

Supplementary Information

Aberrant splicing of Cav1.2 calcium channel induced by decreased Rbfox1 enhances arterial constriction during diabetic hyperglycemia

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Table S1. Clinical characteristics of the patients

Sample	Sex	Age (y)	Relevant Medical History	Glucose level (mmol/L)	Pre-op medications
1	F	58	Hypertension, Diabetes	6.79	/
2	F	64	Hypertension, Diabetes	6.70	Metformin Amlodipine besylate
3	F	66	Cerebral infarction, Diabetes Cholecystectomy, Hysterectomy, Moyamoya disease	10.54	/
4	F	72	Hypertension, Diabetes left middle cerebral occlusion	7.12	Nifedipine, Metformin, Acarbose
5	M	53	Moyamoya disease, Diabetes	7.3	Insulin
6	M	56	Moyamoya disease	4.34	/
7	F	47	Moyamoya disease	5.55	/
8	F	42	Moyamoya disease	3.84	/
9	M	66	Moyamoya disease, Cerebral infarction, Hypertension	5.01	/
10	M	54	Moyamoya disease, Pulmonary emphysema, Duodenal ulcer	4.72	/

Table S2. Sequences of siRNAs targeting with rat Rbfox1 mRNA

siRNAs	Sense/antisense	Sequences
#1 siRNA	Sense	5' CGAGGUUAAUAAUGCGACAdTdT 3'
	Antisense	3' dTdTGCUCCAAUUAUACGCUGU 5'
#2 siRNA	Sense	5' GGAUCCAGACCUCCGACAAAdTdT 3'
	Antisense	3' dTdTCCUAGGUCUGGAGGCUGUU 5'
#3 siRNA	Sense	5' CGGUGUUGUUUACCAGGAUdTdT 3'
	Antisense	3' dTdTGCCACAACAAAUGGUCCUA 5'

Table S3. Primer sequences for PCR

Target genes	Oligonucleotide primers (5'-3')	Product length (bp)
Rat <i>Cacna1c</i> exon 8	FP: TCAATGATGCCGTAGGAAG RP: CGCTAAGAACACCGAGAA	102
Rat <i>Cacna1c</i> exon 8a	FP: ACGCTATGGGCTATGAGT RP: GGTGATCCAGTCCAGGTA	206
Rat <i>Cacna1c</i> exon 9*/Δ9*	FP: TTCCAAAGAGAGGGAGAAAGC RP: CTGCAGAATCTATTCCACCGG	393 (E9*) 318 (ΔE9*)
Rat <i>Cacna1c</i> exon 33/Δ33	FP: GCCTCTTCACGGTGGAG RP: TCCCAATCACTGCATAGATAA	357 (E33) 324 (ΔE33)
Human <i>Cacna1c</i> exon 9*/Δ9*	FP: TTCCAAAGAGAGGGAGAAGGC RP: CTGCAGAACCGATTCCACCGG	393 (E9*) 318 (ΔE9*)
Human <i>Cacna1c</i> exon 33/Δ33	FP: GCCTGTTCAAAATCGCC RP: TCCCGATCACCGCGTAGATGA	402 (E33) 369 (ΔE33)
Rat <i>Gapdh</i>	FP: CAGGGCTGCCTTCTCTTGTTG RP: AACTTGCCGTGGGTAGAGTC	111

Table S4. The information of key antibodies used in this study

Target protein	Western blotting		Immunofluorescence	
	Rbfox1	β-actin	Rbfox1	α-SMA
Vendor	ThermoFisher (MA533104)	Abways Technology (AB0035)	Santa Cruz Biotechnology (sc-135476)	Novus Biologicals (NBP2-33006)
Species reactivity	Human, Mouse, Rat	Human, Mouse, Rat	Mouse, Rat	Mouse, Rat
Host/Subtype	Mouse IgG1	Rabbit IgG	Rabbit IgG	Mouse IgG
Classification	Monoclonal	Monoclonal	Monoclonal	Monoclonal
Purification	Protein G	Affinity- chromatography	Protein G	Protein A
Antigen	Recombinant protein taken from the N-terminus of human FOX1 expressed in and purified from E. Coli	A synthesized peptide derived from human beta Actin	Amino acids 38-118 mapping near the N- terminus of Fox-1 of human	N-Terminal decapeptide of Alpha smooth muscle isoform of actin

Table S5. Summary of Cav1.2 channel electrophysiological characteristics of isolated VSMCs from control or HFD/STZ-treated rats MAs

	$V_{0.5}$ (mV)	E_{rev} (mV)	k	n
Ctrl	-4.74±2.19	54.85±1.57	-12.35±0.92	9
HFD/STZ	-12.13±1.68*	57.08±1.77	-11.19±0.85	9
	$V_{0.5,act}$ (mV)	n	$V_{0.5,inact}$ (mV)	n
Ctrl	18.88±4.42	11	-16.50±1.94	7
HFD/STZ	5.94±3.94*	10	-20.25±2.59	5

$V_{0.5}$: half-activation potential, E_{rev} : reversal potential, k : slope rate. $V_{0.5,act}$: half-activation potential of activation curve, $V_{0.5,inact}$: half-inactivation potential of inactivation curve. * P <0.05 versus Ctrl, unpaired t test.

Table S6. Summary of Cav1.2 channel electrophysiological characteristics of NG, GS or GS plus H-89-treated VSMCs

	$V_{0.5}$ (mV)	E_{rev} (mV)	k	n
NG	-2.14±1.73	55.49±1.72	-10.13±0.79	12
GS	-9.06±1.13**	49.49±1.44*	-7.39±0.65*	12
GS+H-89	-7.68±1.55*	51.51±1.55	-9.50±0.78	15

$V_{0.5}$: half-activation potential, E_{rev} : reversal potential, k : slope rate. * P <0.05, ** P <0.01 versus NG-treated VSMCs, 1-way ANOVA followed by a Tukey's post hoc test.

Table S7. Summary of Cav1.2 channel electrophysiological characteristics of vehicle (Ctrl), MGO or MGO plus H-89-treated VSMCs

	$V_{0.5}$ (mV)	E_{rev} (mV)	k	n
Ctrl	-1.80±2.37	55.56±2.12	-10.95±1.01	8
MGO	-10.23±1.71*	54.86±1.98	-10.04±0.84	9
MGO+H-89	-9.69±1.75*	50.04±1.38	-11.37±0.80	9

$V_{0.5}$: half-activation potential, E_{rev} : reversal potential, k : slope rate. * P <0.05 versus vehicle-treated VSMCs, 1-way ANOVA followed by a Tukey's post hoc test.

Table S8. Summary of Cav1.2 channel electrophysiological characteristics of NT or Rbfox1 siRNA-treated VSMCs

	$V_{0.5}$ (mV)	E_{rev} (mV)	k	n
NT siRNA	-2.51±1.26	49.34±1.15	-8.21±0.66	11
Rbfox1 siRNA	-8.75±1.24**	47.84±1.36	-7.96±0.69	14

$V_{0.5}$: half-activation potential, E_{rev} : reversal potential, k : slope rate. ** P <0.01 versus NT siRNAs-treated VSMCs, unpaired t test.

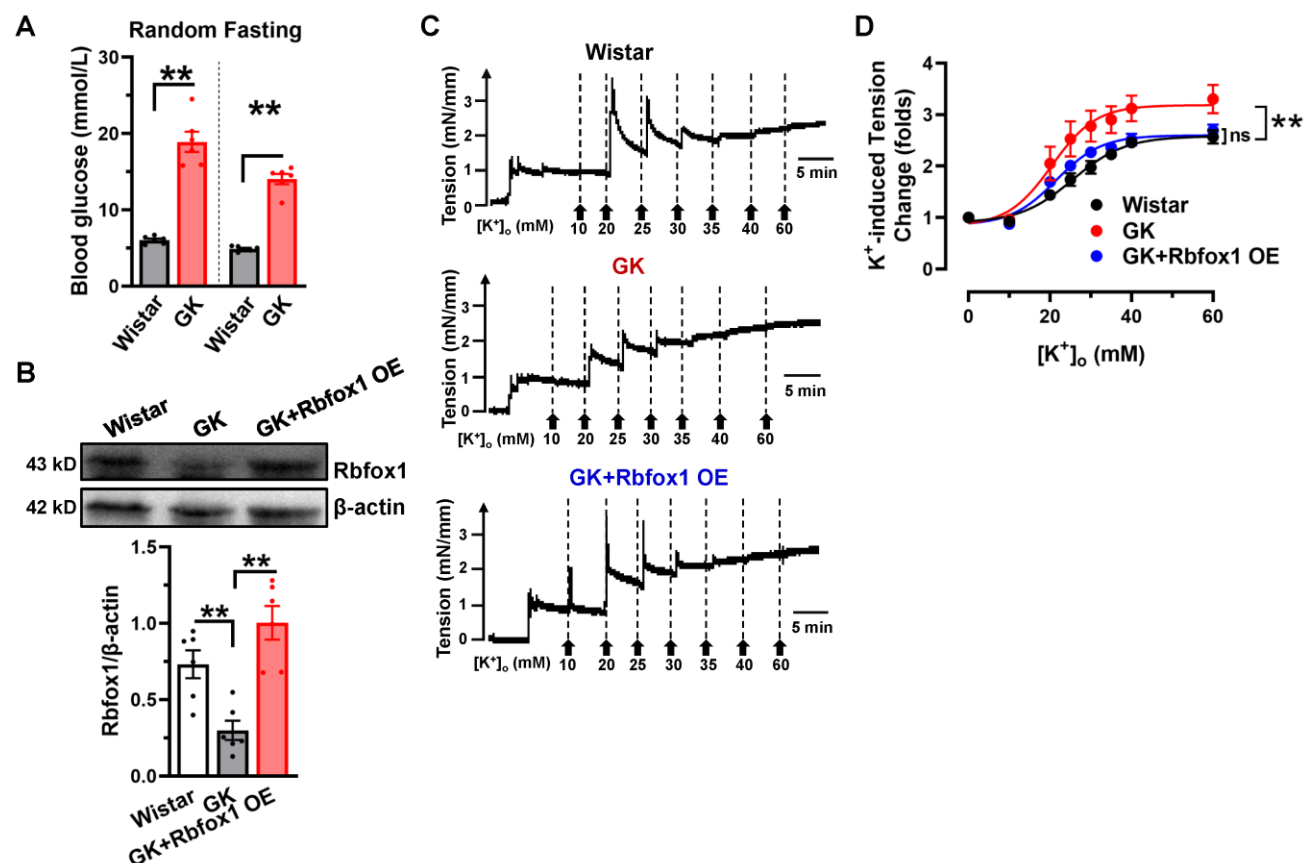


Figure S1. Overexpression of Rbfox1 in mesenteric arteries reduces vasoconstriction in Goto-Kakizaki (GK) rats. (A) Random and fasting blood glucose levels in the rats. (B) Expression of Rbfox1 was detected 3 days after treatment by Rbfox1 expression plasmids (OE) in mesenteric arteries from GK rats. β-actin served as an internal control. Band intensities were analyzed to show the relative expression of Rbfox1 and summarized as a bar chart. n=6 rats for each group, 1-way ANOVA followed by a Tukey's post hoc test. (C) Exemplary traces of mesenteric artery tension responding to increased KCl extracellular concentration (from 0 to 60 mmol/L KCl) using vascular myograph in Wistar, GK and Rbfox1 overexpressed GK rats. (D) Plots of concentration-tension relationship were represented and fitted with Boltzmann equation for differently-treated arteries. n=6 rats for each group. **P*<0.05 vs Wistar rats, 2-way ANOVA followed by Sidak's multiple comparisons.

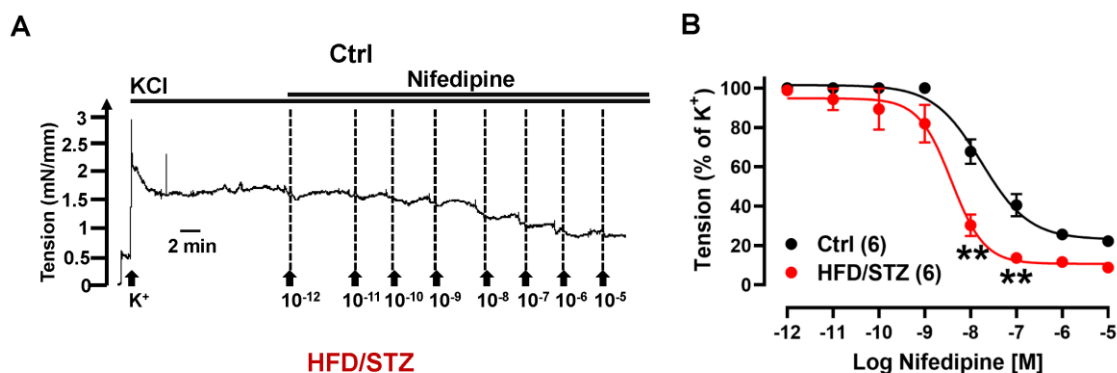


Figure S2. MAs from diabetic rats are more sensitive to nifedipine in comparison to control ones. (A) Arterial tension responding to different concentration of nifedipine under 60 mmol/L KCl bath solution was measured by vascular myography in control (Ctrl) or HFD/STZ-treated rats (one artery for each rat). (B) Plots of concentration-tension relationship were represented for the MAs from control or diabetic rats. ** $P < 0.01$ vs control rats, 2-way ANOVA followed by Sidak's multiple comparisons.

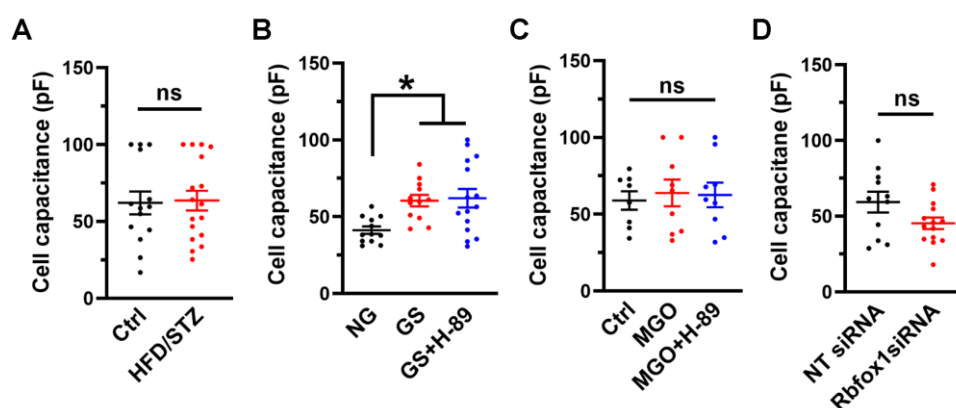


Figure S3. The cell capacitances (C_m) are measured in differentially-treated VSMCs. The C_m were shown as scatter plots in freshly isolated VSMCs of MAs from control or HFD/STZ-treated rats (A), in VSMCs treated with NG, GS or GS plus H-89 (B), in VSMCs treated with vehicle (Ctrl), MGO or MGO plus H-89 (C), and in NT or Rbfox1 siRNA-treated VSMCs (D). * $P < 0.05$, ns indicates no significant differences, unpaired t test or 1-way ANOVA followed by a Tukey's post hoc test.

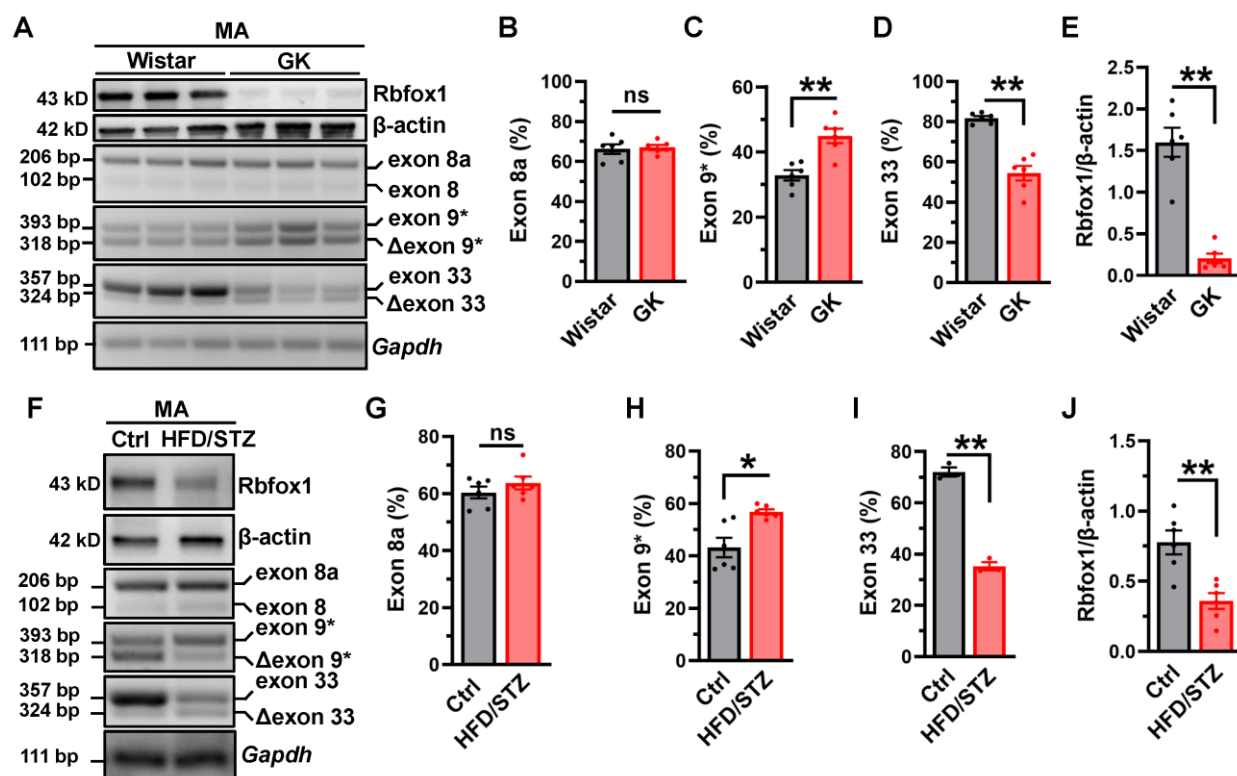


Figure S4. Cav_v1.2 channel is aberrantly spliced in the arteries from GK rats and non-hypertensive HFD/STZ rats. Mesenteric arteries (MAs) from Wistar or GK rats, and Ctrl or HFD/STZ rats were isolated to determine the expression level of Rbfox1 protein by Western blotting. The proportion of Cav1.2 with exon 8/8a, exon 9* or exon 33 were detected by RT-PCR. The relative Rbfox1 expression was normalized to β-actin after treatments (**A**, **F**). The values for percent exon 8/8a (**B**, **G**), exon 9* (**C**, **H**) or exon 33 inclusion (**D**, **I**) of Cav1.2 channels were presented as bar charts. Relative expression of Rbfox1 and summarized as a bar chart (**E**, **J**). n=3-6 rats for each group. ns indicates no significant differences, * $P<0.05$, ** $P<0.01$ vs Wistar or Ctrl rats, unpaired t test.

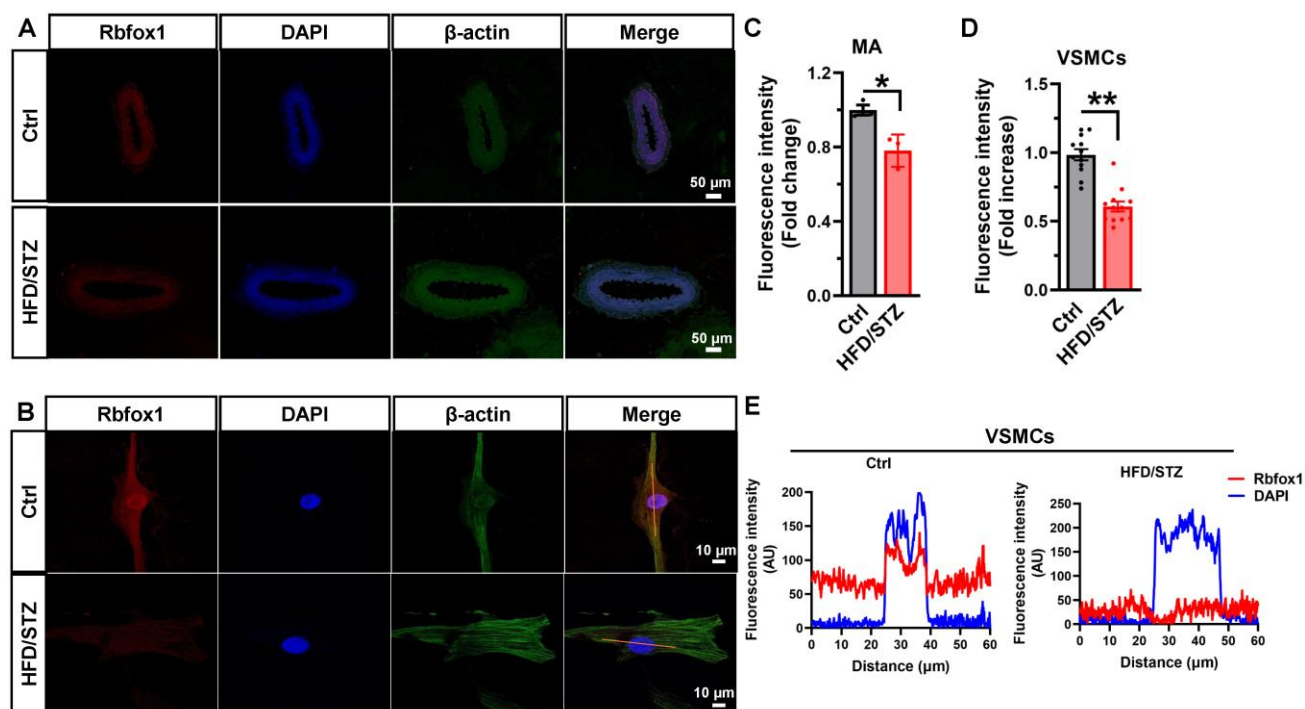


Figure S5. Rbfox1 is downregulated in the arteries/VSMCs from HFD/STZ rats. Immunofluorescence staining was used to detect the expression of Rbfox1 in MAs (A) or isolated VSMCs (B) from Ctrl and HFD/STZ rats. Fluorescence intensities were analyzed (C&D). (E) Fluorescence intensity profiles for Rbfox1 (red) and DAPI (blue) along the yellow line drawn in the pictures, expressed as Arbitrary Unit (AU), in Ctrl and HFD/STZ rats. (n=3 arteries and 12 cells from 3 rats, respectively). * $P < 0.05$, ** $P < 0.01$ vs ctrl, unpaired t test.

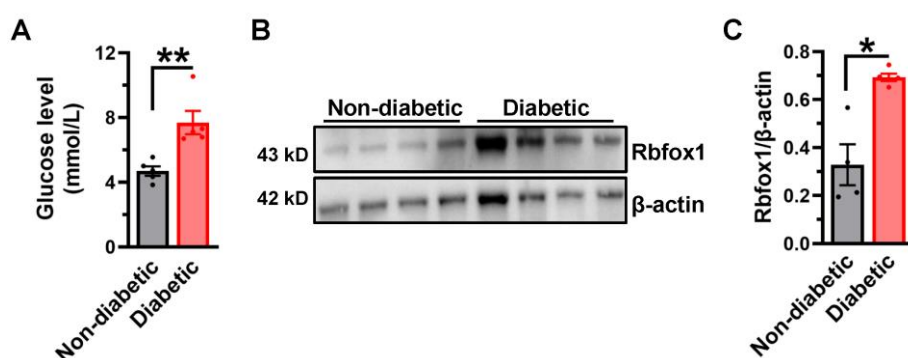


Figure S6. Rbfox1 is upregulated in the arteries from diabetic patients. (A) Blood glucose levels from the patients with or without diabetes. ** $P = 0.0048$ vs non-diabetic patients, unpaired t test. (B) Rbfox1 protein expression detected by Western blotting in diabetic versus nondiabetic cerebral arteries from the patients, β -actin was served as an internal control. (C) The relative Rbfox1 expression was normalized to β -actin, and presented as a bar chart. * $P = 0.022$ vs non-diabetic patients, unpaired t test with Welch's correction.

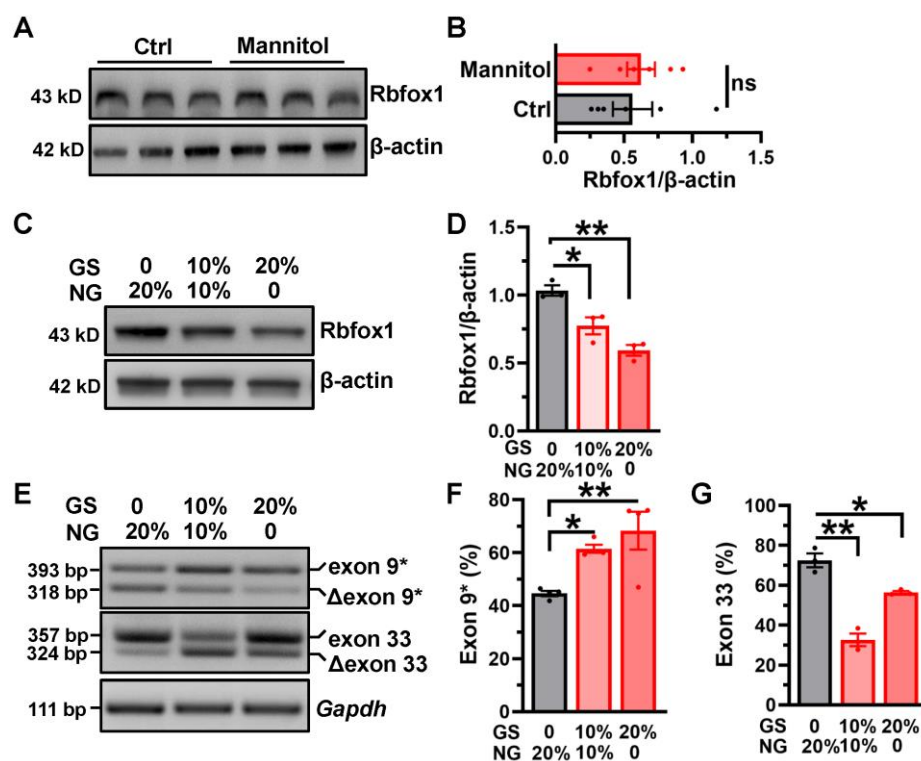


Figure S7. GS decreases Rbfox1 expression and regulates Cav1.2 alternative exons 9* and 33. (A) Mannitol (25 mmol/L) was used to treat isolated VSMCs for 48 hr. The expression of Rbfox1 was checked by Western blotting, and β-actin was detected as internal control. (B) The expression level of Rbfox1 was normalized to β-actin expression after mannitol treatment. $P=0.7304$, unpaired t test. (C) Rbfox1 expression was checked by Western blotting after treating with increasing concentration of GS. (D) The relative Rbfox1 expression levels were normalized by β-actin expression. (E) Expressions of Cav1.2 alternative exon 9* or exon 33 were checked by RT-PCR. The values for percent exon 9* (F) or exon 33 inclusion (G) were analyzed after treating with GS. * $P<0.05$, ** $P<0.01$ vs NG-treated VSMCs, 1-way ANOVA followed by a Tukey's post hoc test.

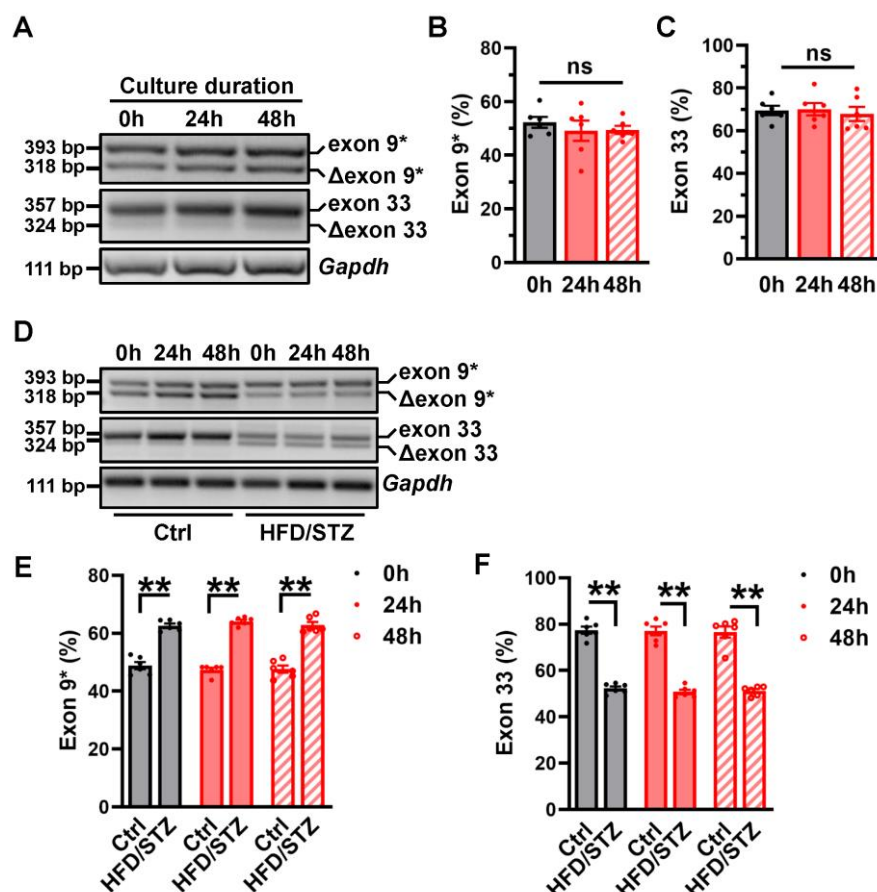


Figure S8. Cell culture does not alter Cav1.2 AS events in isolated VSMCs. (A) Freshly isolated VSMCs from rats (0 hr) were cultured with the medium for 24 or 48 hr. The proportion of Cav1.2 with alternative exon 9* (B) or exon 33 (C) were detected by RT-PCR. *Gapdh* mRNA was detected as loading control. ns indicates no significant differences, 1-way ANOVA followed by a Tukey's post hoc test. (D) Freshly isolated VSMCs from control or HFD/STZ rats (0 hr) were cultured with the medium for 24 or 48 hr. The proportion of Cav1.2 with alternative exon 9* (B) or exon 33 (C) were detected by RT-PCR. *Gapdh* mRNA was detected as loading control. ** $P < 0.01$ vs control rats, 2-way ANOVA followed by a Sidak's multiple comparisons.

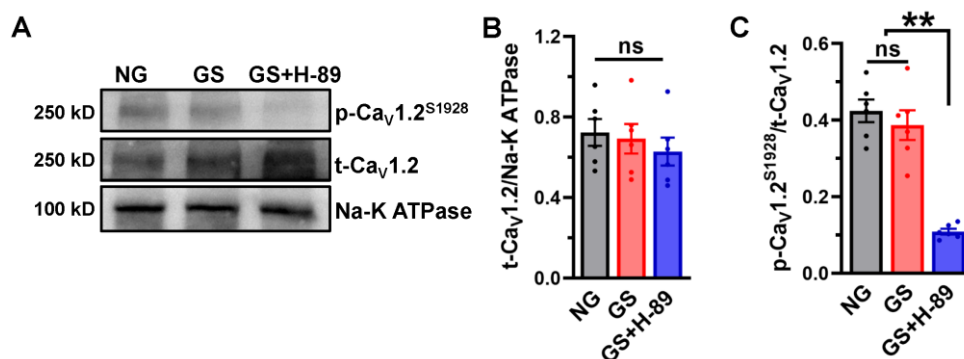


Figure S9. GS application does not increase Cav1.2 expression and S1928 phosphorylation in VSMCs. (A) VSMCs were treated with 20% NG, GS or GS plus 50 $\mu\text{mol/L}$ PKA inhibitor H-89 for 48 hr and Western blotting were performed to detect the expression of total Cav1.2 (B) and S1928 phosphorylated Cav1.2 (C). ** $P < 0.01$ vs GS. ns indicates no significant differences, 1-way ANOVA followed by a Tukey's post hoc test.

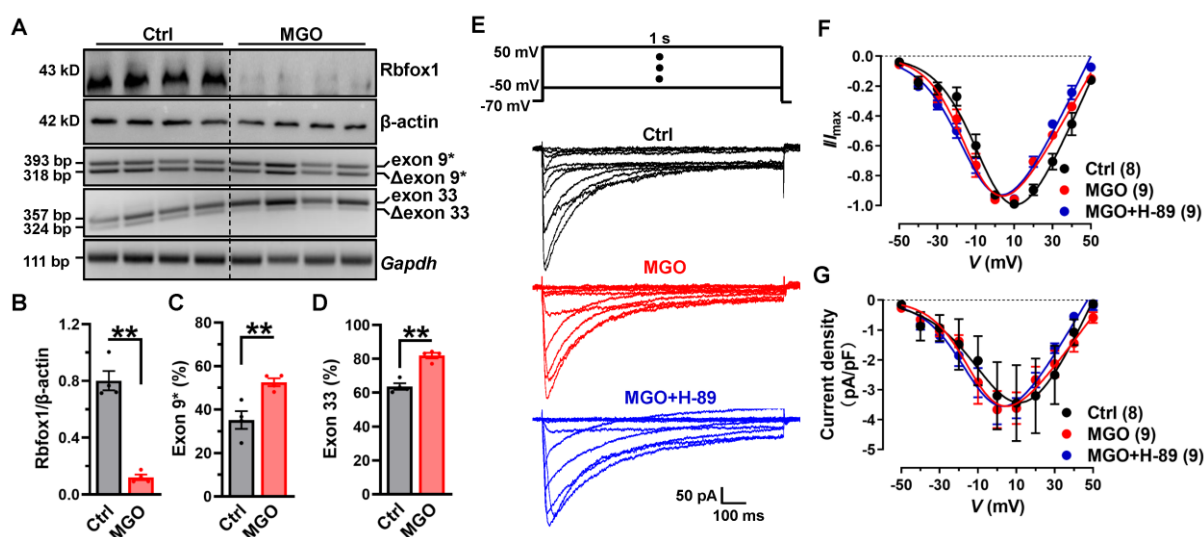


Figure S10. Methylglyoxal (MGO) induces decreased Rbfox1, aberrant Cav1.2 AS and hyperpolarization of current–voltage curve of Cav1.2 in VSMCs. (A) As one of main components in AGEs, MGO (500 $\mu\text{mol/L}$) was used to treat VSMCs for 48 hr and β -actin was detected as internal control. The proportion of Cav1.2 with alternative exon 9* or exon 33 were detected by RT-PCR. (B) The relative Rbfox1 expression level was presented in differently-treated VSMCs. The proportions of Cav1.2 with alternative exon 9* (C) or exon 33 (D) were analyzed in differently treated VSMCs. (E) Whole-cell currents in the Cav1.2 channel were recorded under different potentials, increasing from -50 to 50 mV (10-mV increase per step; $I-V$ protocol) in isolated aortic VSMCs under 10 mmol/L Ba^{2+} bath solution. Additionally, 50 $\mu\text{mol/L}$ PKA inhibitor H-89 was applied 2 hr before the Cav1.2 currents recording in MGO-treated VSMCs. (F) Plots of Cav1.2 current-voltage relationship ($I-V$) curve of VSMCs were analyzed after treating with vehicle (Ctrl), MGO or MGO plus H-89. (G) Current densities of Cav1.2 channel were calculated by currents divided by cell capacitance (C_m) of the VSMCs.

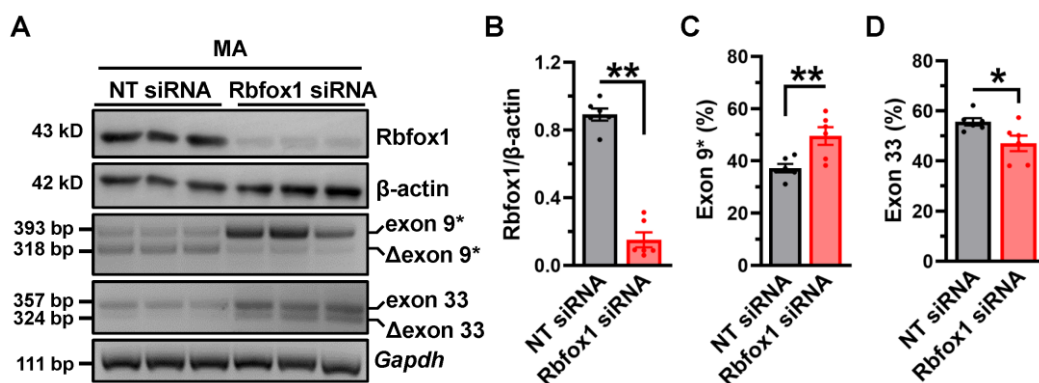


Figure S11. Rbfox1 dynamically regulates Cav1.2 alternative exons 9* and 33 in MAs. (A) NT or Rbfox1 siRNAs were transfected into isolated MAs using reversible permeabilization procedure. After 72 hr culture, Rbfox1 protein and Cav1.2 with alternative exon 9* or exon 33 were detected by Western blotting and RT-PCR, respectively. (B) The relative Rbfox1 expression was presented in MAs after different treatments. The values for percent exon 9* (C) or exon 33 inclusion (D) were presented as bar charts. * $P < 0.05$, ** $P < 0.01$ vs NT siRNA-treated MAs, unpaired t test.

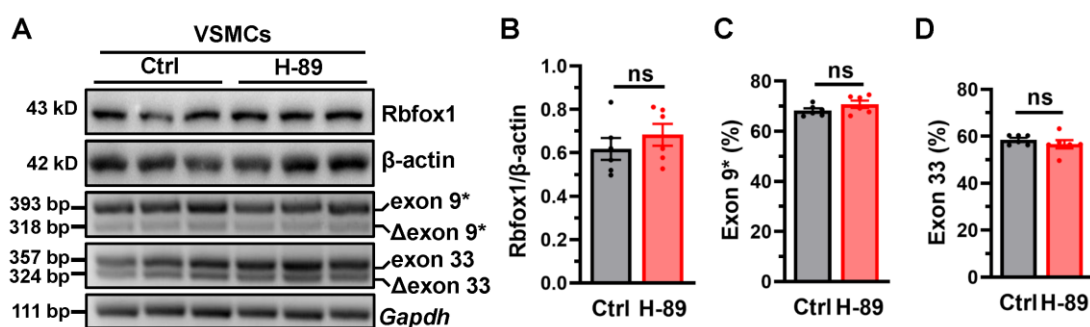


Figure S12. H-89 does not affect Rbfox1 expression and Cav1.2 AS events in NG-treated VSMCs. (A) Vehicle (Ctrl) or H-89 (50 μ mol/L) was used to treat isolated VSMCs for 48 hr. The expression of Rbfox1 was checked by Western blotting, and the proportion of Cav1.2 with alternative exon 9* or exon 33 were detected by RT-PCR. (B) The relative Rbfox1 expression was normalized to β -actin after treatments. The values for percent exon 9* (C) or exon 33 inclusion (D) of Cav1.2 channels were presented as bar charts. ns indicates no significant differences, unpaired t test.

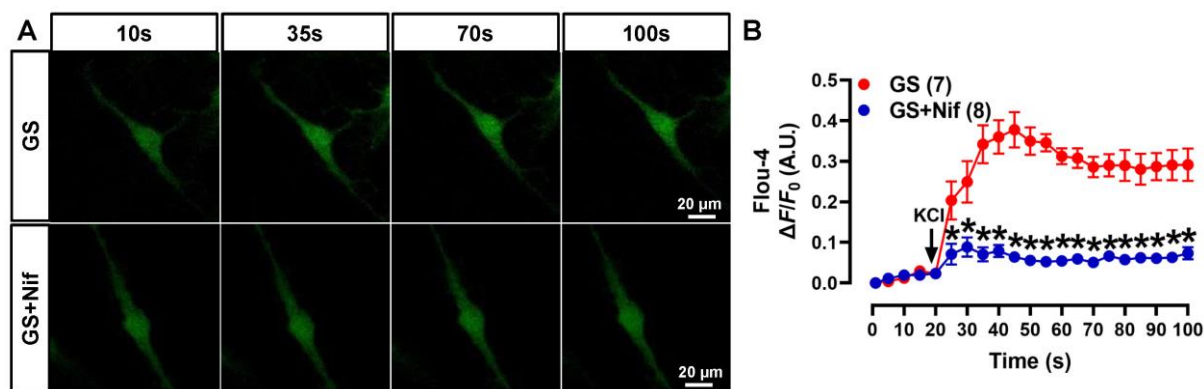


Figure S13. Application with nifedipine blocks GS-induced $[Ca^{2+}]_i$ elevation. (A) VSMCs were treated with 20% GS for 48 hr and 10^{-6} mol/L nifedipine (Nif) were added 10 min before $[Ca^{2+}]_i$ triggered by 60 mmol/L KCl, $[Ca^{2+}]_i$ was monitored by Ca^{2+} fluorescence indicator Fluo-4 AM. The fluorescent intensity was measured by time series scanning mode under a confocal microscope. (B) $\Delta[Ca^{2+}]_i$ was presented as $\Delta F/F_0$ and shown as a line diagram, $*P < 0.01$ vs GS-treated VSMCs, 2-way ANOVA followed by Sidak's multiple comparisons.

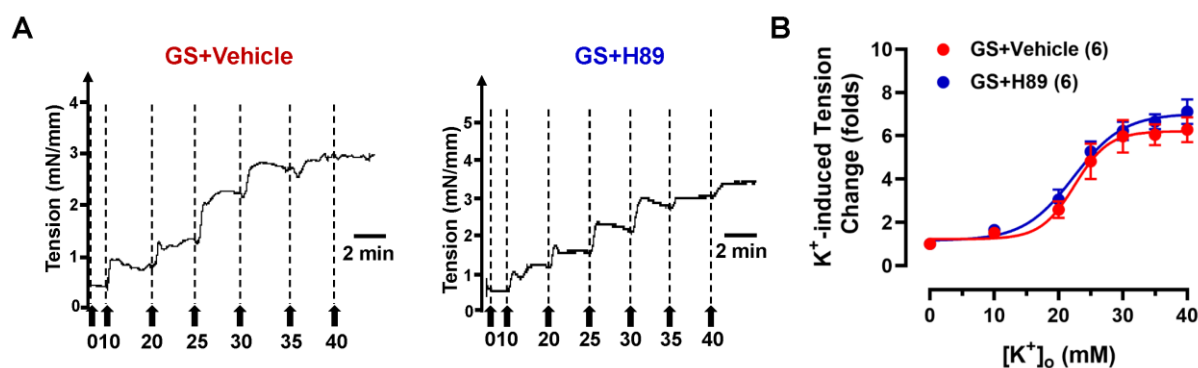


Figure S14. PKA inhibitor H-89 doesn't affect GS-induced vasoconstriction. (A) Isolated MAs were cultured with 20% GS with or without H-89 (50 μ mol/L) for 48 hr, then the forced tension induced by increasing extracellular K^+ concentration was recorded by using vascular myograph. (B) Plots of concentration-tension relationship were represented and fitted with Boltzmann equation for differently-treated arteries. There were no significant statistical differences, 2-way ANOVA followed by Sidak's multiple comparisons.

Fig. 4A

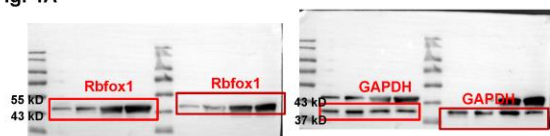


Fig. 5A

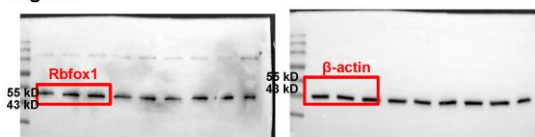


Fig. 4D

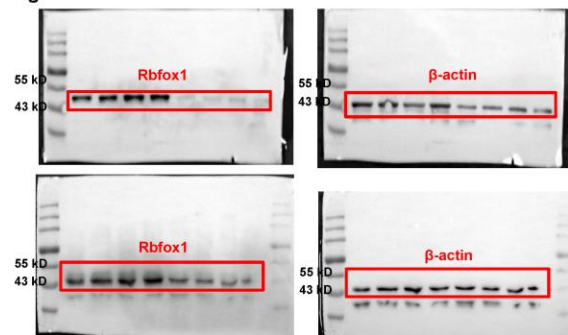


Fig. 5E

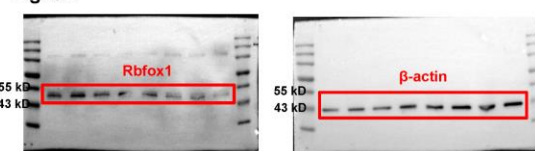


Fig. 5I

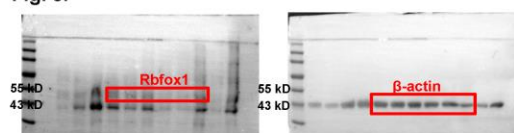


Fig. 6A

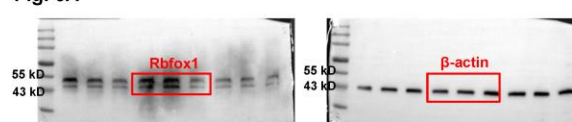


Fig. 6E

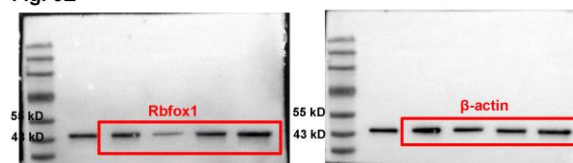


Fig. S1B

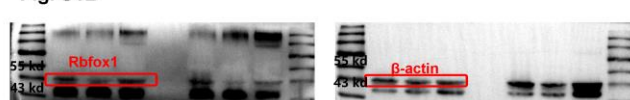


Fig. S7C

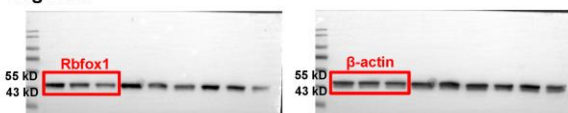


Fig. S4A

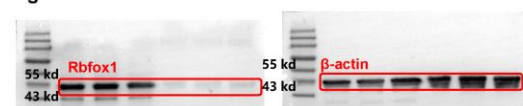


Fig. S9A



Fig. S4F

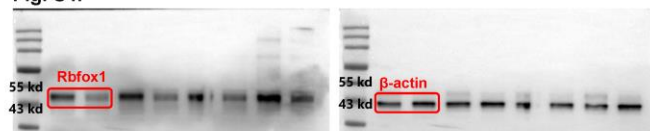


Fig. S10A

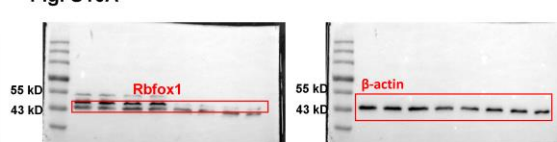


Fig. S6B

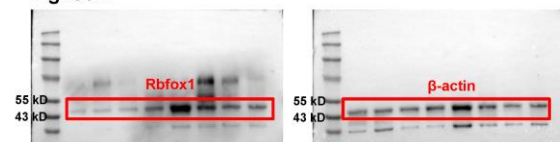


Fig. S11A



Fig. S7A

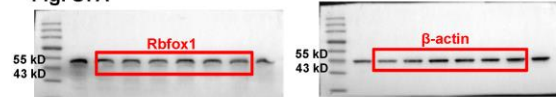


Fig. S12A

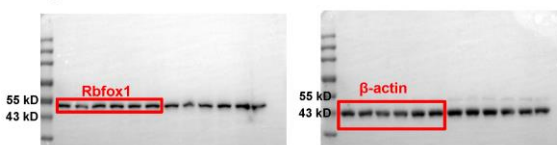


Figure S15. Uncropped Western blotting images.