Supplementary Information

for

Sulforaphane protects microvascular endothelial cells in lower limb

ischemia/reperfusion injury mice

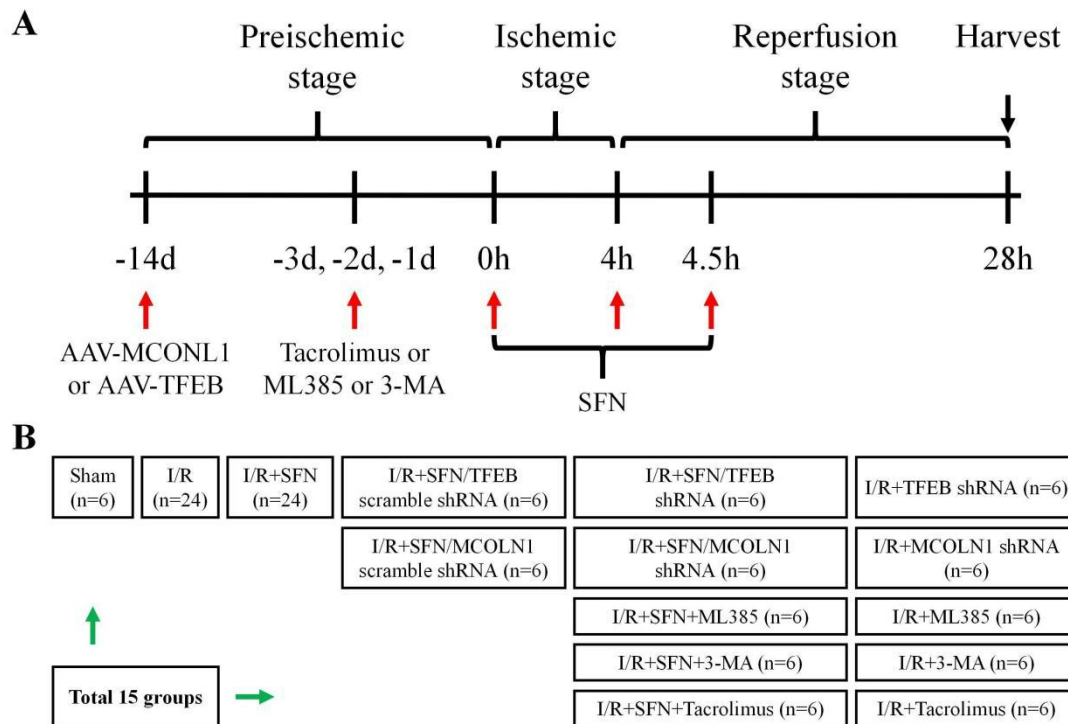Fanfeng Chen *et. al*

Figure S1

Figure S1. Study design and grouping strategy. (**A**) Schematic diagram of the steps of animal experiments. (**B**) Grouping strategy depending on the drug administration method. Abbreviations: SFN, sulforaphane; I/R, ischemia/reperfusion; TFEB, transcription factor EB; AAV, adeno-associated virus; 3MA, 3-methyladenine; MCOLN1, mucolipin 1.

Figure S2
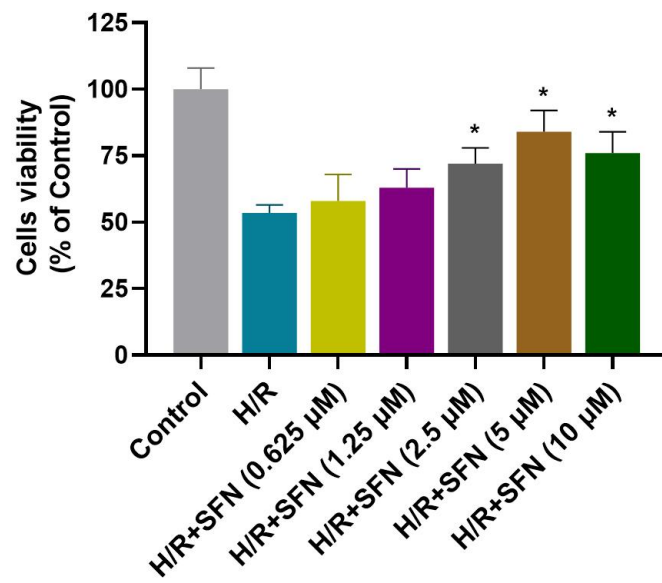
Figure S2. Effect of SFN on H/R-induced alterations in cell viability of HUVECs. Adopting CCK-8 to measure relative cell viability after being treated with SFN in different concentrations. Data are displayed as the means ± SD (n = 4 per group). Significance: $^*P < 0.05$ vs. H/R group. Abbreviations: SFN, sulforaphane; H/R, hypoxia/reoxygenation; HUVECs, human umbilical vein endothelial cells; CCK-8, cell counting kit-8; SD, standard deviation.

Figure S3

Figure S3. SFN enhances autophagy, attenuates oxidative stress and reduces EC death via enhancing TFEB activity in I/R limbs. Mice were treated with saline, SFN, SFN+scrambled shRNA, SFN+TFEB shRNA and TFEB shRNA, then underwent IR. (**A-C**) Images of skeletal muscle sections stained with antibodies against NFE2L2/CD31 (A, scale bars = 100 μm), LC3/CD31 (B, scale bars = 50 μm) and TUNEL/CD31 (C, scale bars = 100 μm), merged images include DAPI staining. (**D-E**) Quantification of immunofluorescence data from (A-B) showing the mean optical density of NFE2L2 (A) and LC3 (B) on vascular endothelium (CD31$^+$ cell) in the skeletal muscle. (**F**) Quantification of TUNEL and CD31 double-positive cells, the percentages of double-positive cells versus total CD31 positive cells are indicated, data from (C). Data are expressed as the means ± SD (n = 4 per group).Significance:

ns stands for not significant, $^{*}P < 0.05$. Abbreviations: SFN, sulforaphane; I/R, ischemia/reperfusion; LC3, microtubule-associated protein 1 light chain 3; TFEB, transcription factor EB; shRNA, short hairpin RNA; NFE2L2, nuclear factor, erythroid 2 like 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; CD31, cluster of differentiation 31; SD, standard deviation; DAPI, 4,6-diamidino-2-phenylindole.
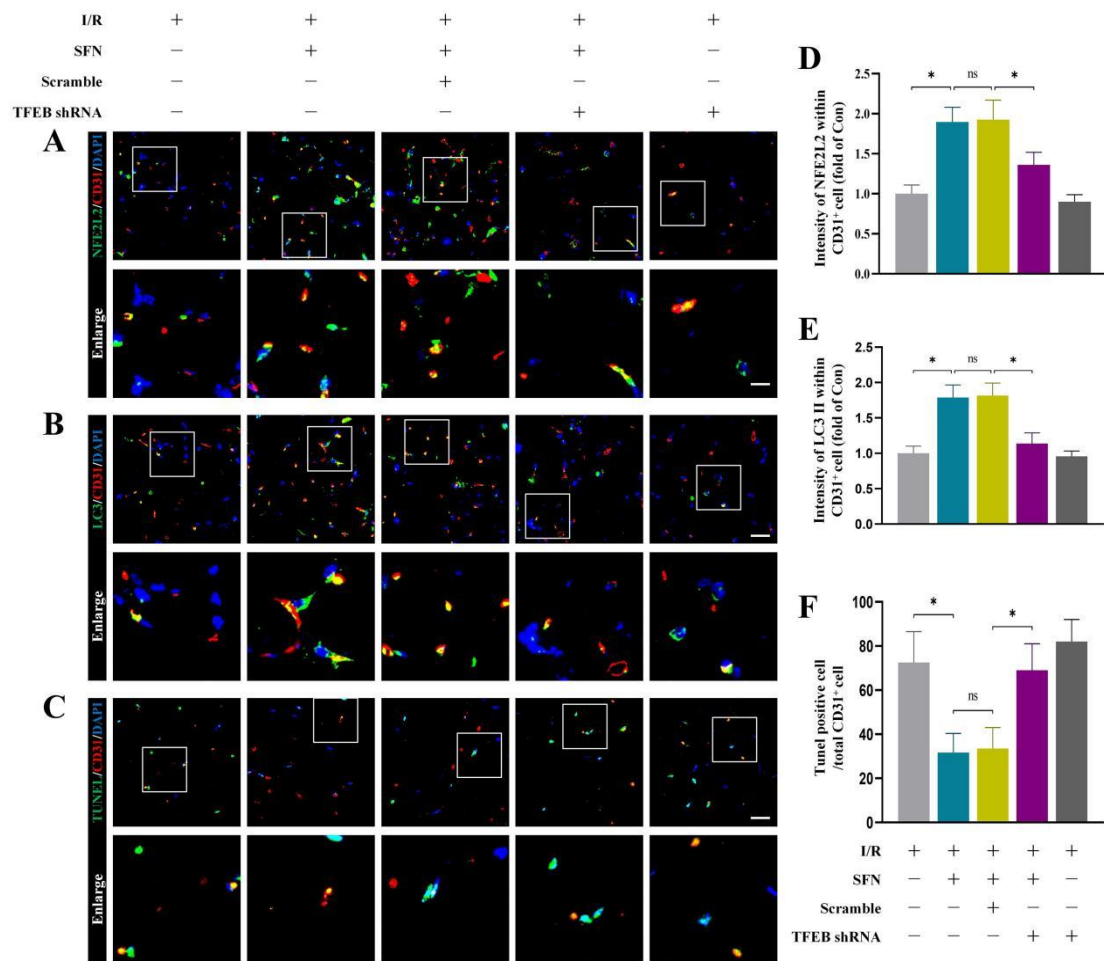
Figure S4
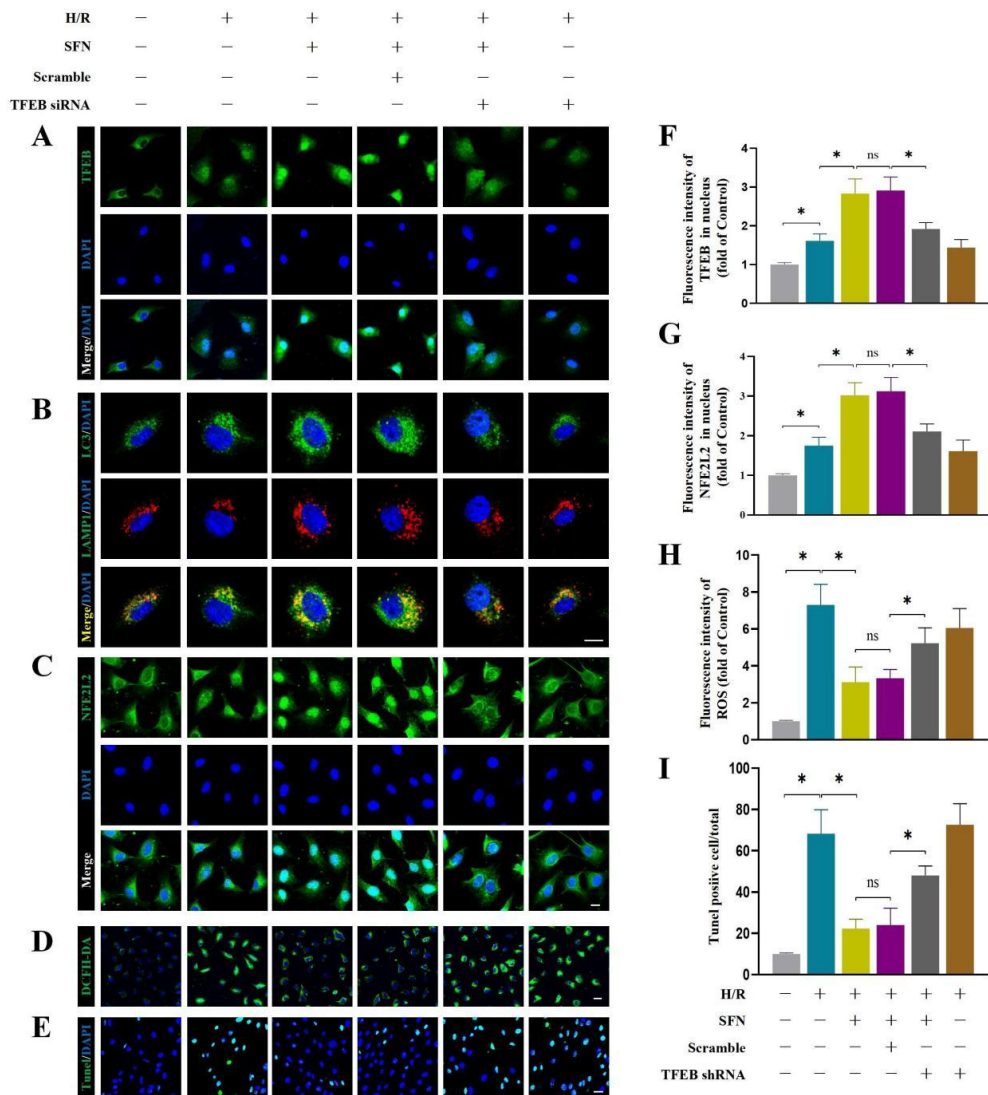
Figure S4. SFN enhances autophagy, attenuates oxidative stress and reduces EC death via enhancing TFEB activity in vitro. (**A**) Representative immunofluorescence images of HUVECs depicting TFEB and DAPI. Scale bars: 25 μm. (**B**) Representative immunofluorescence images of HUVECs depicting LC3 and LAMP1, nuclei were recognized by DAPI staining. Scale bars: 10 μm. (**C**) Representative immunofluorescence images of HUVECs depicting NFE2L2 and DAPI. Scale bars: 25 μm. (**D**) ROS of HUVECs was detected by DCFH-DA assay, Scale bars: 25 μm. (**E**) TUNEL assay of HUVECs, Scale bars: 25 μm. (**F**) Quantification and analysis of immunofluorescence data from (A) displaying the average optical density of TFEB in the nucleus. (**G**) Quantification of immunofluorescence data from (C) displaying the average optical density of NFE2L2 in the nucleus. (**H**) Quantification and analysis of immunofluorescence data from (D) displaying the average optical density of ROS. (**I**)

The percentages of TUNEL-positive cells versus total cells are indicated from (E). Data are displayed as the means ± SD (n = 4 per group). Significance: ns, non-significant; $^*P$ < 0.05. Abbreviations: SFN, sulforaphane; H/R, hypoxia/reoxygenation; HUVECs, human umbilical vein endothelial cells; DCFH-DA, 2'-7'-dichlorodihydrofluorescein-diacetate; ROS, reactive oxygen species; LAMP1, lysosomal associated membrane protein 1; CTSD, cathepsin D; LC3, microtubule-associated protein 1 light chain 3; TFEB, transcription factor EB; siRNA, short interfering RNA; NFE2L2, nuclear factor, erythroid 2 like 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; CD31, cluster of differentiation 31; SD, standard deviation; DAPI, 4,6-diamidino-2-phenylindole.

	IR	+	+	+	+		
	SFN	−	+	+	+		
	ML385	−	−	+	+		
HO1							32kDa
SOD1							16kDa
Bax							20kDa
Bcl-2							26kDa
C-Caspase-3							17kDa
GAPDH							37kDa

	Sham	I/R	I/R+SFN	
Beclin1				60kDa
p62				62kDa
C-CTSD				28kDa
LC3 I				16kDa
LC3 II				14kDa
GAPDH				37kDa

Fig.5E-1

I/R	+	+	+	+
SFN	−	+	+	−
3MA	−	−	+	+

Beclin1 — 60kDa

p62 — 62kDa

C-CTSD — 28kDa

LC3 I — 16kDa
LC3 II — 14kDa

Bax — 20kDa

Bcl-2 — 26kDa

C-Caspase-3 — 17kDa

GAPDH — 37kDa

Sham   IR   I/R+SFN

TFEB — 68kDa

Histone 3 — 17kDa

I/R	+	+	+	+	+
SFN	−	+	+	+	−
Scramble	−	−	+	−	−
TFEB shRNA	−	−	−	+	+

TFEB — 68kDa

NFE2L2 — 100kDa

Histone 3 — 17kDa

Fig.5E-2

I/R	+	+	+	+
SFN	−	+	+	−
3MA	−	−	+	+

Beclin1 — 60kDa

p62 — 62kDa

C-CTSD — 28kDa

LC3 I — 16kDa
LC3 II — 14kDa

Bax — 20kDa

Bcl-2 — 26kDa

C-Caspase-3 — 17kDa

GAPDH — 37kDa

Sham   IR   I/R+SFN

TFEB — 68kDa

Histone 3 — 17kDa

I/R	+	+	+	+	+
SFN	−	+	+	+	−
Scramble	−	−	+	−	−
TFEB shRNA	−	−	−	+	+

TFEB — 68kDa

NFE2L2 — 100kDa

Histone 3 — 17kDa

Fig.5E-3

I/R	+	+	+	+
SFN	−	+	+	−
3MA	−	−	+	+

Beclin1 — 60kDa

p62 — 62kDa

C-CTSD — 28kDa

LC3 I — 16kDa
LC3 II — 14kDa

Bax — 20kDa

Bcl-2 — 26kDa

C-Caspase-3 — 17kDa

GAPDH — 37kDa

Sham   IR   I/R+SFN

TFEB — 68kDa

Histone 3 — 17kDa

I/R	+	+	+	+	+
SFN	−	+	+	+	−
Scramble	−	−	+	−	−
TFEB shRNA	−	−	−	+	+

TFEB — 68kDa

NFE2L2 — 100kDa

Histone 3 — 17kDa

I/R	+	+	+	+	+
SFN	−	+	+	+	−
Scramble	−	−	+	−	−
TFEB shRNA	−	−	−	+	+

p62 — 62kDa

C-CTSD — 28kDa

LC3 I — 16kDa
LC3 II — 14kDa

HO1 — 32kDa

SOD1 — 16kDa

Bax — 20kDa

Bcl-2 — 26kDa

C-Caspase-3 — 17kDa

GAPDH — 37kDa

I/R	+	+	+	+	+
SFN	−	+	+	+	−
Scramble	−	−	+	−	−
MCOLN1 shRNA	−	−	−	+	+

MCOLN1 — 64kDa

Calcineurin — 62kDa

GAPDH — 37kDa

I/R	+	+	+	+	+
SFN	−	+	+	+	−
Scramble	−	−	+	−	−
MCOLN1 shRNA	−	−	−	+	+

TFEB — 68kDa

NFE2L2 — 100kDa

Histone 3 — 17kDa

Top-left panel

I/R   +   +   +   +   +
SFN   −   +   +   +   −
Scramble   −   −   +   −   −
MCOLN1 shRNA   −   −   −   +   +

Protein	Size
HO1	32kDa
SOD1	16kDa
p62	62kDa
LC3 I	16kDa
LC3 II	14kDa
Bax	20kDa
Bcl-2	26kDa
C-Caspase 3	17kDa
GAPDH	37kDa

Top-middle panel

I/R   +   +   +   +   +
SFN   −   +   +   +   −
Scramble   −   −   +   −   −
MCOLN1 shRNA   −   −   −   +   +

Protein	Size
HO1	32kDa
SOD1	16kDa
p62	62kDa
LC3 I	16kDa
LC3 II	14kDa
Bax	20kDa
Bcl-2	26kDa
C-Caspase 3	17kDa
GAPDH	37kDa

Top-right panel

I/R   +   +   +   +   +
SFN   −   +   +   +   −
Scramble   −   −   +   −   −
MCOLN1 shRNA   −   −   −   +   +

Protein	Size
HO1	32kDa
SOD1	16kDa
p62	62kDa
LC3 I	16kDa
LC3 II	14kDa
Bax	20kDa
Bcl-2	26kDa
C-Caspase 3	17kDa
GAPDH	37kDa

Bottom-left panel

I/R   +   +   +   +
SFN   −   +   +   +
Tacrolimus   −   −   +   +

Protein	Size
Calcineurin	52kDa
GAPDH	37kDa
TFEB	68kDa
Histone 3	17kDa

Bottom-middle panel

I/R   +   +   +   +
SFN   −   +   +   +
Tacrolimus   −   −   +   +

Protein	Size
Calcineurin	62kDa
GAPDH	37kDa
TFEB	68kDa
Histone 3	17kDa

Bottom-right panel

I/R   +   +   +   +
SFN   −   +   +   +
Tacrolimus   −   −   +   +

Protein	Size
Calcineurin	62kDa
GAPDH	37kDa
TFEB	68kDa
Histone 3	17kDa