant difference was evaluated by Mann-Whitney's *U* test or Kruskal-Wallis's test followed by post hoc Bonferroni's test. Error bar = ± SD. $^{*}P < 0.05$ compared with Cre group (A-C) or DR+Scr shRNA (D). $^{\#}P < 0.05$ between the marked groups.

Supplemental Figure 12

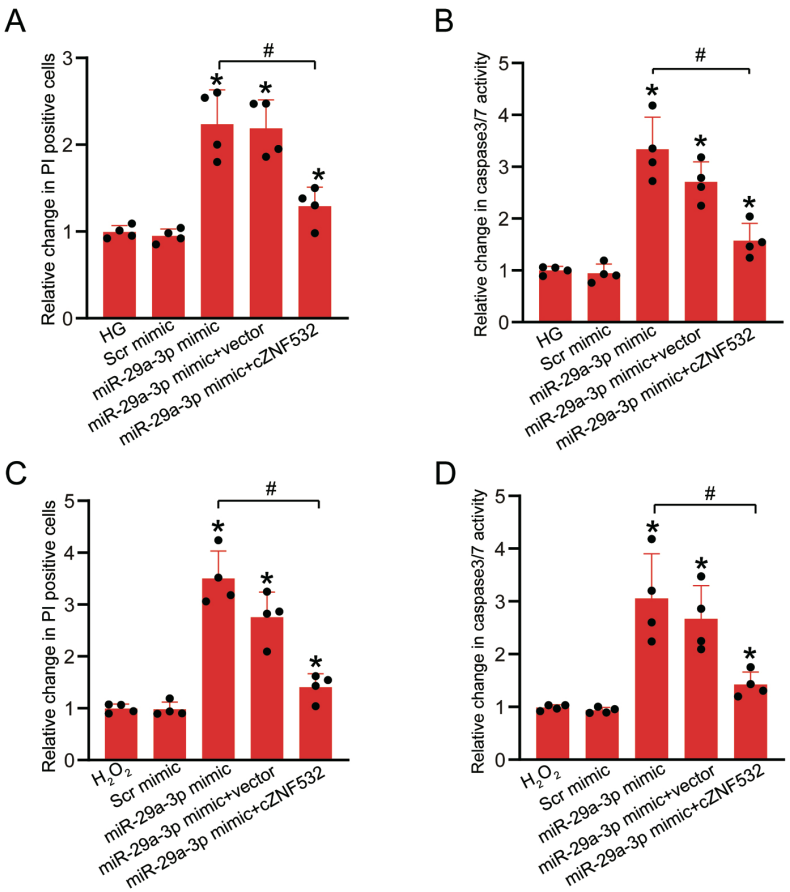


Supplemental Figure 12: cZNF532-miR-29a-3p-NG2/LOXL2/CDK2 network regulates pericyte function in vitro

(A) Pericytes were transfected with miR-29a-3p mimic or left untreated (Ctrl) for 48 hours. qRT-PCRs were conducted to detect the expression of CSPG4, LOXL2, and CDK2 (n=4, **P*<0.05 versus Ctrl group). (B) HEK293T cells were co-transfected wild-type (Wt) or mutant (Mut) LUC-CSPG4, LUC- LOXL2, or LUC-CDK2 with or without miR-29a-3p mimic. pRL-TK vector was transfected as the internal transfection control. Luciferase activity was detected 48 hours after transfection (n=4, **P*<0.05 versus Wt group without miR-29a-3p mimic group). (C) Pericytes were transfected with cZNF532 siRNA, scrambled (Scr) siRNA, or left untreated (Ctrl). Twenty-four hours after transfection, qRT-PCRs were conducted to detect CSPG4,

LOXL2, and CDK2 expression (n=4, *P<0.05 versus Ctrl group). (D) Pericytes were treated as shown for 48 hours. qRT-PCRs were conducted to detect the expression of pericyte markers, including PDGFR-β, α-SMA, desmin, and NG2 (n=4). (E) Pericytes were stained with NG2 and CD31 to label pericytes and HRVECs. After 12-hour migration, the number of recruited pericytes towards HRVECs was quantified (n=4). (F) Cell viability was detected using MTT method at 24-hour post-transfection (n=4). (G) Cell proliferation was detected using Ki67 staining at 24-hour post-transfection (n=4). Representative images and statistical result were shown. Scale bar: 20 μm. For figure D-G: *P<0.05 versus Ctrl group; $^{\#}P$ < 0.05 between the marked group. The significant difference was evaluated by Student *t* test or one-way ANOVA followed by Bonferroni's post-hoc test, Error bar = ± SD.

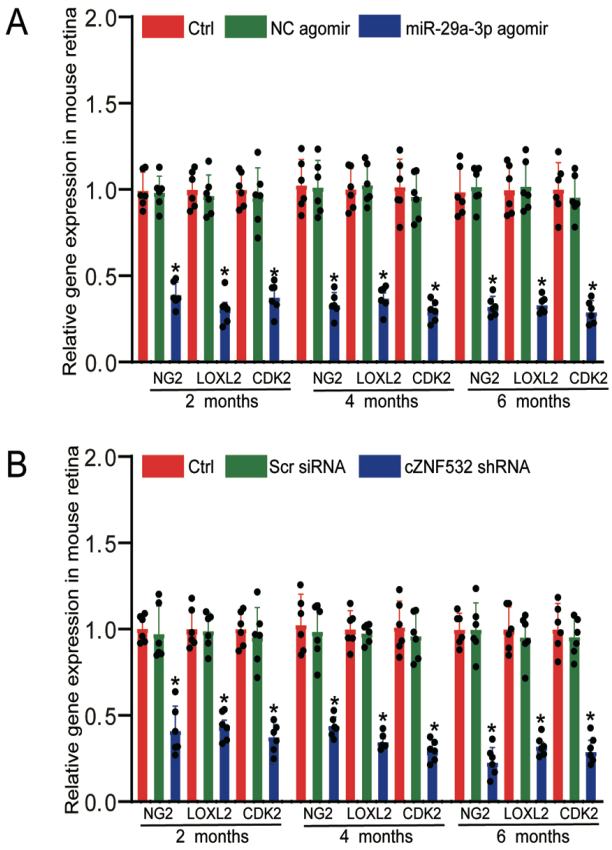
Supplemental Figure 13



Supplemental Figure 13: cZNF532 overexpression partially alleviates miR-29a-3p-mediated pro-apoptotic effect in pericytes

Pericytes were treated as shown, and then exposed to high glucose (30 mM, A and B) or H_2O_2 (100 μm, C and D) for 48 hours. Cell apoptosis was detected by PI staining (n=4, A and C) or caspase 3/7 activity (n=4, B and D). All significant difference was evaluated by one-way ANOVA followed by Bonferroni's post-hoc test. Error bar = ± SD.

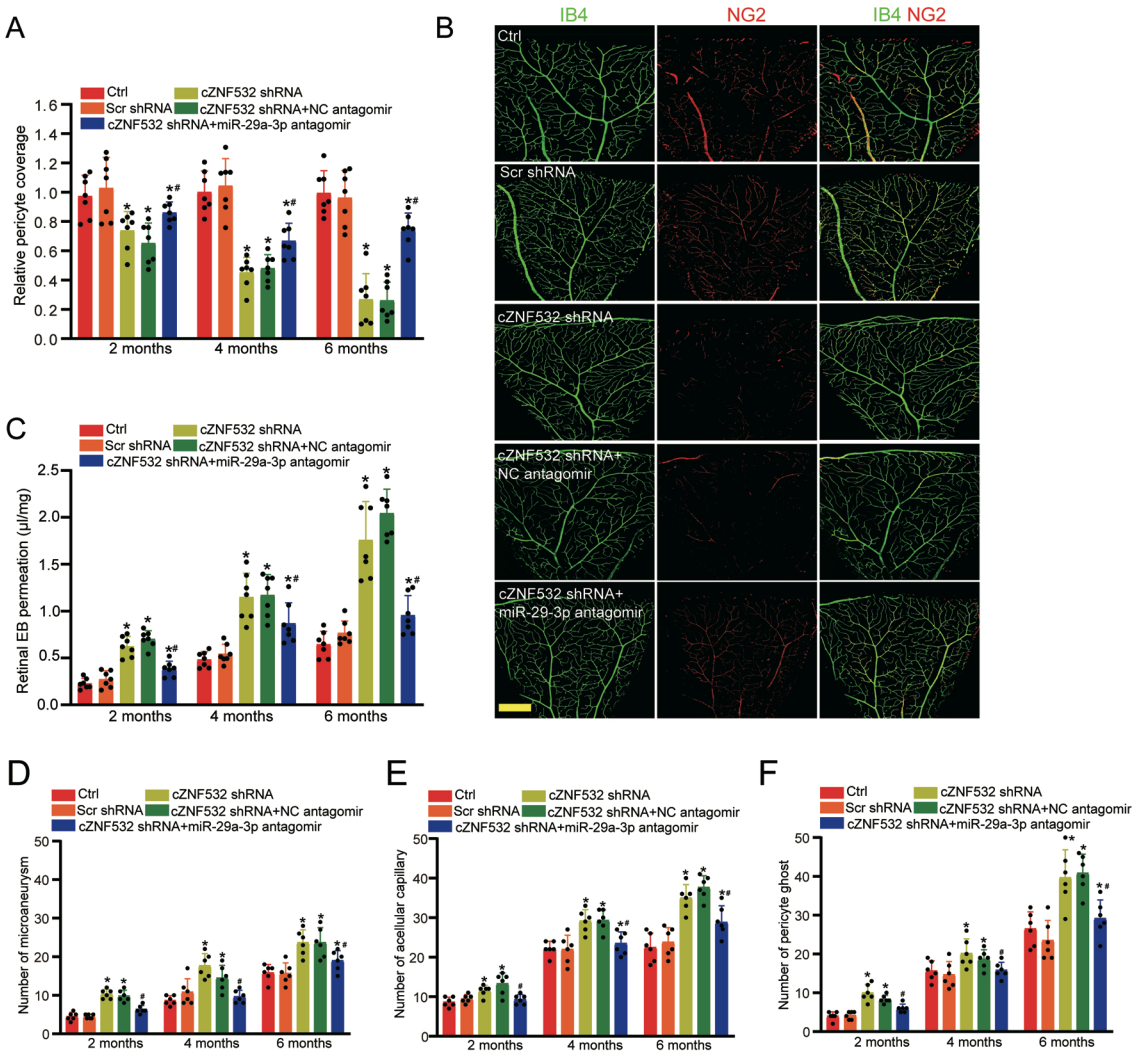
Supplemental Figure 14



Supplemental Figure 14: Identification of cZNF532-miR-29a-3p-NG2/LOXL2/ CDK2 network in mouse retina

(A) qRT-PCRs were conducted to detect retinal NG2, LOXL2 and CDK2 expression in diabetic C57BL/6 mice (2-month old, male) injected with miR-29a-3p antagomir, negative control (NC) antagomir, or left untreated (Ctrl) after 2-month, 4-month, and 6-month treatment (n=6). (B) qRT-PCRs were conducted to detect retinal NG2, LOXL2, and CDK2 expression in diabetic mice (Ctrl), diabetic mice injected with scrambled shRNA or cZNF532 shRNA after 2-month, 4-month, and 6-month treatment (n=6, **P*<0.05 versus Ctrl group). All significant difference was evaluated by Kruskal-Wallis test followed by post-hoc Bonferroni's test. Error bar = ± SD.

Supplemental Figure 15



Supplemental Figure 15: miR-29a-3p inhibition reverses cZNF532 silencing-induced retinal vascular dysfunction in vivo

(A and B) Diabetic C57BL/6 mice (3-month old, male) received an intravitreous injection of scrambled (Scr) shRNA, cZNF532 shRNA, cZNF532 shRNA plus NC antagomir, cZNF532 shRNA plus miR-29a-3p antagomir, or left untreated (Ctrl). Pericyte coverage was quantified by staining the whole-mount retinas with IB4 and NG2 after 2-month, 4-month, and 6-month treatment. The statistical result and representative images after 6-month treatment are shown (n=7; Scale bar: 100 μm). (C) The above-mentioned mice were infused with EB dye for 2 hours. The statistical result of EB extravasation were shown after 2-month, 4-month, and 6-month

treatment (n=7). (D-F) Retinal trypsin digestion and PAS staining was conducted to detect the number of microaneurysm (D, n=6, per mm^2 retina), acellular capillary (E, n=6, per mm^2 retina), and pericyte ghost (F, n=6, per per mm^2 retina). Blood glucose levels of diabetic mice were above 300 mg/dl. All significant difference was evaluated by Kruskal-Wallis's test followed by post hoc Bonferroni's test. Error bar = ± SD. *P<0.05.

Supplemental Table 1: Physiological parameters of non-diabetic and diabetic mice

	Initial		3 months		6 months	
	Body weight (g)	Glucose (mmol/L)	Body weight (g)	Glucose (mmol/L)	Body weight (g)	Glucose (mmol/L)
Non-diabetic (Ctrl)	23.7±2.42	6.98±0.95	40.80±5.42	6.33±0.78	68.30±4.05	6.32±0.52
Diabetic+Scr shRNA	24.50±4.36	25.44±2.56*	32.22±3.12*	24.09±5.03*	45.20±1.39*	28.72±1.04*
Diabetic+ cZNF532 shRNA	23.90±3.21	28.02±1.72*	34.20±3.12*	27.83±3.23*	44.80±3.62*	29.94±2.13*

All data were shown as mean±SD. The difference of physiological parameters between non-diabetic and diabetic mice was determined by repeated measures ANOVA at different time points. "*" indicated significant difference compared with Ctrl group.

Supplemental Table 2: Prediction of cZNF532-interacting miRNAs

miRNA	**Number of binding sites**
hsa-miR-1184	2
hsa-miR-1205	2
hsa-miR-1286	2
hsa-miR-1287	5
hsa-miR-182	3
hsa-miR-1827	3
hsa-miR-29a-3p	3
hsa-miR-370	2
hsa-miR-450b-3p	2
hsa-miR-498	2
hsa-miR-520f	2
hsa-miR-616	2
hsa-miR-637	2
hsa-miR-652	2
hsa-miR-758	2
hsa-miR-767-3p	2
hsa-miR-769-3p	2
hsa-miR-885-3p	2
hsa-miR-892b	3
hsa-miR-942	2

Supplemental Table 3: Characteristic data from the patients with diabetic retinopathy and macular hole

Characteristic	**MH**	**DR**	***P* value**
Number of patients	8	28	<0.001
Age (years)	66.3±6.6	63.8±5.1	0.051
Male (% percentage)	5/3 (62.5%)	18/10 (64.3%)	0.216
Number of diabetes mellitus	8	28	<0.001
Total cholesterol, mg/dL	182.7 ± 23.5	192.2 ± 11.8	0.0612
Creatinine, mg/dL	1.91 ± 0.73	1.31 ± 0.57	0.0325
Triglyceride, mg/dL	79.5 ± 11.3	138.5 ± 16.6	0.0054
HbA1c, %	3.92±1.72	7.36 ±1.52	0.0028
Macular hole	8	0	<0.001
Macular edema			
+Proliferative membrane	0	16	<0.001
Alone	0	12	<0.001
Proliferative membrane			
+retinal detachment	0	6	<0.001
Alone	0	10	<0.001
NVI	0	4	<0.001

MH, macular hole; DR, diabetic retinopathy; NVI, neovascularization of the iris.

Data were expressed as mean±SD or percentage. *P* values were determined by the Student *t* test or Fisher exact test.

Supplemental Table 4: Detection of NG2, LOXL2, and CDK2 expression in vitreous samples by ELISA assays

	Non-DR (n=8 eyes)	DME Only (n=12 eyes)	DME with PDR (n=12 eyes)	NVI (n=4 eyes)
NG2	156.3±6.4 pg/ml	226.3±15.1 pg/ml	338.5±10.2 pg/ml	364.9±6.8 pg/ml
LOXL2	Not detected	257.3±25.8 pg/ml	685.3±42.5 pg/ml	767.9±38.2 pg/ml
CDK2	92.5±12.7 pg/ml	613.3±65.3 pg/ml	872.9±84.4 pg/ml	1026.3±93.5 pg/ml