Supplemental information

Hyperglycemia-regulated tRNA-derived fragment

tRF-3001a propels neurovascular

dysfunction in diabetic mice

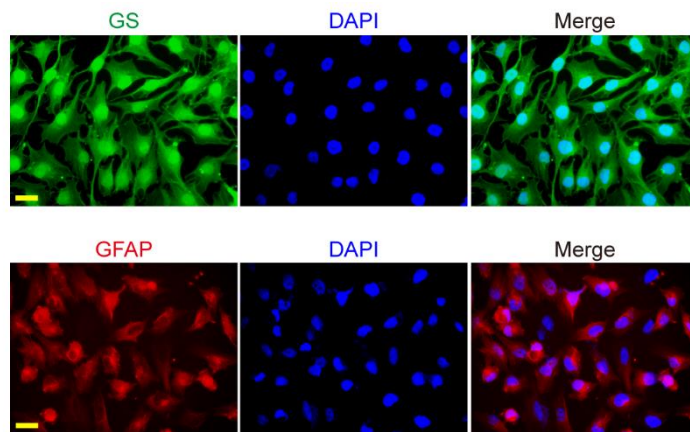Jun-Ya Zhu, Wen Yao, Xi-Sen Ni, Mu-Di Yao, Wen Bai, Tian-Jing Yang, Zi-Ran Zhang, Xiu-Miao Li, Qin Jiang, and Biao Yan

Figure S1. Identification and purity detection of primarily isolated Müller cells. Related to Figure 2

Immunostaining of GS and GFAP were conducted to label Müller cells. DAPI was stained to label cell nuclei. Green, GS; Red, GFAP; Blue, DAPI. Scale bar, 20 μm.
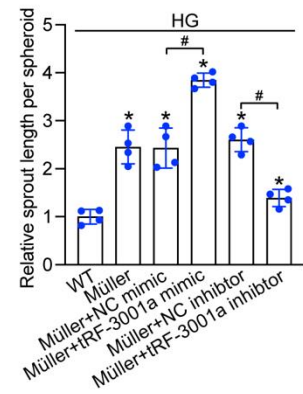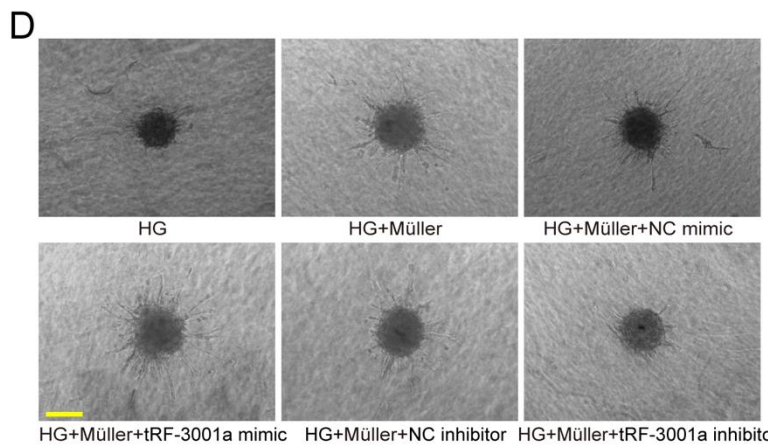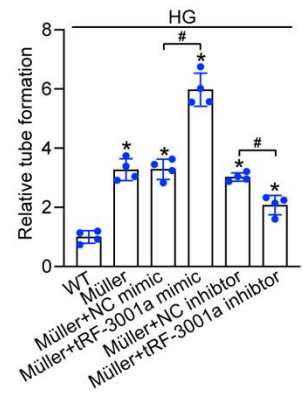
A

EdU DAPI

HG   HG+Müller   HG+Müller+NC mimic

HG+Müller+tRF-3001a mimic   HG+Müller+NC inhibitor   HG+Müller+tRF-3001a inhibitor

B

HG   HG+Müller   HG+Müller+NC mimic

HG+Müller+tRF-3001a mimic   HG+Müller+NC inhibitor   HG+Müller+tRF-3001a inhibitor

C

HG   HG+Müller   HG+Müller+NC mimic

HG+Müller+tRF-3001a mimic   HG+Müller+NC inhibitor   HG+Müller+tRF-3001a inhibitor

D

HG   HG+Müller   HG+Müller+NC mimic

HG+Müller+tRF-3001a mimic   HG+Müller+NC inhibitor   HG+Müller+tRF-3001a inhibitor

Figure S2. tRF-3001a regulates Müller cell-EC crosstalk in vitro. Related to Figure 2

(A-D) HRVECs were co-cultured without or with Müller cells transfected with the negative control (NC) mimics, tRF-3001a mimics, NC inhib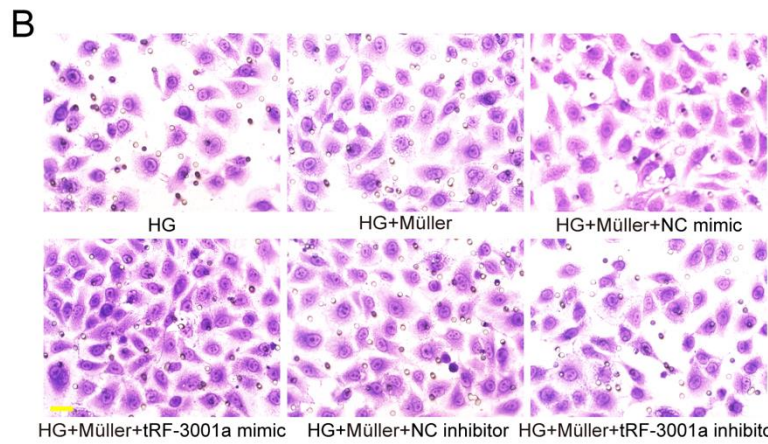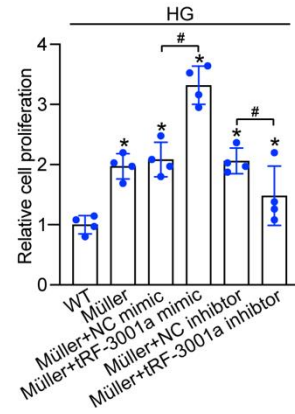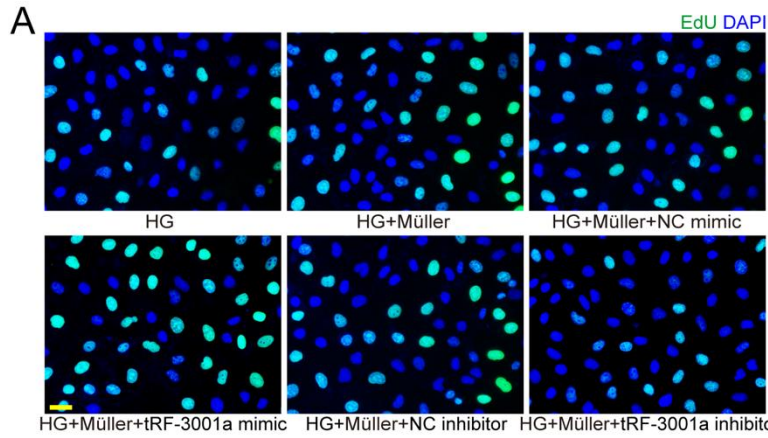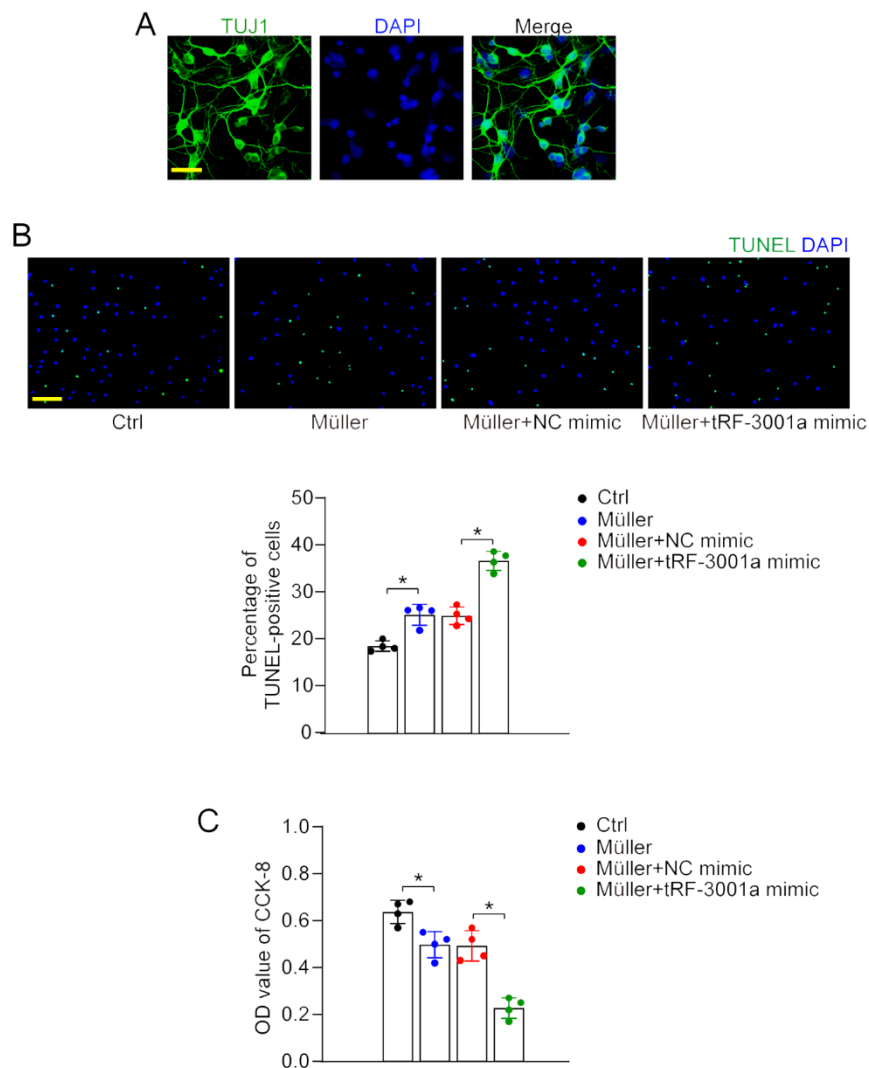itors, tRF-3001a inhibitors, or left untreated (Ctrl), and then exposed to high glucose (25 mM, HG) for 48 h. Cell proliferation was examined by EdU staining after 48 h co-culture. EdU, green; DAPI, blue. Scale bar, 20 μm (A, n = 4). The migration ability of HRVECs was examined by transwell assays after 24 h co-culture. Scale bar, 20 μm (B, n = 4). The tube formation ability of HRVECs was observed at 12 h following cells seeding on the matrix. The average length of tube formation for each field was statistically analyzed. Scale bar, 100 μm (C, n = 4). The sprouting ability of HRVECs was examined by spheroid sprouting assays after 48 h co-culture. Scale bar, 100 μm (D, n = 4). *$P < 0.05$ versus HG; #$P < 0.05$ between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.

Figure S3. tRF-3001a regulates Müller cell-RGC crosstalk in vitro. Related to Figure 2

(A) Primary RGCs were isolated from C57BL/6 mouse pups at postnatal day 0-3. Immunostaining of the primary RGCs was conducted using TUJ1 antibody. TUJ1, green; DAPI, blue. Scale bar, 20 μm. (B and C) Primary RGCs were co-cultured without or with Müller cells after the transfection of negative control (NC) mimics, tRF-3001a mimics, or left untreated (Ctrl), and then exposed to high glucose (25 mM, HG) for 24 h. Cell apoptosis was examined by TUNEL assays. TUNEL, green; DAPI, blue. Scale bar, 50 μm (B, n = 4). The viability of primary RGCs was examined by CCK-8 assays (C, n = 4). *$P < 0.05$ between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
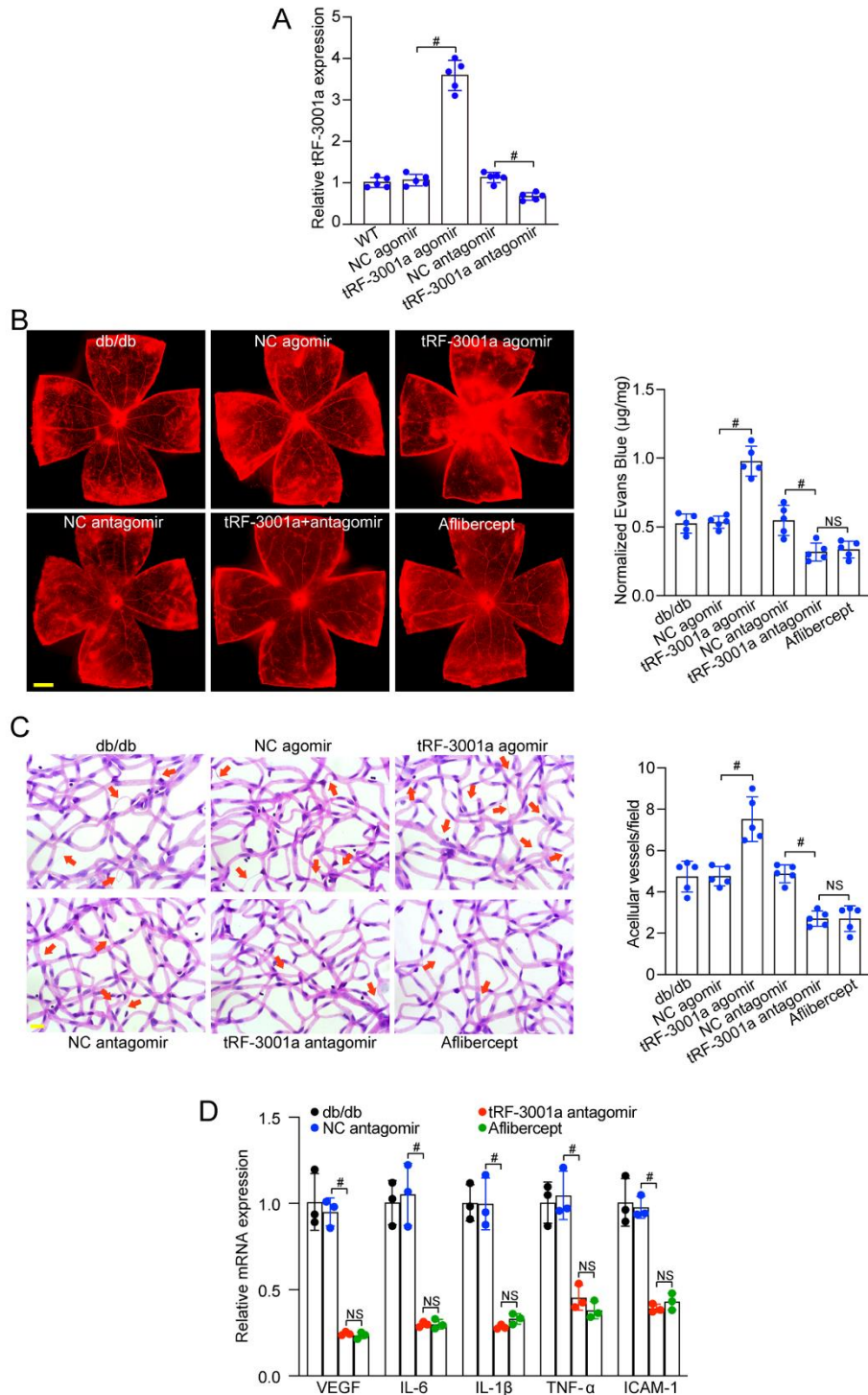
Figure S4. tRF-3001a regulates diabetes-induced retinal vascular dysfunction in diabetic db/db mice. Related to Figure 3

(A) The diabetic db/db mice (male, 3-month old) received intravitreal injections of negative control (NC) agomir, tRF-3001a agomir, NC antagomir, tRF-3001a antagomir, Aflibercept, or left untreated (WT) for one month. qRT-PCRs were used to examine tRF-3001a expression (n = 5). (B) These mice were infused with Evans blue dye for 2 h. The fluorescence signal of flat-mounted retina was observed under a 4 × lens. Evans blue leakage was quantified and the representative images were shown (n = 5). Scale bar, 500 μm. (C) Retinal trypsin digestion was used to detect retinal acellular capillaries. Red

arrow indicated acellular capillaries. Quantification analysis was averaged from 15 randomly selected fields per retina. The representative images were shown (n = 5). Scale bar, 10 μm. (D) qRT-PCR assays were used to detect the expression of VEGF, IL-6, IL-1β, ICAM-1, and TNF-α mRNA (n = 3). $^{\#}P <$ 0.05 between the marked groups; *NS*, no significant difference. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
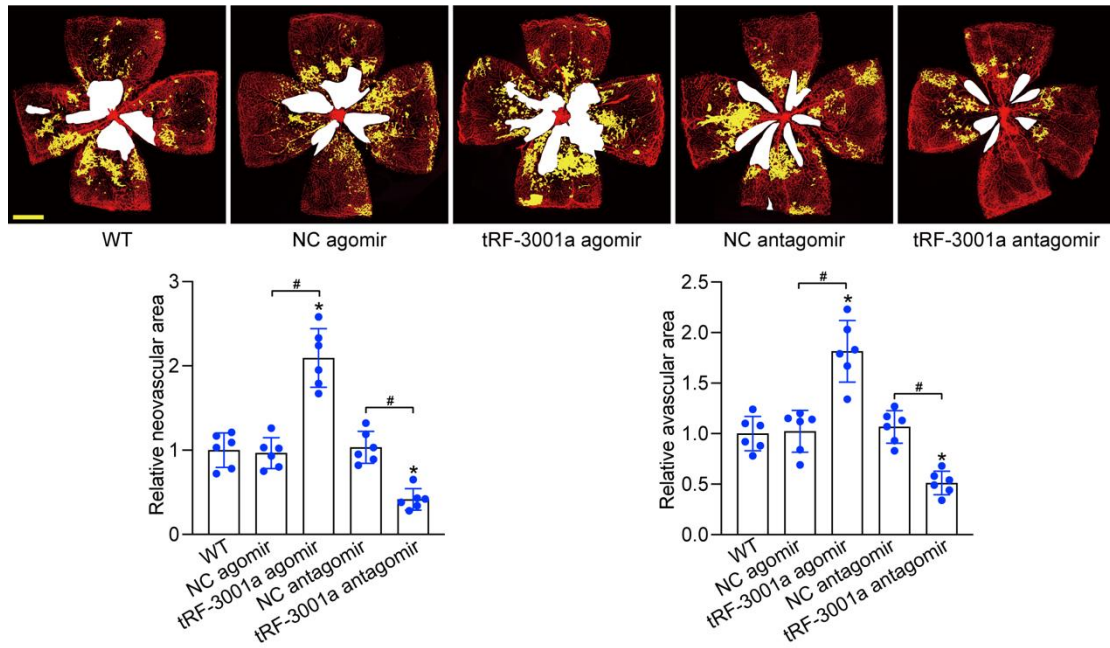
Figure S5. tRF-3001a regulates pathological retinal neovascularization in OIR model. Related to Figure 3

Seven-day-old C57BL/6J mice with their mothers were exposed to 75% oxygen for 5 days following intravitreous injections of negative control (NC) antagomir, tRF-3001a antagomir, NC agomir, tRF-3001a agomir, or left untreated (WT), and were then exposed to RA (21% oxygen) until P17. The retinas were collected on P17 and stained with Isolectin B4 to label retinal vessels. Yellow staining indicated the neovascular area; white area indicated the avascular area. Scale bar: 500 μm. Pathological neovascular area and avascular area were statistically analyzed (n = 5). *$P < 0.05$ versus WT; #$P < 0.05$ between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
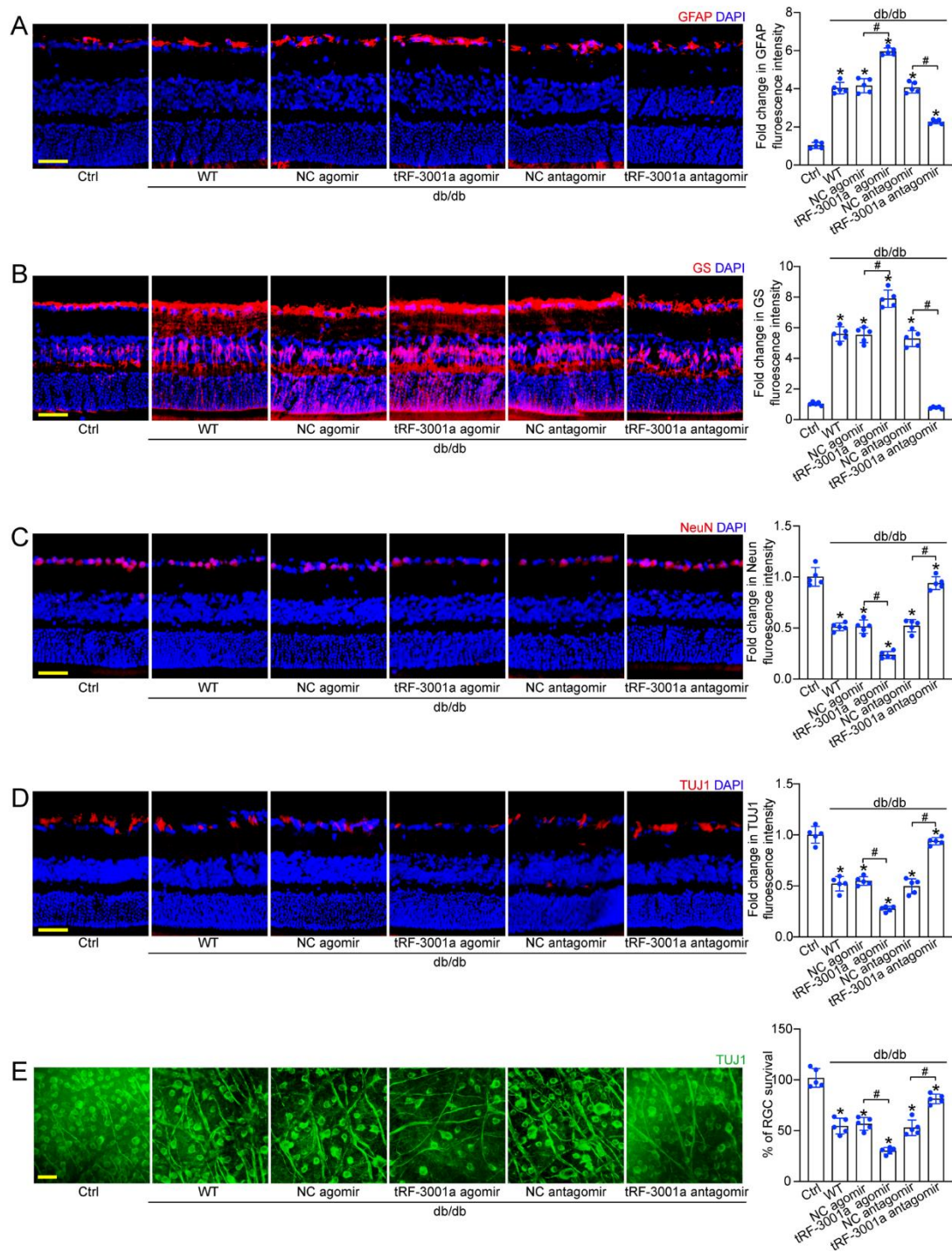
Figure S6. tRF-3001a regulates retinal neuronal dysfunction in db/db diabetic mice. Related to Figure 4

(A and B) The diabetic db/db mice (male, 3-month old) received intravitreal injections of negative control (NC) agomir, tRF-3001a agomir, NC antagomir, tRF-3001a antagomir, or left untreated (WT) for one month. db/m mice were taken as the control group (Ctrl). Immunofluorescence and quantitative analysis of GFAP staining (A) or GS staining (B) was conducted to detect retinal reactive gliosis along with the representative images (n = 5). Scale bar, 50 μm. (C and D) Immunofluorescence and quantitative analysis of NeuN staining (C) or TUJ1 staining (D) was conducted to detect RGC survival. The representative images were shown (n = 5). Scale bar, 50 μm. (E) Retinal whole-mounts following

TUJ1 staining were observed from the peripheral area. RGC survival rate was calculated by dividing the average number of TUJ1-positive cells in one field in the injured retina by that in the uninjured (Ctrl) retina (n = 5). Scale bar, 20 μm. *$P < 0.05$ versus Ctrl; #$P < 0.05$ between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
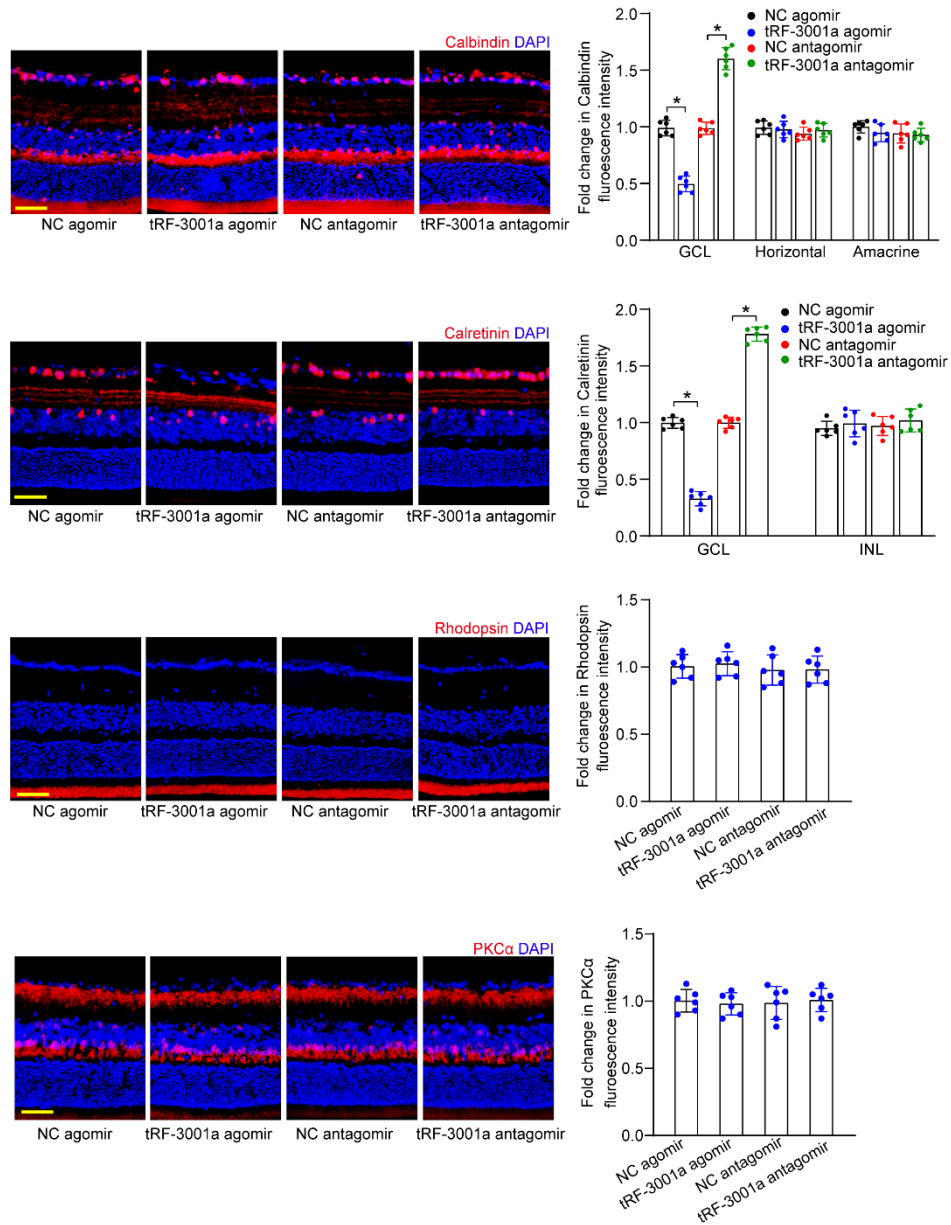
Figure S7. tRF-3001a has no effect on the label signal of amacrine cells, horizontal cells, rod and cone photoreceptors, and bipolar cells in STZ-induced diabetic mice. Related to Figure 4

C57BL/6J mice received intravitreal injections of negative control (NC) agomir, tRF-3001a agomir, NC antagomir, or tRF-3001a antagomir following 4-month diabetes induction. Immunofluorescence staining of calbindin, calretinin, rhodopsin, and PKCα were conducted to label amacrine cells, horizontal cells, rod and cone photoreceptors, and bipolar cells. The representative images and quantification results were shown (n = 6). Scale bar, 50 μm. *$P < 0.05$ between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
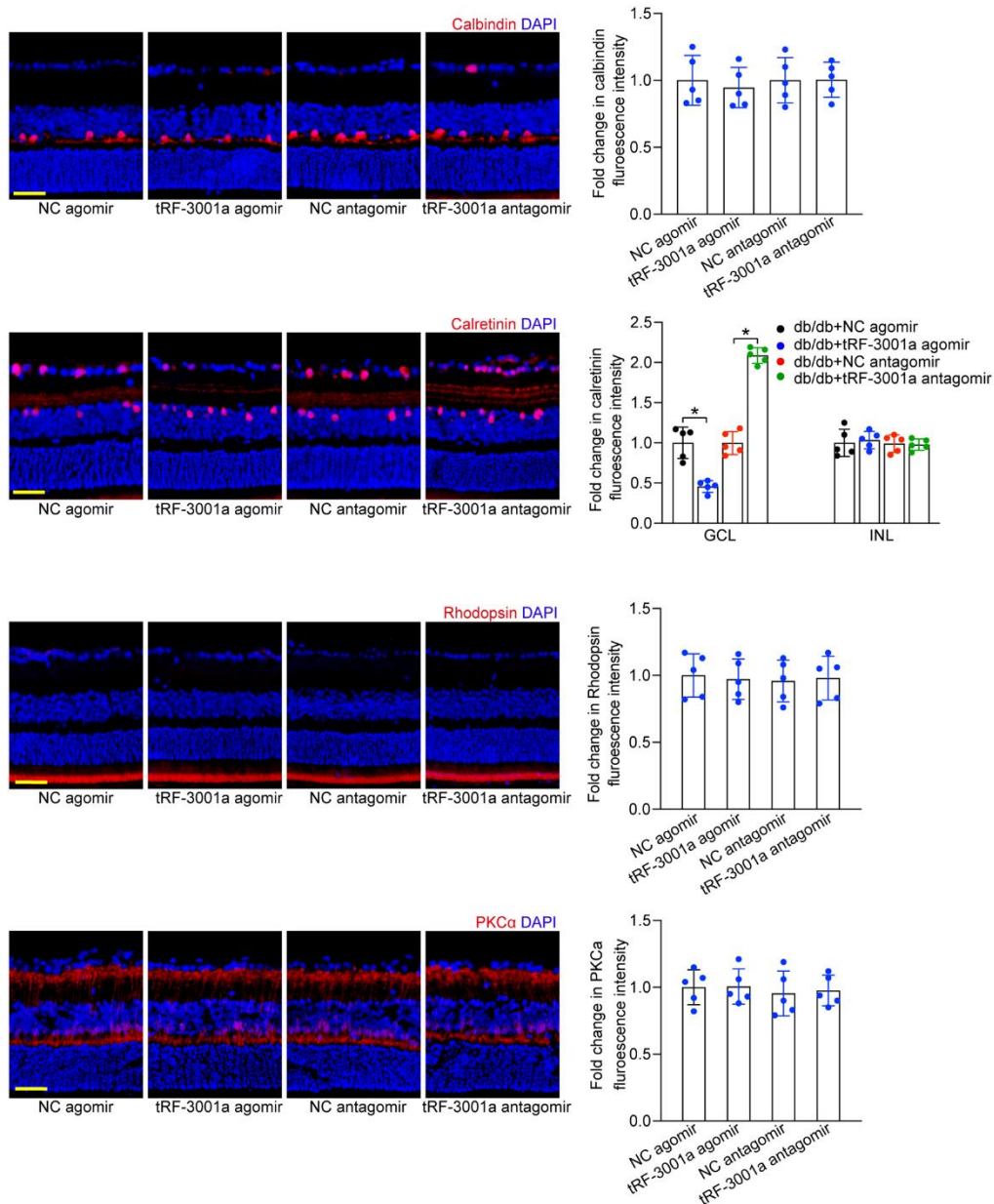
Figure S8. tRF-3001a has no effect on the label signal of amacrine cells, horizontal cells, rod and cone photoreceptors, and bipolar cells in db/db diabetic mice. Related to Figure 4

The diabetic db/db mice (male, 3-month old) received intravitreal injections of negative control (NC) agomir, tRF-3001a agomir, NC antagomir, tRF-3001a antagomir, or left untreated (WT) for one month. Immunofluorescence staining of calbindin, calretinin, rhodopsin, and PKCα were conducted to label amacrine cells, horizontal cells, rod and cone photoreceptors, and bipolar cells. The representative images and quantification results were shown (n = 5). Scale bar, 50 μm. *$P < 0.05$ between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
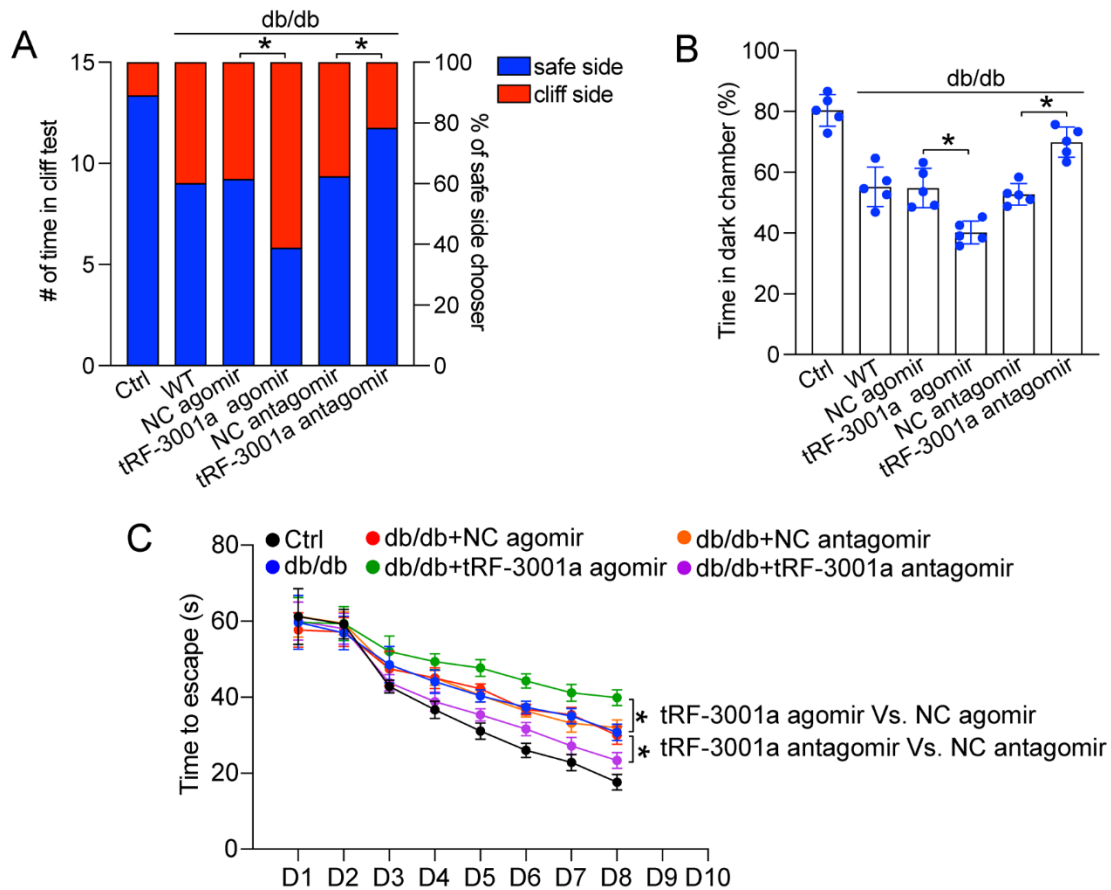
Figure S9. tRF-3001a regulates visual impairment in db/db diabetic mice. Related to Figure 5
(A-C) The diabetic db/db mice (male, 3-month old) received intravitreal injections of negative control (NC) agomir, tRF-3001a agomir, NC antagomir, tRF-3001a antagomir, or left untreated (WT) for one month. db/m mice were taken as the control group (Ctrl). The statistical results displayed the number (left y axis) and percentage (right y axis) of cliff/safe side choosers (A, n = 5). The statistical result showed the percentage of time in dark chamber within 5 min (B, n = 5). The statistical result showed the time required until the mice reached at the visible platform for 8 consecutive days (C, n = 5). *$P <$ 0.05 between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
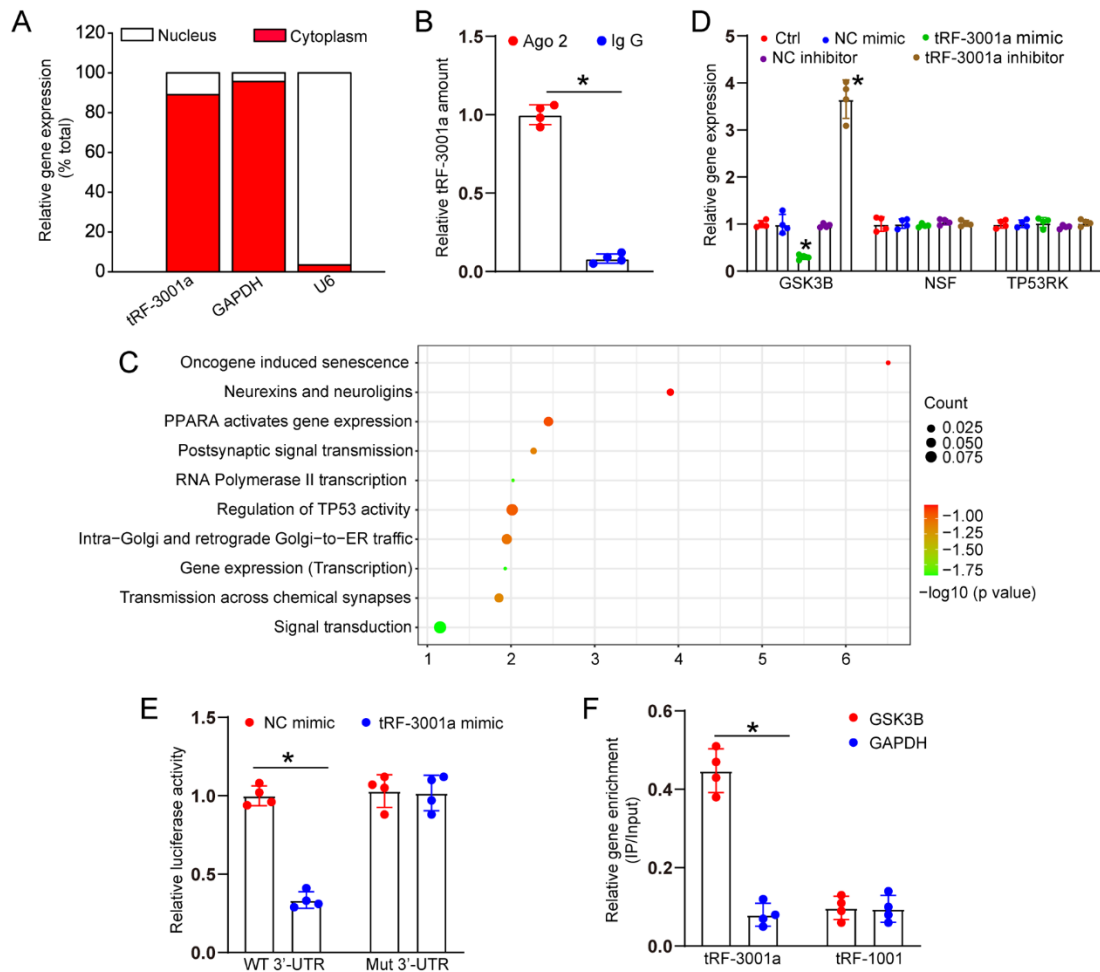
Figure S10: tRF-3001a regulates neurovascular dysfunction by targeting GSK3B. Related to Figure 6

(A) The expression distribution of U6, GAPDH, and tRF-3001a was examined by qRT-PCRs in the nucleus fractions and cytoplasm fractions of Müller cells (n = 4). (B) The fractions from Müller cells were immunoprecipitated using Ago2 or IgG antibody. The amounts of tRF-3001a in immunoprecipitates were examined by qRT-PCRs (n = 4, *$P < 0.05$). (C) The target genes of tRF-3001a were predicted by miRDB database. Pathway analysis of these target genes were conducted by the REACTOME_PATHWAY database. (D) The levels of GSK3B, NSF, and TP53RK expression were examined by qRT-PCRs in Müller cells after the transfection of negative control (NC) mimics, tRF-3001a mimics, NC inhibitors, tRF-3001a inhibitors, or left untreated (Ctrl) (n = 4, *$P<0.05$ versus Ctrl group). (E) The luciferase activity of wild-type GSK3B 3′-UTR or mutant GSK3B 3′-UTR following the transfection with NC mimics or tRF-3001a mimics in Müller cells were detected (n = 4, *$P<0.05$ versus tRF-3001a mimics). (F) The 3′-end biotinylated tRF-3001a or biotinylated tRF-1001 was transfected into Müller cells. After streptavidin capture, the amounts of GSK3B and GAPDH in the input and bound fractions were examined by qRT-PCRs (n = 4, *$P<0.05$ versus GAPDH group). The significant difference was evaluated by Student's t test or 1-way ANOVA followed by post hoc Bonferroni test.
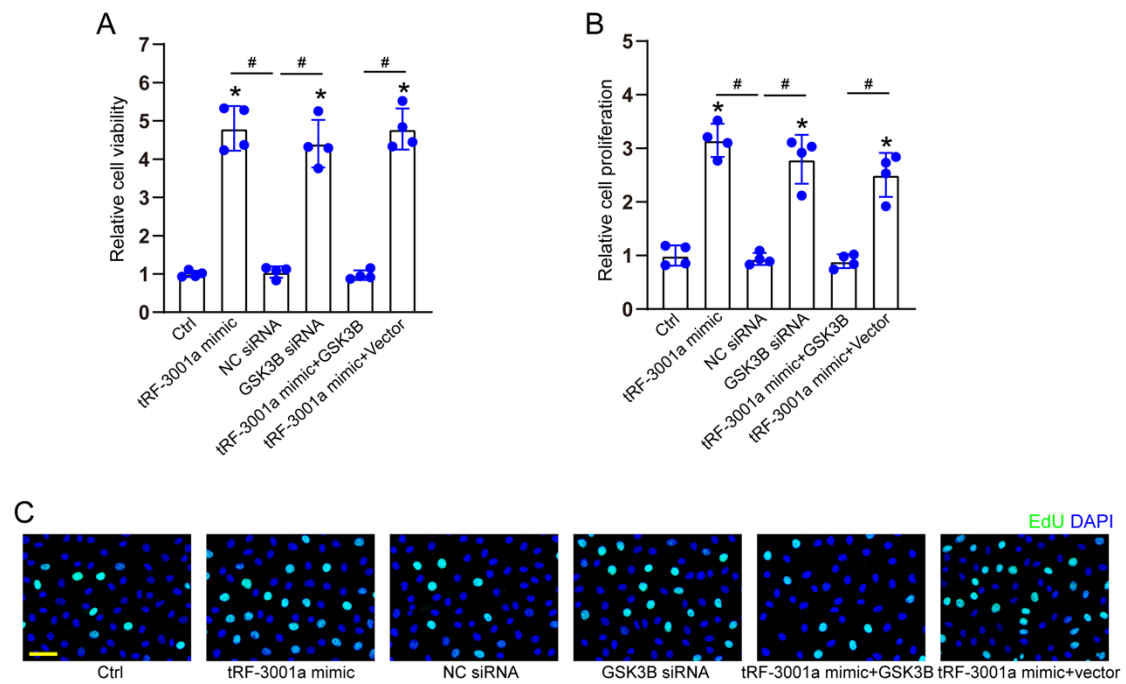
Figure S11. tRF-3001a-GSK3B axis regulates retinal endothelial cell function in vitro. Related to Figure 6

(A-C) HRVECs were transfected with the negative control (NC) mimics (Ctrl), tRF-3001a mimics, NC siRNA, GSK3B siRNA, tRF-3001a mimics plus GSK3B overexpression vector, or tRF-3001a mimics plus null vector for 24 h. Cell viability was examined by CCK-8 assays (A, n = 4). Cell proliferation was examined by EdU staining and EdU-positive cells were quantitated. EdU, green; DAPI, blue. Scale bar, 20 μm (B and C, n = 4). *$P < 0.05$ versus Ctrl; #$P < 0.05$ between the marked groups. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni test.
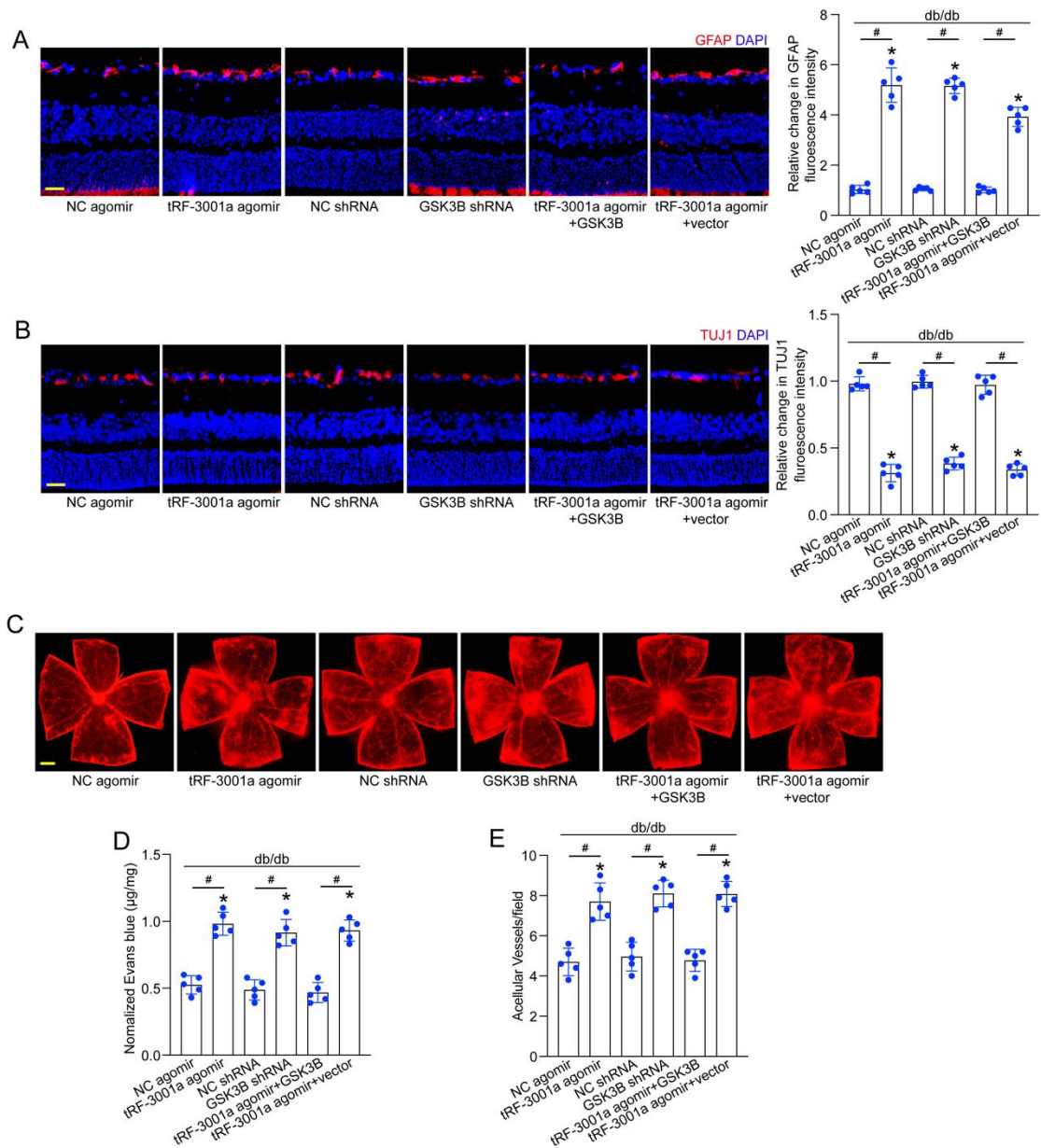
Figure S12. tRF-3001a-GSK3B axis regulates retinal neurovascular dysfunction in db/db diabetic mice. Related to Figure 6

(A-E) The diabetic db/db mice (male, 3-month old) received intravitreal injections of negative control (NC) agomir, tRF-3001a agomir, NC shRNA, GSK3B shRNA, tRF-3001a plus GSK3B overexpression vector, or tRF-3001a plus null vector for one month. Retinal reactive gliosis (A; Scale bar, 50 μm), RGC degeneration (B; Scale bar, 50 μm), retinal vasopermeability (C and D; Scale bar, 500 μm), and retinal acellular capillaries (E) was determined to evaluate the role of tRF-3001a-GSK3B axis in retinal neurovascular dysfunction *in vivo* (n = 5). *$P < 0.05$ versus Ctrl group, #$P < 0.05$ between the marked groups; One-way ANOVA followed by post hoc Bonferroni test.

Table S1. Baseline characteristics of the patients for fibrovascular membrane collection. Related to Figure 1 and STAR Methods.

	Proliferative diabetic retinopathy (n = 10)	Idiopathic epiretinal membranes (n = 10)	*P* value
Age, y	58.40±3.12	59.4±2.95	0.82
Male, %	40	40	1
Total cholesterol, mmol/L	4.83±0.35	5.19±0.22	0.39
Creatinine, μmol/L	63.90±3.38	52.00±3.71	0.029
Triglyceride, mmol/L	2.14±0.22	1.29±0.15	0.005
HbAlc, %	8.01±0.29	5.81±0.11	0.0001

Data were expressed as mean ±SD or percentage. *P* values were determined by Student's *t* test or Fisher exact test.

Table S2. Baseline characteristics of the patients with DR and cataract for AH sample collection. Related to Figure 8 and STAR Methods.

	DR	Cataract	*P* value
Number	30	30	
Gender (Male/Female)	18/12	16/14	0.673
Age	65.6±7.9	68.2±9.5	0.186
Hypercholesterolemia	41±5.12	26±4.16	0.023
Hypertriglyceridemia	32±4.72	21±2.96	0.048
Blood glucose level	8.7±2.68	4.9±0.83	<0.001
Glycosylated hemoglobin	7.9±1.93	5.1±1.12	<0.001

Data were expressed as mean ±SD or percentage. *P* values were determined by Student's *t* test or Fisher exact test.

Table S3. The sequence information for the relevant RNAs. Related to STAR Methods.

Name	Sequence (5'-3')
tRF-3001a mimic	AUCCCACCGCUGCCACCA
NC mimic	CCCUAGCCAAUCGACCCC
tRF-3001a inhibitor	UGGUGGCAGCGGUGGGAU
NC inhibitor	CCCCUACCCCCGCUAAGA

Table S4. Primer sequences for qPCR assays. Related to STAR Methods

Primer sequence		
tRF-3001a	Forward	5'-CGCGCGACCCACCGCG-3'
	Reverse	5'-AGTGCAGGGTCCGAGGTATT-3'
U6	Forward	5'-TCAAATGATTAGGTTTATCTTTCAATTAT-3'
	Reverse	5'-AGTGCAGGGTCCGAGGTATT-3'
VEGF	Forward	5'-GTCCTCTCCTTACCCCACCTCCT-3'
	Reverse	5'-CTCACACACACAGCCAAGTCTCCT-3'
IL-6	Forward	5'-TCCATCCAGTTGCCTTCTTG-3'
	Reverse	5'-TTCCACGATTTCCCAGAGAAC-3'
IL-1β	Forward	5'-GCCTGTGTTTTCCTCCTTGC-3'
	Reverse	5'-TGCTGCCTAATGTCCCCTTG-3'
ICAM-1	Forward	5'-GGAAGGGAGCCAAGTAACTGTGAAG-3'
	Reverse	5'-GAGCGGCAGAGCAAAAGAAGC-3'
TNF-α	Forward	5'-CAGGCGGTGCCTATGTCTC-3'
	Reverse	5'-CGATCACCCCGAAGTTCAGTAG-3'
β-actin	Forward	5'-GGGAAATCGTGCGTGAC-3'
	Reverse	5'-AGGCTGGAAAAGAGCCT-3'