Supplementary information

Materials and methods

Cell culture and treatment

THP-1 monocytes were purchased from BIO-HIGH Technology (Hebei, China) and maintained in medium with 10% fetal bovine serum (Gemini, USA) and 1% (volume fraction) penicillin-streptomycin in a humidified incubator at 37 °C with 95% air/5% CO_2. Before the experiment, the THP-1 cells were treated with 100 ng/mL phorbol myristate (PMA) and incubated for more than 24 h to induce the differentiation of monocytes; then treated with 50 μg/mL oxidized low-density lipoprotein (ox-LDL) for 48 h alone or in combination with 1000 μg/mL flavone of *Polygonatum odoratum* (PAOA-flavone) (30%, China) purchased from Wuhan Dongkang Source Technology Co., Ltd. Ox-LDL-treated THP-1 macrophages were inoculated into 6-well plates, and the transfection mixture consisted of 4 μl/well Lipofectamine™ 3000 reagent (Invitrogen, USA) and 4 μg/well RNA oligo added to 125 μL RPMI 1640 medium, which was used to transfect the cells for 48 h for the subsequent experiments. All the recombinant viruses and expressional plasmids were obtained commercially.

RNA extraction and RT-qPCR

TRIzol reagent (Invitrogen, USA) was used to extract total RNA from the treated macrophages. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed in a 20 μL reaction system using the BeyoFast™ SYBR Green One Step RT-qPCR Kit (Beyotime, China). The relative messenger RNA (mRNA) levels of the target genes were quantified using the comparative cycle threshold ($2^{-\Delta\Delta C_T}$) method. The mRNA level of *β-actin* was used as the content standardization. The primer sequences used in this study were shown in Table S2.

Western blot analysis

The treated THP-1 macrophages were rinsed twice with cold PBS and then lysed with RIPA+PSMF buffer (96:4, volume ratio). The cell lysates were centrifuged at 12 000 rpm for 10 min at 4 ℃, and the supernatant was retained and boiled with a denaturing buffer in a metal bath for 5–8 min. Equivalent amounts of protein samples were subjected to 10% (volume fraction) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer. PVDF membranes (0.45 μm) were incubated with anti-sortilin (1:2500; volume ratio, the same as below), anti-HNF1b (1:1000) or anti-SAE1 (1:1000) antibodies overnight at 4 ℃. After rinsing three times, the membrane was incubated in an appropriate HRP-conjugated secondary antibody at room temperature for 1 h. An enhanced chemiluminescence (ECL) detection reagent (Shanghai Life iLab Biotechnology Co., Ltd., China) was used to detect the protein bands, and imaging was performed by a Bio-Rad gel imager (BIORAD, USA). The optical density of the objective bands was normalized to that of β-actin.

Bioinformatics analysis

The upstream transcription factors of *sort1* were predicted by PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and the Alibaba website (http://gene-

regulation.com/pub /programs/alibaba2/index.html), and the conserved HNF1b-binding sites in the *sort1* promoter (human and mouse) were predicted by the JASPAR database (http://jaspar.genereg.net). The GeneMANIA website (http://genemania.org) was used to analyze the relationship among HNF1b, sortilin, SAE1 and SUMO1. The GEO datasets of the Bioinformatics website (https://www.aclbi.com/static/index.html#/geo) were used to explore the expression of SUMO1 and SAE1 in patients with atherosclerotic plaques, and ASSISTANT was used for the clinical bioinformatics website (https://www.aclbi.com/static/index.html#) to analyze the relationship between HNF1b and SUMO1 and between SAE1 and SUMO1. The SUMO1 binding sites within the HNF1b protein were predicted with the SUMOplot™ Analysis program (https://www.abcepta.com/sumoplot) and JASSA website (human and mouse) (http://www.jassa.fr).

Dual-luciferase assay

The HNF1b lentivirus and the psiCHECK™-2-SORT1 promoter-WT or psiCHECK™-2-SORT1 promoter-MUT (849-GTTTATAATTAAA-861 was mutagenized to 849-GTTCGCCTTGAAA-861) were co-transfected into 293T cells with Lipofectamine™ 3000 for 24 h. After removing the reagents and rinsing with PBS, the cells were subsequently lysed, and the fluorescence intensity was measured by dual-luciferase reporter assay to calculate the ratio of firefly Luc/Renilla Luc to demonstrate the regulatory role of HNF1b in sortilin expression.

Chromatin immunoprecipitation assay (ChIP)

Cells were crosslinked with 1% (volume fraction) formaldehyde for 10 min at room temperature, after which IP lysis buffer containing protease inhibitors was added to the cells for lysis, followed by fragmentation by sonication. The lysates were then centrifuged at 12 000 rpm at 4 ℃ for 10 min, and the supernatant was collected and sampled for the input experiments. The complexes were co-incubated with anti-HNF1b or immunoglobulin G (IgG) antibodies, after which Protein A+G agarose/salmon sperm DNA was added and spun at 4 ℃ for 1 h. The complexes were eluted in elution buffer and treated with proteinase K. DNA was purified with a quick PCR purification kit, followed by real-time qPCR analysis. The primers used were as follows: human *sortilin* F, TCGGGTTGGGTGCATTGTATC; human *sortilin* R, TGCAAGTCATTACACTCAAAGCAAG.

Oil red O staining and the determination of total cholesterol and triglyceride contents

The ox-LDL-treated THP-1 macrophages were washed twice and then fixed in 4% tissue fixative at room temperature for 30 min. The cells were soaked with 60% (volume fraction) isopropanol for 2 min, stained with freshly diluted Oil Red O (ORO) solution at 37 ℃ for 1 h, soaked with 60% isopropanol for 2 min, stained with hematoxylin for 15 min, rinsed with PBS and observed under a microscope (MShot, China). To determine the cellular contents of triglyceride (TG), total cholesterol (TC), the culture medium was discarded, and the treated macrophages were rinsed with PBS and lysed with RIPA+PSMF (96:4). The lysed cell solution was collected after centrifugation, and the total protein concentration of the cell lysate was determined by the BCA method. Test standards for TC (A111-1-1; Nanjing Jiancheng Bioengineering Institute, China) and TG (A110-1-1; Nanjing Jiancheng Bioengineering Institute, China) were used to measure the absorbance values of the calibration standard tubes and the sample tubes, respectively, by using a microplate reader colorimetric assay, and the content of TG and TC in the sample was calculated based on the obtained absorbance values.

Coimmunoprecipitation assay (CoIP)

Cells were grown until approximately 90% (volume fraction) confluence, added to an appropriate amount of precooled IP cell lysis buffer (supplemented with protease inhibitor), lysed on ice for 20 min, and centrifuged at 12 000 rpm and 4 ℃ for 10 min. Cell lysates were collected, the protein concentration was determined by the BCA method, and a small amount of supernatant was denatured and used for input experiments. Then, the lysates were incubated with an anti-HNF1b antibody (1:500; #720259; Invitrogen, USA) and protein A/G magnetic beads overnight at 4 ℃, followed by washing, resuspending in SDS sample buffer and western blot analysis.

Mice and treatments

Two spontaneous atherosclerosis mouse models were used in our study, male $LDLR^{-/-}$ mice (5-week-old) and $ApoE^{-/-}$ mice (5-week-old), which were purchased from Cavens Laboratory Animal Co., Ltd. (Nanjing, China). All animals were placed in a barrier environment with a controlled temperature ((22±0.8) ℃), a 12-h light/dark cycle and free access to food and water. After a wk of standard diet for adaptation, the model animals were randomly divided into groups and changed to a Western diet (20% fat, 45% carbohydrates, 23% protein, and 1.25% cholesterol) for 10 weeks. $LDLR^{-/-}$ mice (6-week-old) were divided into three groups: control, HNF1b, and HNF1b+sortilin groups. At the end of the fifth week, a recombinant HNF1b adeno-associated virus (AAV) alone or in combination with a recombinant sortilin AAV was injected into $LDLR^{-/-}$ mice via the tail vein (1×10^{11} GC/mice; Jikai Gene, China). $LDLR^{-/-}$ mice were sacrificed, and blood and aortic samples were collected after eight weeks. $ApoE^{-/-}$ mice were randomly divided into four groups (control, PAOA-flavone, PAOA-flavone+SAE1 siRNA, and PAOA-flavone+SAE1 siRNA+HNF1b oe groups) and fed a western diet alone or supplemented with PAOA-flavone (75 mg/(kg·d)). AAV-shControl (nontargeting control; Hanbio Biotechnology, Shanghai, China), AAV-shSAE1 (4.08×10^{13} viral genomes/mL per mice; Jikai Gene, Shanghai, China), AAV-HNF1b (1.5×10^{11} viral genomes/mL per mice), or AAV-shSAE1 were injected through the tail vein. All mice were sacrificed at 10 weeks, and serum and aortic samples were collected for subsequent determination. All the animal experiments were performed by following the Guidelines for Laboratory Animals of the National Institute of Health and approved by the Committee on the Ethics of Animal Experiments of Guilin Medical University (GLMC-IACUC-2022011).

Analysis of atherosclerotic lesions

The hearts were embedded into the OCT complex (Sakura, USA), and serially frozen-sliced (8-μm thick) throughout the three aortic valves. The ten frozen sections of each mouse were collected for analysis. The lipidosis and plaque area were evaluated by ORO and hematoxylin eosin (HE) staining and photographed with an inverted microscope (MShot). The percentage of lesion areas was quantified with Image-Pro Plus 5.1 software (Media Cybernetics, USA).

Immunofluorescence staining

The frozen sections of arterial tissue were incubated with anti-sortilin (1:600), anti-HNF1b (1:400) and anti-SAE1 (1:400) antibodies and a fluorescent secondary antibody (1:500), sealed with sealing liquid containing an antifade mounting medium with DAPI, and observed and captured under a fluorescence microscope (BioTek, USA). The expression of SAE1, HNF1b and sortilin was quantified with ImageJ software (National Institutes of Health, Germany).

Statistical analysis

All statistical analyses were performed using Prism version 9.0.1 software (GraphPad, USA). The unpaired Student's *t*-test was utilized to evaluate the statistical differences between two groups, and one-way analysis of variance (ANOVA) to evaluate more than two groups. Statistical significance was indicated when the *P* value was <0.05. The result data were presented as mean±standard deviation (SD). The number of independent experiments and the other specific statistical methods used for each panel were provided in the corresponding figure legends.

Supplementary Figures

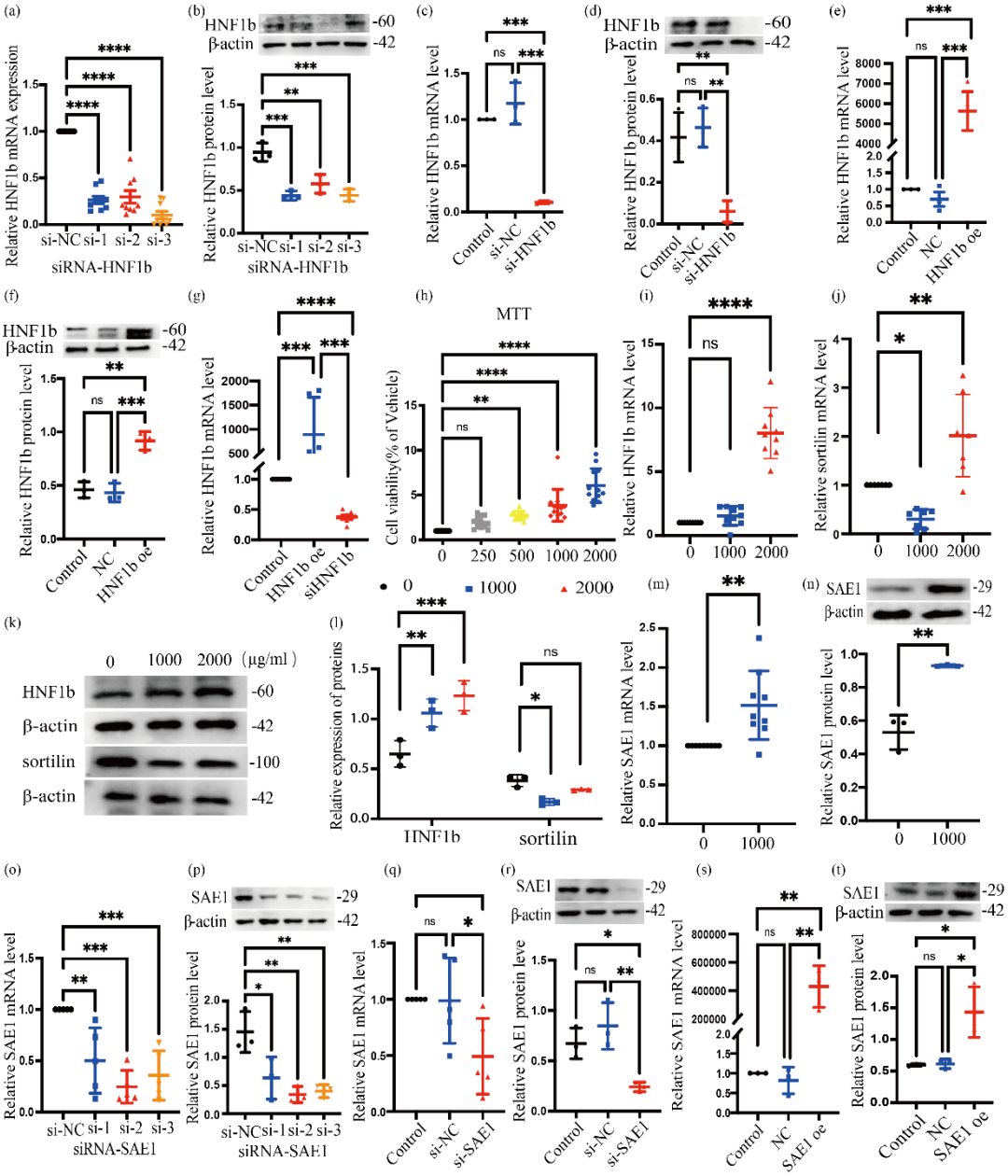
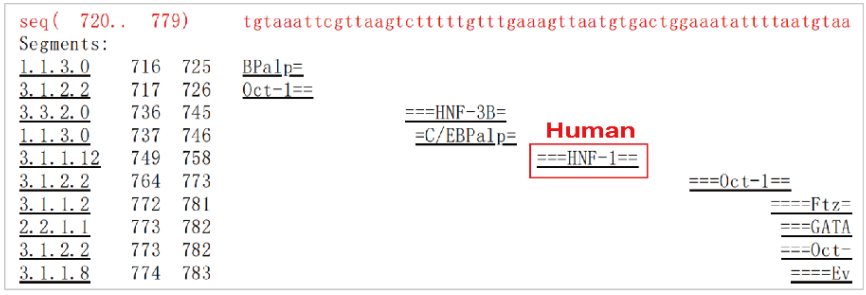


Fig. S1 (a, b) The expression of hepatocyte nuclear factor 1β (HNF1b) was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting in THP-1 macrophages treated with NC small interfering RNA (siRNA), HNF1b siRNA1, siRNA2, or siRNA3 for 48 h. (c, d) The expression of HNF1b was measured by RT-qPCR and western blotting (WB) in THP-1 macrophages treated with 50 μg/mL oxidized low-density lipoprotein (ox-LDL) in combination with NC siRNA or HNF1b siRNA for 48 h. (e, f) The overexpression efficiency of HNF1b was determined by RT-qPCR and WB in THP-1 macrophages treated with 50 μg/mL ox-LDL in combination with NC or HNF1b for 48 h. (g) The level of HNF1b messenger RNA (mRNA) was determined by RT-qPCR in ox-LDL-treated THP-1 macrophages transfected with HNF1b lentivirus or HNF1b siRNA, respectively. (h) Cell viability was measured by MTT assay in THP-1 macrophages treated with Flavone of *Polygonatum odoratum* (PAOA-flavone) (0, 250, 500, 1000, and 2000 μg/mL) for 48 h. (i–l) The mRNA and protein levels of HNF1b and sortilin were measured by RT-qPCR and western blot in THP-1 macrophages treated with 50 μg/mL ox-LDL alone or in combination with PAOA-flavone (1000 or 2000 μg/mL) for 48 h. (m, n) The protein and mRNA levels of SAE1 were determined by RT-qPCR and western blot in THP-1 macrophages incubated with 50 μg/mL ox-

LDL alone or in combination with PAOA-flavone (1000 μg/mL) for 48 h. (o, p) The expression of SAE1 was measured by RT-qPCR and western blot in THP-1 macrophages treated with NC siRNA or SAE1 siRNA1, siRNA2, or siRNA3 for 48 h. (q, r) The expression of SAE1 was measured by RT-qPCR and western blot in THP-1 macrophages treated with NC siRNA or SAE1 siRNA lentiviruses for 48 h. (s, t) The overexpression efficiency was determined by RT-qPCR and WB in THP-1 macrophages transfected with NC or SAE1 lentiviruses for 48 h. All results are expressed as the mean±standard deviation (SD) from at least triplicate experiments independently. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$; ns, non-significant.

(a)

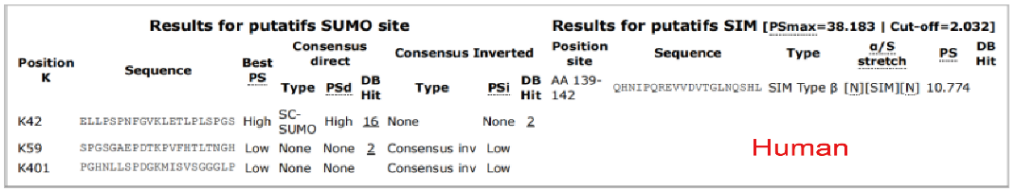


(b)

(c)

(d)

(e)



Results for putatifs SUMO site

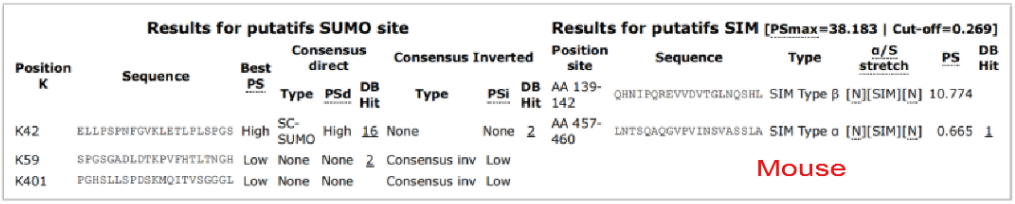
Position K	Sequence	Best PS	Consensus direct Type	Consensus direct PSd	Consensus direct DB Hit	Consensus Inverted Type	Consensus Inverted PSi	Consensus Inverted DB Hit
K42	ELLPSPNFGVKLETLPLSPGS	High	SC-SUMO	High	16	None	None	2
K59	SPGSGAEPDTKPVFHTLTNGH	Low	None	None	2	Consensus inv	Low	
K401	PGHNLLSPDGKMISVSGGGLP	Low	None	None		Consensus inv	Low	

Results for putatifs SIM [PSmax=38.183 | Cut-off=2.032]

Position site	Sequence	Type	α/S stretch	PS	DB Hit
AA 139-142	QHNIPQREVVDVTGLNQSHL	SIM Type β	[N][SIM][N]	10.774	

Human

(f)



Results for putatifs SUMO site

Position K	Sequence	Best PS	Consensus direct Type	Consensus direct PSd	Consensus direct DB Hit	Consensus Inverted Type	Consensus Inverted PSi	Consensus Inverted DB Hit
K42	ELLPSPNFGVKLETLPLSPGS	High	SC-SUMO	High	16	None	None	2
K59	SPGSGADLDTKPVFHTLTNGH	Low	None	None	2	Consensus inv	Low	
K401	PGHSLLSPDSKMQITVSGGGL	Low	None	None		Consensus inv	Low	

Results for putatifs SIM [PSmax=38.183 | Cut-off=0.269]

Position site	Sequence	Type	α/S stretch	PS	DB Hit
AA 139-142	QHNIPQREVVDVTGLNQSHL	SIM Type β	[N][SIM][N]	10.774	
AA 457-460	LNTSQAQGVPVINSVASSLA	SIM Type α	[N][SIM][N]	0.665	1

Mouse

Fig. S2 (a, b) The binding prediction of the human/mouse *sort1* promoter with transcription factors using the Alibaba online website. (c, d) The conserved sequences within the human/mouse *sort1* promoter bound by hepatocyte nuclear factor 1β (HNF1b) using the online JASPAR prediction. (e, f) The prediction of small ubiquitin-like modifier 1 (SUMO1)ylation sites in human and mouse HNF1b proteins with JASSA website.

Supplementary Tables

Table S1 Conservative HNF1b-binding sites at 849–861 bp in human and 334–346 bp in mouse within *sort1* promoter by bioinformatic analysis

Pattern name	Score	Start (bp)	End (bp)	Strand	HNF1b-binding site
Hsa_sort1	10.998635	89	100	+	TTTATATTTAAT
	9.33695	849	861	+	GTTTATAATTAAA
	8.515744	849	861	−	TTTAATTATAAAC
	9.951329	156	167	−	TTATTATTTCAC
	9.609174	849	860	−	TTAATTATAAAC
	9.159572	850	861	+	TTTATAATTAAA
	5.967767	1065	1077	−	TCTAATGAATAAA
Mus_sort1	7.249544	334	346	+	GTGAATAATTCAA
	6.1270547	1147	1159	−	CTAAATCCTTAAC
	6.0220833	334	346	−	TTGAATTATTCAC
	5.59854	1147	1159	+	GTTAAGGATTTAG

HNF1b: hepatocyte nuclear factor 1β.

Table S2 Sequences of primers used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Primer name	Primer sequence (5′→3′)
HNF1b-F	TGGTCACTGAGGTCCGTGTCTAC
HNF1b-R	GTTCAGGCTGTGAGTCTGGTTGG
sort1-F	AGCACCACCAATGTCCAGAG
sort1-R	GTTCAGGCTGTGAGTCTGGTTGG
SAE1-F	AGGACTGACCATGCTGGATCAC
SAE1-R	CTCAGTGTCCACCTTCACATCC
β-actin-F	GGCACCAGGGCGTGATG
β-actin-R	AGGTCTCAAACATGATCTGGGTC

HNF1b: hepatocyte nuclear factor 1β; F: forward; R: reverse; SAE1: small ubiquitin-related modifier (SUMO)-activating enzyme subunit 1.