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**Research Article**

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# Sortilin-induced lipid accumulation and atherogenesis are suppressed by HNF1b SUMOylation promoted by flavone of *Polygonatum odoratum*

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**Abstract:** This study aims to investigate the impact of hepatocyte nuclear factor 1β (HNF1b) on macrophage sortilin-mediated lipid metabolism and aortic atherosclerosis and explore the role of the flavone of *Polygonatum odoratum* (PAOA-flavone)-promoted small ubiquitin-related modifier (SUMO) modification in the atheroprotective efficacy of HNF1b. HNF1b was predicted to be a transcriptional regulator of sortilin expression via bioinformatics, dual-luciferase reporter gene assay, and chromatin immunoprecipitation. HNF1b overexpression decreased sortilin expression and cellular lipid contents in THP-1 macrophages, leading to a depression in atherosclerotic plaque formation in low-density lipoprotein (LDL) receptor-deficient ($LDLR^{-/-}$) mice. Multiple SUMO1-modified sites were identified on the HNF1b protein and co-immunoprecipitation confirmed its SUMO1 modification. The SUMOylation of HNF1b protein enhanced the HNF1b-inhibited effect on sortilin expression and reduced lipid contents in macrophages. PAOA-flavone treatment promoted SUMO-activating enzyme subunit 1 (SAE1) expression and SAE1-catalyzed SUMOylation of the HNF1b protein, which prevented sortilin-mediated lipid accumulation in macrophages and the formation of atherosclerotic plaques in apolipoprotein E-deficient ($ApoE^{-/-}$) mice. Interference with SAE1 abrogated the improvement in lipid metabolism in macrophage cells and atheroprotective efficacy in vivo upon PAOA-flavone administration. In summary, HNF1b transcriptionally suppressed sortilin expression and macrophage lipid accumulation to inhibit aortic lipid deposition and the development of atherosclerosis. This anti-atherosclerotic effect was enhanced by PAOA-flavone-facilitated, SAE1-catalyzed SUMOylation of the HNF1b protein.

**Key words:** Atherosclerosis; Lipid accumulation; Hepatocyte nuclear factor 1β (HNF1b); Flavone of *Polygonatum odoratum*; SUMOylation

## 1 Introduction

Atherosclerosis (AS) is a lipid disorder-related pathology characterized by subintimal lipid deposition and foam cell aggregation in the arterial wall, leading to the development of cardiovascular disease (CVD) (Ji et al., 2021; Björkegren and Lusis, 2022; Susser and Rayner, 2022). Macrophages derived from the circulation infiltrate the subintima and continuously ingest the invaded low-density lipoprotein (LDL), which ultimately gives rise to excessive lipid accumulation in macrophages and foam cell formation (Cahill et al., 2019; Lin et al., 2022). The prevention of lipid accumulation in macrophages has been well established to effectively combat atherogenesis and atherosclerotic CVD. Sortilin, a vacuolar protein encoded by the *sort1* gene, is strongly associated with hyperlipidemia and the risk of cardiovascular disease (Sun et al., 2020; di Pietro et al., 2022). Abundant studies have revealed that sortilin manipulates the vesicular transport and lysosomal degradation of lipid-related proteins to accelerate macrophage cholesterol accumulation and the progression of AS (Patel et al., 2015; Goettsch et al., 2018; Lv et al., 2019). Unfortunately, the upstream regulatory mechanisms that control the expression and biological function of macrophage sortilin remain unclear.

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Hepatocyte nuclear factor 1β (HNF1b), a promising transcription factor encoded by the *hnf1b* gene, plays a protective role in lipid dysmetabolism diseases such as coronary heart disease and type 2 diabetes mellitus (Johnstone et al., 2011; Kornfeld et al., 2013; Wu et al., 2017). Distinct from other family members, HNF1b lacks the main transactivation motif (activation domain I (ADI) and ADIII) within the carboxyl terminus, leading to the inability of HNF1b to activate the transcription of target genes. HNF1b also heterodimerizes with HNF1a, causing HNF1b to potentially function as a negative regulator of HNF1a transcriptional activity (Mendel et al., 1991; Toniatti et al., 1993). HNF1b transcriptionally suppresses a variety of lipid-related genes to influence intracellular processes of lipid metabolism, which eventually restricts lipid accumulation and the formation of lipid droplets (LDs) in hepatocytes and adipocytes (Hu et al., 2020; Li H et al., 2020; Watanabe et al., 2020; Kato et al., 2022; Liu et al., 2022). However, the effects and underlying mechanisms of HNF1b in macrophage lipid metabolism and the development of AS have not yet been reported. Our laboratory has observed that the expression of HNF1b is reduced while sortilin is upregulated in oxidized LDL (ox-LDL)-treated macrophages and atherosclerotic lesions, implying that HNF1b acts as a suppressor of sortilin. Moreover, a pilot bioinformatics analysis revealed a highly conservative HNF1b-binding site within the promotor of the *sort1* gene, indicating the potential regulatory role of HNF1b in sortilin expression. Further research is required to elucidate the exact mechanisms of HNF1b in lipid metabolism and its potential anti-atherosclerotic action through sortilin.

Small ubiquitin-related modifier 1 (SUMO1) protein modification involves a series of enzymatic reactions, including those induced by SUMO-activating enzyme E1 (SAE1)/SAE2, to reversibly, post-translationally modify the peptide chain of transcription factors. SUMOylation impacts the levels and nuclear localizations of related transcription factors, ultimately altering the activity of those nuclear receptors/proteins involved in the transcription of target genes (Melchior, 2000; Liu et al., 2021; Yu CX et al., 2021; Vertegaal, 2022; Yu K et al., 2023). To date, the precise regulatory mechanisms of SUMOylation in AS pathogenesis are still poorly understood. SUMO1-modified substrates are closely associated with intracellular cholesterol contents, and the SUMOylation of nuclear factors upregulates some lipid-related proteins to resist the occurrence of AS plaques (Miranda et al., 2010; Stein et al., 2014). Since there are several predicted SUMO1 modification sites in HNF1b, further exploration of SUMOylation in AS pathogenesis via this lipid-manipulated transcription factor is warranted.

A prospective study authenticated a negative association between the dietary intake of flavanone-rich foods and the risk of death from coronary heart diseases. *Polygonatum odoratum* (PAOA), as a medicinal and edible plant, is rich in flavones among its extracted components (Mink et al., 2007). Flavones of PAOA (PAOA-flavones) are a promising class of dietary antioxidants that change the expression levels of genes related to fatty acid oxidation and lipogenesis, showing hypolipidemic efficacy (Chanet et al., 2012; Zhuo et al., 2019; Meng et al., 2022). These flavones have been confirmed to specifically increase the level of SUMO1 and to limit intracellular cholesterol accumulation in vitro (Rodrigues et al., 2005; Pawellek et al., 2017). Based on this, it is inferred that PAOA-flavones also improve lipid metabolism processes to benefit individuals with atherosclerotic CVD through SUMOylation, which warrants further investigation.

To verify that PAOA-flavone-promoted HNF1b SUMOylation suppresses macrophage sortilin expression and lipid accumulation, ultimately antagonizing AS progression, we first examined whether HNF1b transcriptionally inhibits macrophage sortilin expression to improve intracellular lipid accumulation and the lesions of aortic AS. Then, we investigated the effect of SUMOylation on HNF1b-controlled sortilin expression and macrophage lipid accumulation. Finally, we explored the role of PAOA-flavone-enhanced SAE1-catalyzed SUMOylation in HNF1b levels and lipid content in macrophages, as well as lipid deposition and AS in the aortic wall. This research will provide a novel therapeutic strategy for the prevention of atherosclerotic CVD.

## 2 Results

### 2.1 Inhibition of sortilin expression and alleviation of macrophage cholesterol accumulation by HNF1b in vitro

When compared to the control group, the messenger RNA (mRNA) and protein levels of HNF1b were significantly suppressed, while the expression of sortilin was evidently increased in THP-1 macrophages

incubated with 50 μg/mL ox-LDL for 48 h (Figs. 1a–1c). Subsequently, the lentiviruses expressing HNF1b and its small interfering RNA (siRNA) were transfected into THP-1 macrophages treated with ox-LDL. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting determination results showed that the HNF1b lentivirus induced overexpression of HNF1b (HNF1b oe), which elevated the levels of HNF1b mRNA and protein, while siRNA-induced HNF1b silencing (siHNF1b) resulted in a significant decrease in HNF1b mRNA and protein levels. HNF1b oe reduced the levels of sortilin mRNA and protein, whereas siHNF1b heightened sortilin expression in THP-1 macrophages, which indicated a probable negative role of HNF1b in macrophage sortilin expression at the transcriptional level (Figs. 1d–1f and S1a–S1g). To verify our speculation, the FASTA sequences of the human/mouse *sort1* promotor were scanned using the online Alibaba database, which suggested that HNF1b was a potential transcription factor of the human/mouse *sort1* gene (Figs. S2a and S2b). An analysis of the JASPAR website (http://jaspar.genereg.net) demonstrated that 849–861 bp or 334–346 bp within the human or mouse *sort1* promoter are conserved sites for the binding of HNF1b (Figs. S2c and S2d, Table S1). The dual-luciferase reporter assays further established that HNF1b could bind to the *sort1*-wild type (WT) promoter, which led to a significant decrease in luciferase activity, whereas this effect disappeared in the presence of *sort1*-mutant (MUT) (Fig. 1g). The chromatin immunoprecipitation (ChIP) assays confirmed that HNF1b directly bound to the *sort1* promoter in THP-1 macrophages (Figs. 1h and 1i). HNF1b-suppressed sortilin expression affected macrophage lipid contents. HNF1b reduced the contents of triglyceride (TG) and total cholesterol (TC) in THP-1 macrophages treated with 50 μg/mL ox-LDL for 48 h (Fig. 1j). A significant increase in lipid content was observed upon interference by HNF1b siRNA. Oil Red O (ORO) staining showed that the number and size of intracellular LDs were significantly decreased in the HNF1b oe group compared with the control group, whereas co-transfection with HNF1b oe and overexpression of sortilin (sortilin oe) increased intracellular LDs in THP-1 macrophages (Fig. 1k). These data identified that HNF1b inhibited sortilin expression and alleviated lipid accumulation in macrophages.

### 2.2 Suppression of sortilin expression and amelioration of aortic atherogenesis by HNF1b in vivo

After infection with recombinant adeno-associated viruses (AAVs) expressing HNF1b and sortilin in atherosclerosis model LDL receptor-deficient ($LDLR^{-/-}$) mice fed a high-fat diet (HFD) for eight weeks, the changes in the rodent plasma lipid profile were measured using the enzymatic oxidation method. The plasma levels of TG, TC, and LDL-cholesterol (LDL-C) were significantly decreased in $LDLR^{-/-}$ mice overexpressing HNF1b, and co-infection with the sortilin AAV significantly offset the decrease in plasma lipid levels mediated by HNF1b (Figs. 2a–2d). Histological staining revealed that HNF1b reduced lipid deposition and the area of atherosclerotic plaques in aortic root cross-sections, and the simultaneous overexpression of sortilin significantly counteracted the anti-atherosclerotic effect of HNF1b (Figs. 2e and 2f). The aforementioned results suggested that HNF1b protected the aortic wall against lipid deposition and atherogenesis by suppressing sortilin in vivo. Immunofluorescence determination further verified that the expression of sortilin was dramatically downregulated after the injection of the HNF1b AAV. However, the expression of sortilin vigorously inhibited HNF1b-suppressed sortilin expression and its anti-atherosclerotic effect in the atherosclerosis model $LDLR^{-/-}$ mice fed an HFD (Figs. 2g–2i).

### 2.3 Elevation of HNF1b expression and inhibition of sortilin levels and lipid accumulation by PAOA-flavone in macrophages

The PAOA-flavone was reported to have cardiovascular protection activity. The viability of ox-LDL-treated THP-1 macrophages was not affected by PAOA-flavone at a concentration range of 0–2000 μg/mL for 48 h, which showed no toxicity in the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay (Fig. S1h). When incubated with 1000 or 2000 μg/mL PAOA-flavone, the protein level of HNF1b was increased in a dose-dependent manner, and its mRNA level did not change at 1000 μg/mL PAOA-flavone but did increase at 2000 μg/mL (Figs. S1i–S1l). The mRNA and protein levels of sortilin were decreased at 1000 μg/mL PAOA-flavone. When THP-1 macrophages were treated with 2000 μg/mL PAOA-flavone, the mRNA level of *sortilin* inexplicably increased and its protein level did not change

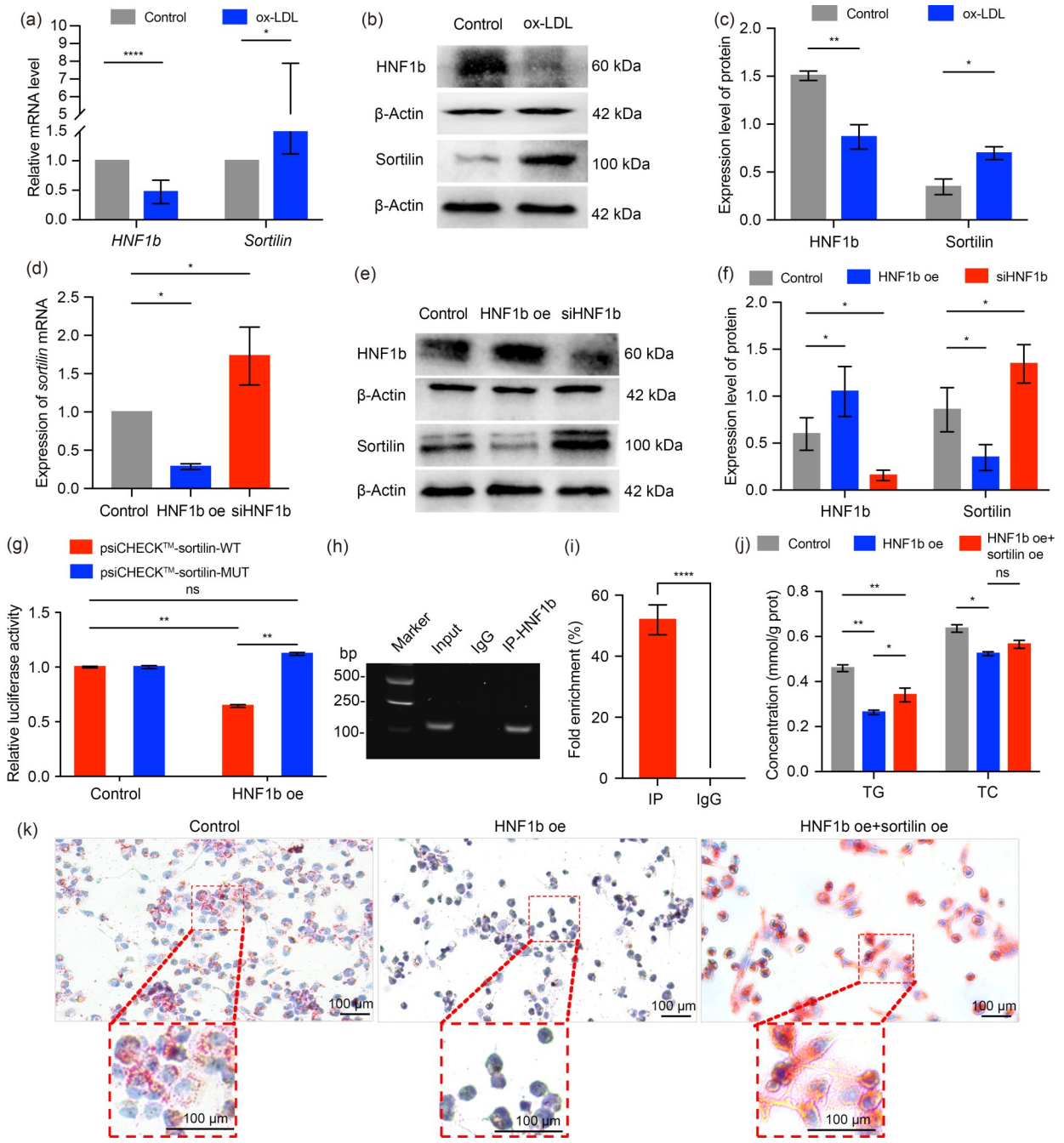


**Fig. 1 Hepatocyte nuclear factor 1β (HNF1b) inhibited sortilin expression and alleviated macrophage cholesterol accumulation in vitro. (a–c) The expression levels of HNF1b and sortilin measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting (WB) in THP-1 macrophages incubated with 50 μg/mL oxidized low-density lipoprotein (ox-LDL) for 48 h. (d–f) The expression of HNF1b and sortilin determined by WB and RT-qPCR in ox-LDL-treated THP-1 macrophages transfected with HNF1b lentivirus or HNF1b small interfering RNA (siRNA). (g) The interaction between HNF1b and the *sort1* promoter identified with a dual-luciferase reporter assay in 293T cells. (h, i) Direct binding of HNF1b to the *sort1* promoter validated by chromatin immunoprecipitation (ChIP). (j) The intracellular contents of triglyceride (TG) and total cholesterol (TC) determined by colorimetry in 50 μg/mL ox-LDL-treated THP-1 macrophages transfected with the HNF1b lentivirus alone or in combination with the sortilin lentivirus. (k) Intracellular lipid droplets (LDs) stained with Oil Red O (ORO) in THP-1 macrophages treated with 50 μg/mL ox-LDL in combination with HNF1b oe, or HNF1b oe+sortilin oe for 48 h. All results are expressed as mean±standard deviation (SD) from triplicate experiments independently. $^{*}$ $P$<0.05; $^{**}$ $P$<0.01; $^{****}$ $P$<0.0001; ns, non-significant. prot: protein; mRNA: messenger RNA; HNF1b oe: overexpression of HNF1b; siHNF1b: siRNA-induced HNF1b silencing; WT: wild type; MUT: mutant; IP: immunoprecipitation; IgG: immunoglobulin G; sortilin oe: overexpression of sortilin.**

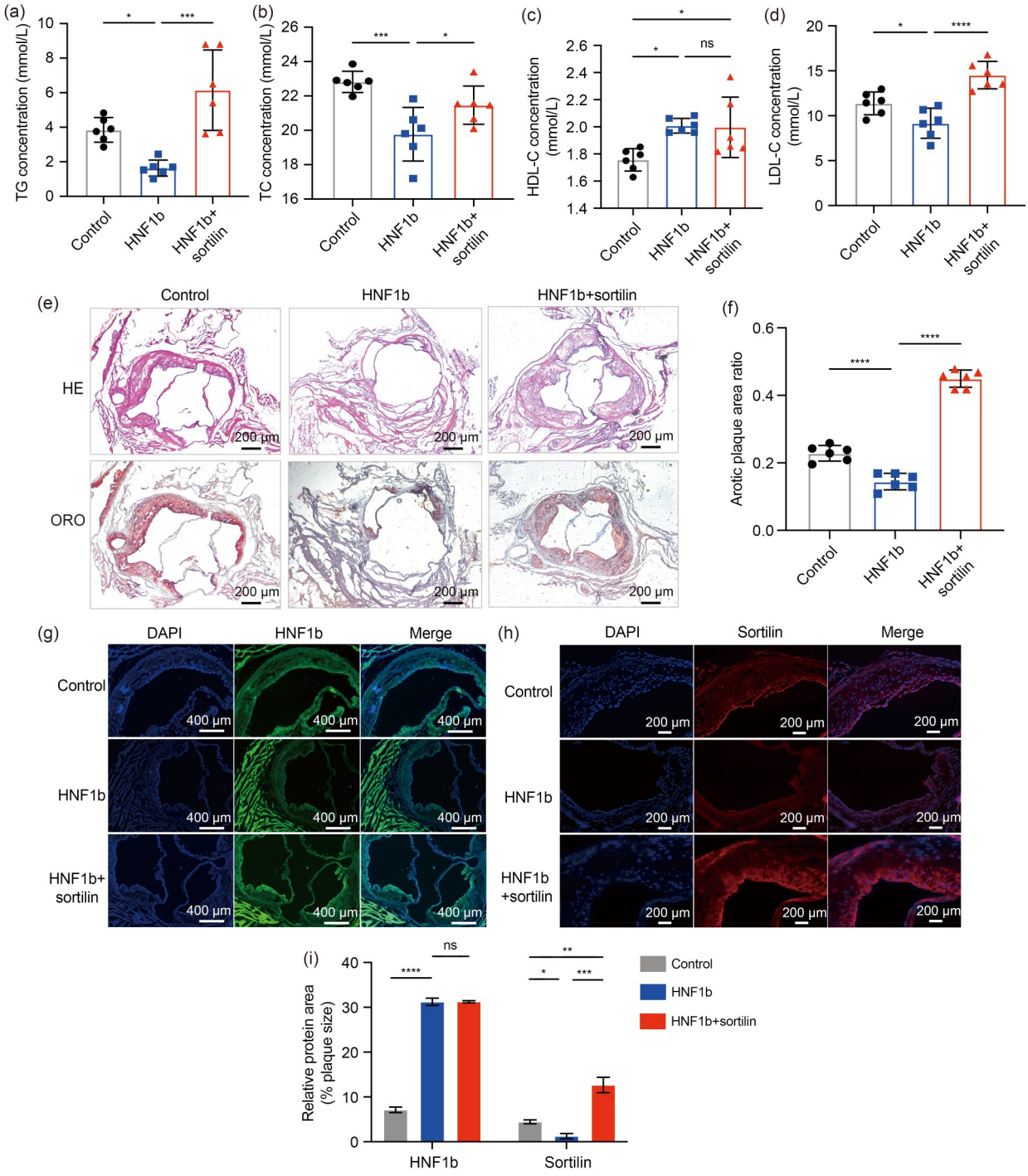


**Fig. 2 Hepatocyte nuclear factor 1β (HNF1b) suppressed sortilin expression and ameliorated aortic atherogenesis in vivo. (a–d) Plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) were determined using the enzymatic oxidation method in high-fat diet (HFD)-fed LDL receptor-deficient ($LDLR^{-/-}$) mice infected with the HNF1b adeno-associated virus (AAV) alone or co-infected with the recombinant HNF1b and sortilin AAVs. (e, f) Cross-sections of the aortic sinus from HFD-fed $LDLR^{-/-}$ mice were stained with hematoxylin eosin (HE) and Oil Red O (ORO), and the aortic plaque area was quantified in ORO-stained frozen sections. (g–i) Aortic expression of HNF1b and sortilin and quantitative analysis were measured by immunofluorescence in HFD-fed $LDLR^{-/-}$ mice transfected with HNF1b AAV alone or co-transfected with recombinant sortilin AAVs. All results are expressed as mean±standard deviation (SD), with $n$=6 (a–d, f) or $n$=3 (i). $^{*}$ $P$<0.05; $^{**}$ $P$<0.01; $^{***}$ $P$<0.001; $^{****}$ $P$<0.0001; ns, non-significant. DAPI: 4',6-diamidino-2-phenylindole.**

significantly. Therefore, the treatment with 1000 μg/mL PAOA-flavone produced findings consistent with our expectations and was therefore used in subsequent experiments. Compared with the control group, treatment with 1000 μg/mL PAOA-flavone enhanced HNF1b protein expression but had no significant effect on *HNF1b* mRNA level (Figs. S1j–S1l). HNF1b siRNA abolished PAOA-flavone-enhanced HNF1b expression, while HNF1b oe augmented the PAOA-flavone-enhanced expression of HNF1b mRNA and protein in THP-1 macrophages (Figs. 3a–3c). Intervention with HNF1b was proven to affect PAOA-flavone-inhibited sortilin expression. HNF1b siRNA in combination with PAOA-flavone was shown to increase the levels of sortilin mRNA and protein, but HNF1b oe in combination with PAOA-flavone was

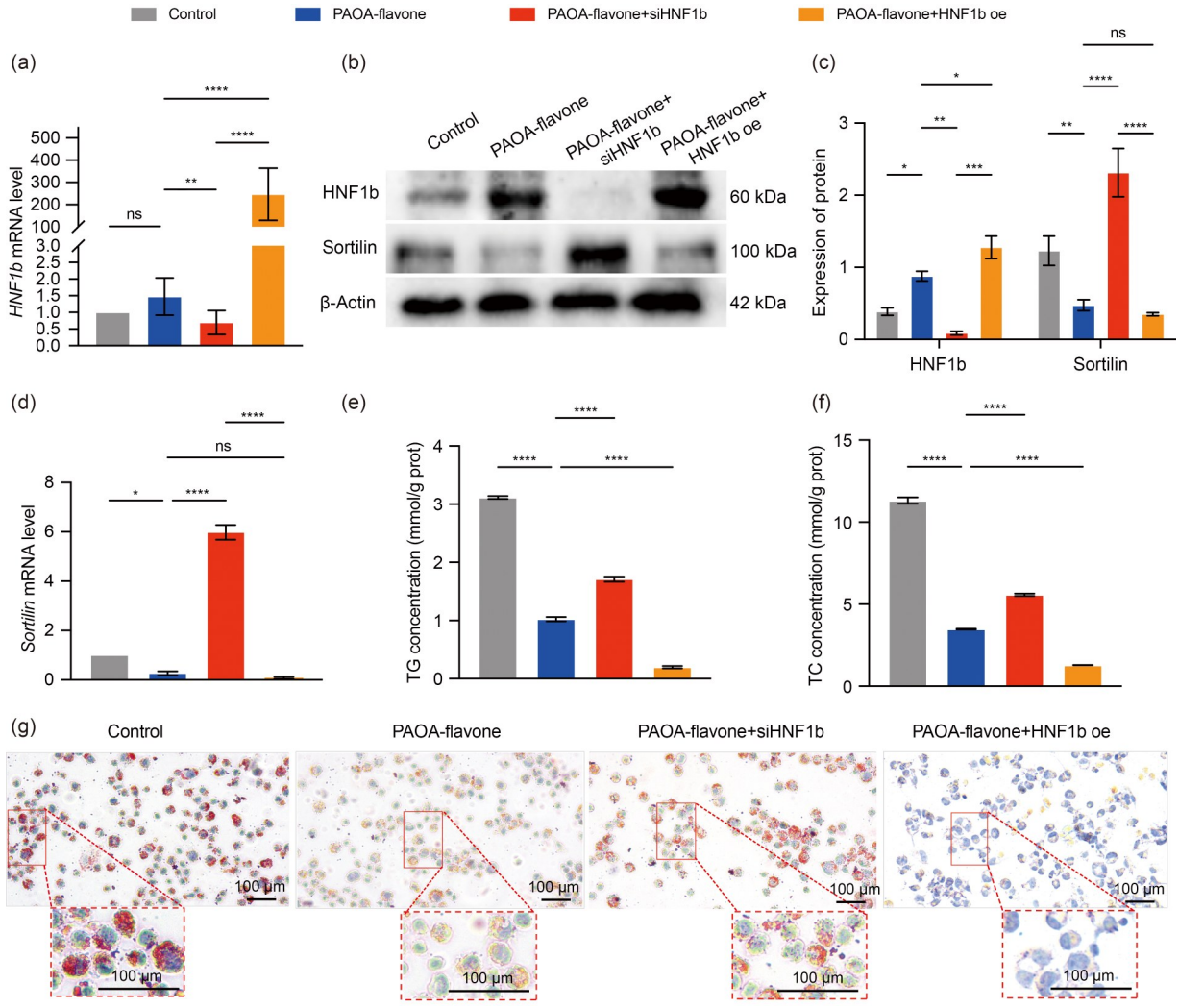


**Fig. 3 Flavone of *Polygonatum odoratum* (PAOA-flavone) elevated hepatocyte nuclear factor 1β (HNF1b) expression and inhibited macrophage sortilin levels and lipid accumulation. (a–d) The mRNA and protein levels of HNF1b and sortilin were measured using reverse transcription quantitative polymerase chain reaction (RT-qPCR) or western blotting in THP-1 macrophages treated with 50 μg/mL oxidized low-density lipoprotein (ox-LDL) in combination with 1000 μg/mL PAOA-flavone, PAOA-flavone+siHNF1b, or PAOA-flavone+HNF1b oe for 48 h. Values were normalized to the expression of the *β-actin* gene. (e, f) The cellular triglyceride (TG) and total cholesterol (TC) contents were assessed in THP-1 macrophages treated with 50 μg/mL ox-LDL in combination with 1000 μg/mL PAOA-flavone, PAOA-flavone+siHNF1b or PAOA-flavone+HNF1b oe for 48 h. (g) Intracellular lipid droplets (LDs) were stained with Oil Red O (ORO) in THP-1 macrophages treated with 50 μg/mL ox-LDL in combination with 1000 μg/mL PAOA-flavone, PAOA-flavone+siHNF1b, or PAOA-flavone+HNF1b oe for 48 h; cells were photographed at 200× magnification. The results are presented as mean± standard deviation (SD) of three independently repeated experiments. $^{*}$ $P$<0.05; $^{**}$ $P$<0.01; $^{***}$ $P$<0.001; $^{****}$ $P$<0.0001; ns, non-significant. siHNF1b: siRNA-induced HNF1b silencing; HNF1b oe: overexpression of HNF1b; mRNA: messenger RNA.**

shown to decrease sortilin expression in THP-1 macrophages (Figs. 3b–3d). These experiments illustrated that PAOA-flavone inhibited macrophage sortilin expression via HNF1b, which probably affects lipid accumulation in macrophages. The colorimetry determination and ORO staining showed that PAOA-flavone pronouncedly decreased the TG and TC contents and the number and volume of LDs in THP-1 macrophages. HNF1b siRNA abrogated PAOA-flavone-suppressed intracellular lipid accumulation, while HNF1b oe enhanced it in THP-1 macrophages (Figs. 3e–3g). The above data demonstrated that PAOA-flavone post-transcriptionally manipulated HNF1b to suppress macrophage sortilin expression and cellular lipid accumulation.

### 2.4 Enhancement of HNF1b SUMOylation by PAOA-flavone in macrophages

In the preceding section, 1000 μg/mL PAOA-flavone was verified to increase HNF1b protein expression rather than its mRNA level. GeneMANIA network (http://genemania.org) analysis revealed strong correlations between HNF1b, sortilin, and SUMO1 (Fig. 4a). SUMO1 levels were significantly lower in individuals with carotid plaques than in the normal population (Fig. 4b). A positive correlation between SUMO1 and HNF1b expression was further revealed in human carotid plaques by the ASSISTANT for Clinical Bioinformatics website (https://www.aclbi.com/static/index.html) (Fig. 4c). The peptide sequences of human and mouse HNF1b proteins had multiple sites for SUMOylation modification, as calculated by the SUMOplot™ Analysis Program and the Joined Advanced Sumoylation Site and Sim Analyser (JASSA) website (http://www.jassa.fr) (Figs. 4d, 4e, S2e, and S2f). These bioinformatics results suggested a crucial role of SUMOylation in HNF1b expression and activity. Intriguingly, an enzyme of SUMOylation, SUMO-activating enzyme subunit 1 (SAE1), was discovered to strongly correlate with HNF1b and SUMO1 through a GeneMANIA network analysis (Fig. 4f). The ASSISTANT for Clinical Bioinformatics website displayed a positive association between SAE1 and SUMO1 and downregulated SAE1 expression in carotid plaques (Figs. 4g and 4h). A co-immunoprecipitation (Co-IP) assay confirmed that PAOA-flavone facilitated the SUMOylation of HNF1b protein, and SAE1 intensified this modification (Fig. 4i). These findings suggested that the SAE1-catalyzed SUMO1ylation of HNF1b protein was enhanced by PAOA-flavone, which may be involved in sortilin suppression and macrophage lipid metabolism.

### 2.5 Facilitation of SAE1-catalyzed SUMOylation of the HNF1b protein by PAOA-flavone for suppressing macrophage sortilin expression and lipid accumulation

PAOA-flavone increased the levels of SAE1 mRNA and protein in THP-1 macrophages treated with 1000 μg/mL PAOA-flavone for 48 h (Figs. 5a–5c and S1m–S1t). Regardless of whether SAE1 was overexpressed (SAE1 oe) or silenced (siSAE1) in THP-1 macrophages, the level of *HNF1b* mRNA was not significantly different from that in the control group (Fig. 5d). SAE1 siRNA led to HNF1b suppression and sortilin upregulation, and SAE1 oe led to HNF1b upregulation and sortilin suppression in THP-1 macrophages treated with 1000 μg/mL PAOA-flavone for 48 h (Figs. 5b, 5c, and 5e). A Co-IP assay confirmed that SAE1 siRNA restrained the SUMO1 modification of HNF1b protein under the PAOA-flavone administration (Fig. 5f). The above experiments showed that PAOA-flavone enhanced SAE1 expression and SAE1-catalyzed SUMOylation of the HNF1b protein to inhibit macrophage sortilin expression. PAOA-flavone was observed to reduce the contents of TG and TC, and the size and number of LDs in ox-LDL-treated THP-1 macrophages. PAOA-flavone-reduced cellular lipid contents were further strengthened by SAE1 oe, as the cellular contents of TC and TG decreased markedly and the number and size of LDs also decreased. SAE1 siRNA inhibited PAOA-flavone-reduced cellular lipid contents, promoting intracellular lipid accumulation and LD generation in ox-LDL-treated THP-1 macrophages (Figs. 5l–5n). These data showed that PAOA-flavone promoted the SAE1-catalyzed SUMOylation of the HNF1b protein to reduce sortilin expression and lipid accumulation in macrophages. Furthermore, it was necessary to determine whether PAOA-flavone-promoted SAE1-catalyzed SUMOylation inhibits macrophage sortilin expression and lipid accumulation via the HNF1b protein. SAE1 siRNA has been proven to antagonize PAOA-flavone-induced upregulation of SAE1 and HNF1b and the suppression of sortilin in ox-LDL-treated THP-1 macrophages. Based on this, when

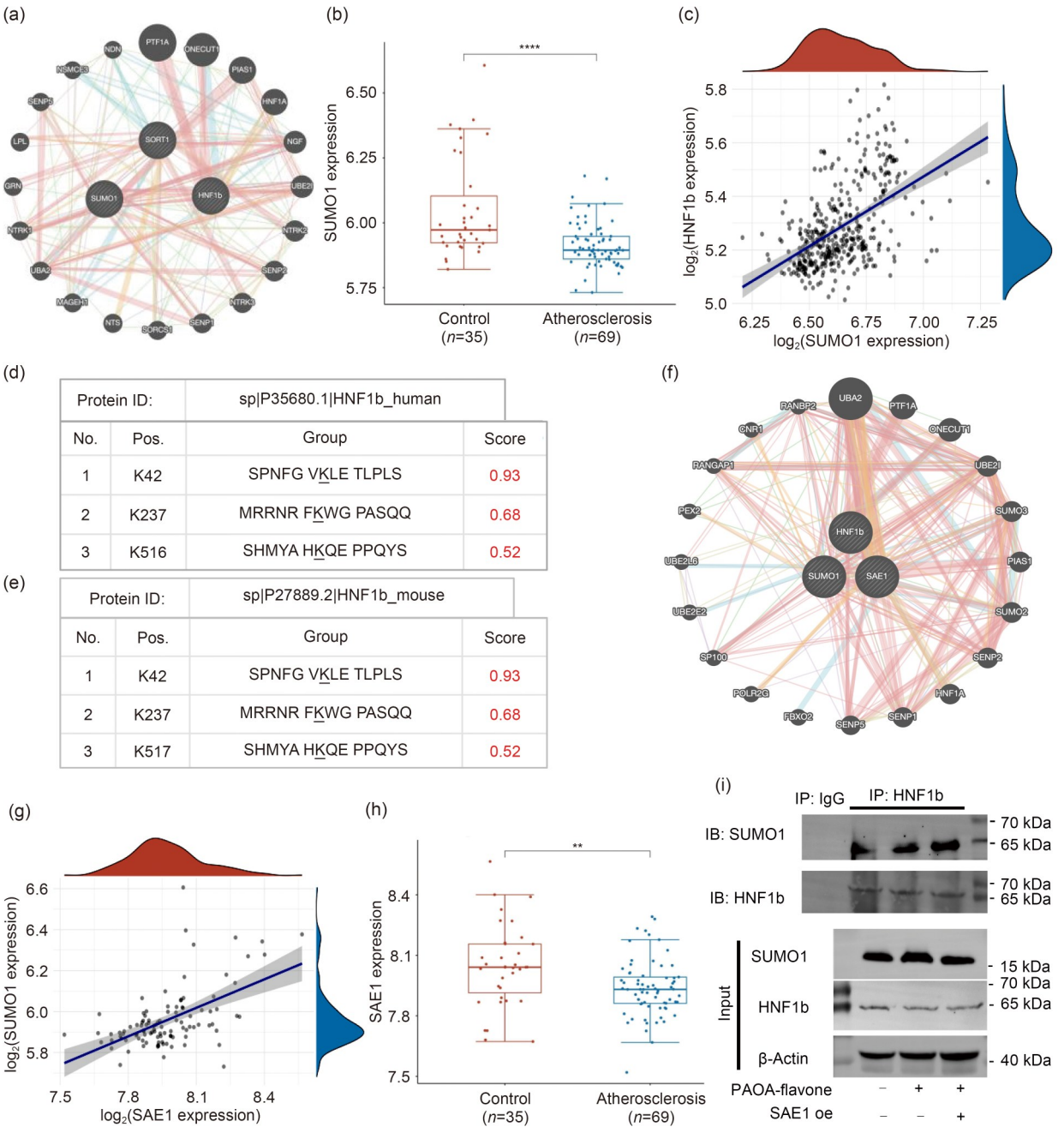


**Fig. 4 Flavone of *Polygonatum odoratum* (PAOA-flavone) enhanced the SUMO1ylation of hepatocyte nuclear factor 1β (HNF1b) protein. (a) The correlation among HNF1b, sortilin, and SUMO1 expression from the GeneMANIA network analysis. (b) The SUMO1 level of human carotid plaque in the Gene Expression Omnibus (GEO) datasets of the Bioinformatics website. Control (*n*=35) and atherosclerosis (*n*=69) (GSE100927). (c) The expressional correlations between HNF1b and SUMO1 were identified by the ASSISTANT for Clinical Bioinformatics website (GSE100927 and GSE40237; *n*=382; $\rho_{Spearman}$=0.51). (d, e) The prediction of SUMOylation sites in human and mouse HNF1b proteins by the SUMOplot™ Analysis Program. (f) The correlation among HNF1b, SUMO-activating enzyme subunit 1 (SAE1), and SUMO1 expression from the GeneMANIA network analysis. (g) The expressional correlation between SAE1 and HNF1b was established by the ASSISTANT for Clinical Bioinformatics website (GSE100927; *n*=104; $\rho_{Spearman}$=0.48). (h) The alteration of SAE1 expression in human carotid plaque in the GEO datasets of the Bioinformatics website. Control (*n*=35) and atherosclerosis (*n*=69) (GSE100927). (i) PAOA-flavone facilitated the SUMOylation of HNF1b protein in oxidized low-density lipoprotein (ox-LDL)-treated THP-1 cells, as shown by co-immunoprecipitation (Co-IP) detection. ** *P*<0.01; **** *P*<0.0001. SUMO1: small ubiquitin-like modifier 1; IB: immunoblotting; IgG: immunoglobulin G; IP: immunoprecipitation; Pos.: position; SAE1 oe: overexpressed SAE1.**

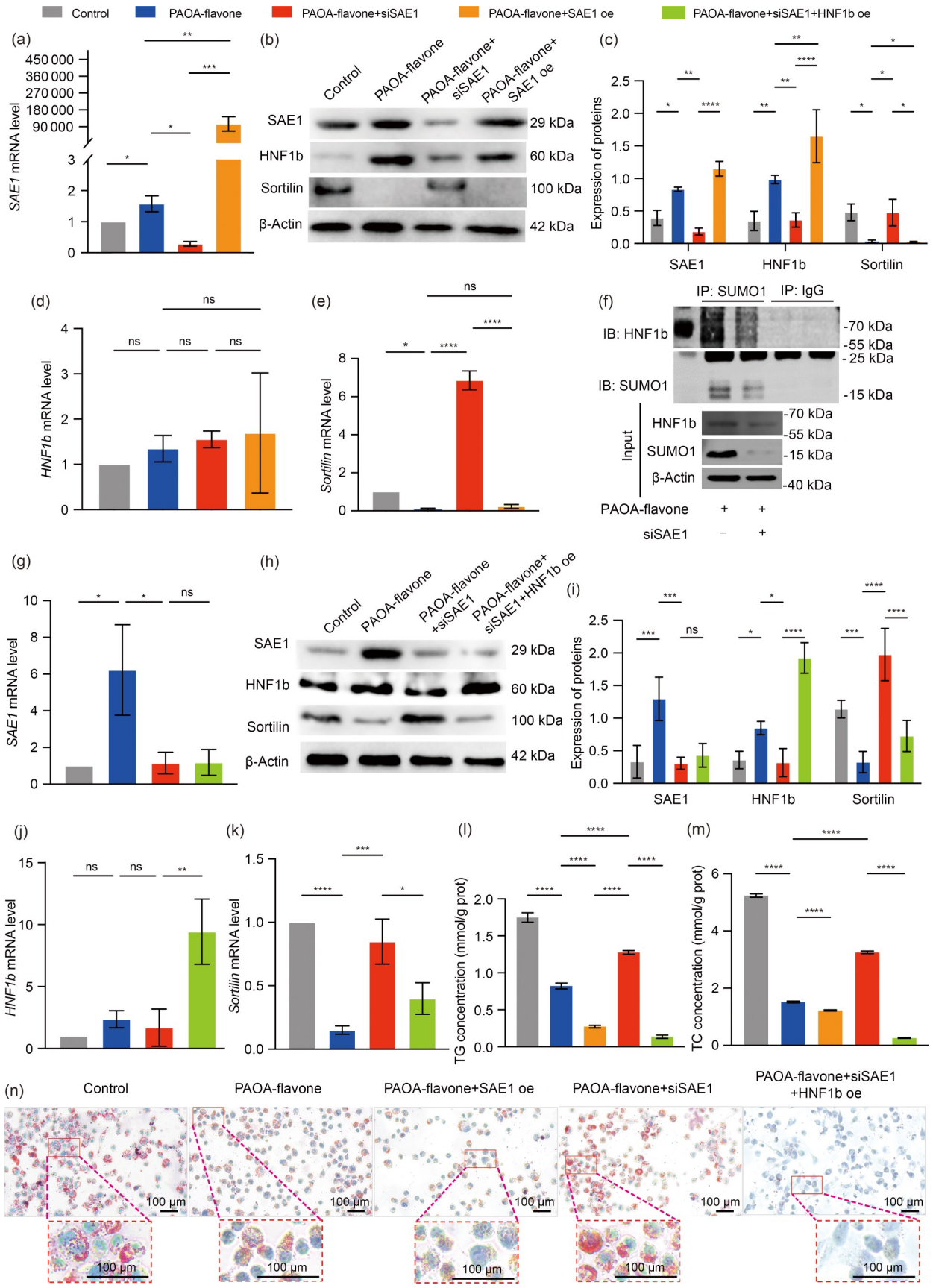
Control
PAOA-flavone
PAOA-flavone+siSAE1
PAOA-flavone+SAE1 oe
PAOA-flavone+siSAE1+HNF1b oe
(a)
SAE1 mRNA level
(b)
SAE1
HNF1b
Sortilin
β-Actin
29 kDa
60 kDa
100 kDa
42 kDa
(c)
Expression of proteins
(d)
HNF1b mRNA level
(e)
Sortilin mRNA level
(f)
IP: SUMO1
IP: IgG
IB: HNF1b
IB: SUMO1
Input
-70 kDa
-55 kDa
-25 kDa
-15 kDa
-40 kDa
PAOA-flavone
siSAE1
(g)
(h)
(i)
(j)
(k)
(l)
TG concentration (mmol/g prot)
(m)
TC concentration (mmol/g prot)
(n)
100 μm

**Fig. 5 Flavone of *Polygonatum odoratum* (PAOA-flavone) facilitated small ubiquitin-related modifier (SUMO)-activating enzyme subunit 1 (SAE1)-catalyzed SUMOylation of the hepatocyte nuclear factor 1β (HNF1b) protein to repress macrophage sortilin expression and lipid accumulation. (a–e) The mRNA and protein levels of SAE1, HNF1b, and sortilin were measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) or western blotting in THP-1 macrophages treated with 50 μg/mL oxidized low-density lipoprotein (ox-LDL) and 1000 μg/mL PAOA-flavone alone or in combination with silenced SAE1 (siSAE1) or SAE1 lentiviruses for 48 h. (f) The combination between SUMO1 and HNF1b protein in THP-1 macrophages incubated with 1000 μg/mL PAOA-flavone treated alone or together with siSAE1, from the co-immunoprecipitation (Co-IP) analysis. (g–k) The mRNA and protein levels of SAE1, HNF1b, and sortilin were measured using RT-qPCR and western blotting in THP-1 macrophages treated with 50 μg/mL ox-LDL and 1000 μg/mL PAOA-flavone alone or in combination with siSAE1 or siSAE1+HNF1b lentiviruses for 48 h. Values were normalized to the expression of the *β-actin* gene. (l, m) The cellular contents of triglyceride (TG) and total cholesterol (TC) were determined in THP-1 macrophages treated with 50 μg/mL ox-LDL and 1000 μg/mL PAOA-flavone alone or in combination with siSAE1 or SAE1 lentiviruses for 48 h. (n) The intracellular lipid droplets (LDs) were stained with Oil Red O (ORO) in THP-1 macrophages incubated with 50 μg/mL ox-LDL and 1000 μg/mL PAOA-flavone alone or in combination with siSAE1 or siSAE1+HNF1b lentiviruses for 48 h; cells were photographed at 200× magnification. All results were expressed as mean±standard deviation (SD) from three independent experiments in duplicate. $^{*}$ $P$<0.05; $^{**}$ $P$<0.01; $^{***}$ $P$<0.001; $^{****}$ $P$<0.0001; ns, non-significant. IgG: immunoglobulin G; mRNA: messenger RNA; HNF1b oe: overexpression of HNF1b; IB: immunoblotting; IP: immunoprecipitation.**

co-transfected with SAE1 siRNA and HNF1b-oe lentivirus in THP-1 macrophages incubated with 1000 μg/mL PAOA-flavone for 48 h, HNF1b oe negated the antagonistic role of SAE1 siRNA in the PAOA-flavone-induced upregulation of SAE1 and HNF1b and the suppression of sortilin, resulting in a decrease in the number and size of intracellular LDs in ox-LDL-treated THP-1 macrophages (Figs. 5g–5n). These results demonstrated that PAOA-flavone promoted the SAE1-catalyzed SUMOylation of the HNF1b protein and inhibited sortilin expression in macrophages, thereby inhibiting intracellular lipid accumulation and LD formation.

### 2.6 Upregulation of SAE1 and HNF1b expression levels by PAOA-flavone for inhibiting aortic lipid deposition and AS in vivo

To verify the anti-atherosclerotic effect of PAOA-flavone in vivo, the AAVs for HNF1b and SAE1 short hairpin RNA (shSAE1) were used to infect apolipoprotein E-deficient ($ApoE^{-/-}$) mice fed an HFD supplemented with PAOA-flavone for 10 weeks. PAOA-flavone significantly decreased the plasma levels of TG, TC, and LDL-C, which were inhibited by the administration of shSAE1 AAV. After co-infection with recombinant shSAE1 and HNF1b AAVs in $ApoE^{-/-}$ mice, the antagonistic role of shSAE1 in the lipid-lowering effect of PAOA-flavone was apparently offset by HNF1b, resulting in a decrease in circulating lipid levels (Figs. 6a–6d). Histological staining revealed that shSAE1 AAV strongly inhibited PAOA-flavone-reduced lipid deposition and plaque area in cross-sections of aortic root, and HNF1b AAV evidently resumed the anti-atherosclerotic effect of PAOA-flavone under the suppression of SAE1 shRNA (Figs. 6e and 6f). These results demonstrated that HNF1b AAV counteracted the antagonistic effect of SAE1 shRNA on PAOA-flavone-prevented aortic walls against lipid deposition and atherogenesis in vivo. The immunofluorescence detection further verified that PAOA-flavone induced the upregulation of SAE1 and HNF1b and the depression of sortilin protein in HFD-fed mice. The PAOA-flavone-elevated protein levels of SAE1 and HNF1b were dramatically reduced upon the injection of shSAE1 AAV. However, simultaneous HNF1b oe obviously recovered the stimulative activity of PAOA-flavone on the expression of HNF1b and the inhibitory activity on sortilin levels in the aortic sinus of $ApoE^{-/-}$ mice fed an HFD (Figs. 6g–6j).

## 3 Discussion

Reduced lipid content in macrophages has been proven to suppress the generation of foam cells and the progression of atherosclerosis (Barish et al., 2012). Our study elucidated an atheroprotective mechanism by ameliorating macrophage lipid accumulation and the formation of foam cells to prevent the development of atherosclerosis. HNF1b transcriptionally inhibited expression of sortilin, alleviating macrophage lipid accumulation and aortic atherosclerotic lesions. SUMOylation was discovered to modify HNF1b protein and to enhance HNF1b-inhibited sortilin expression and lipid accumulation in macrophages. PAOA-flavone administration promoted the SAE1-catalyzed

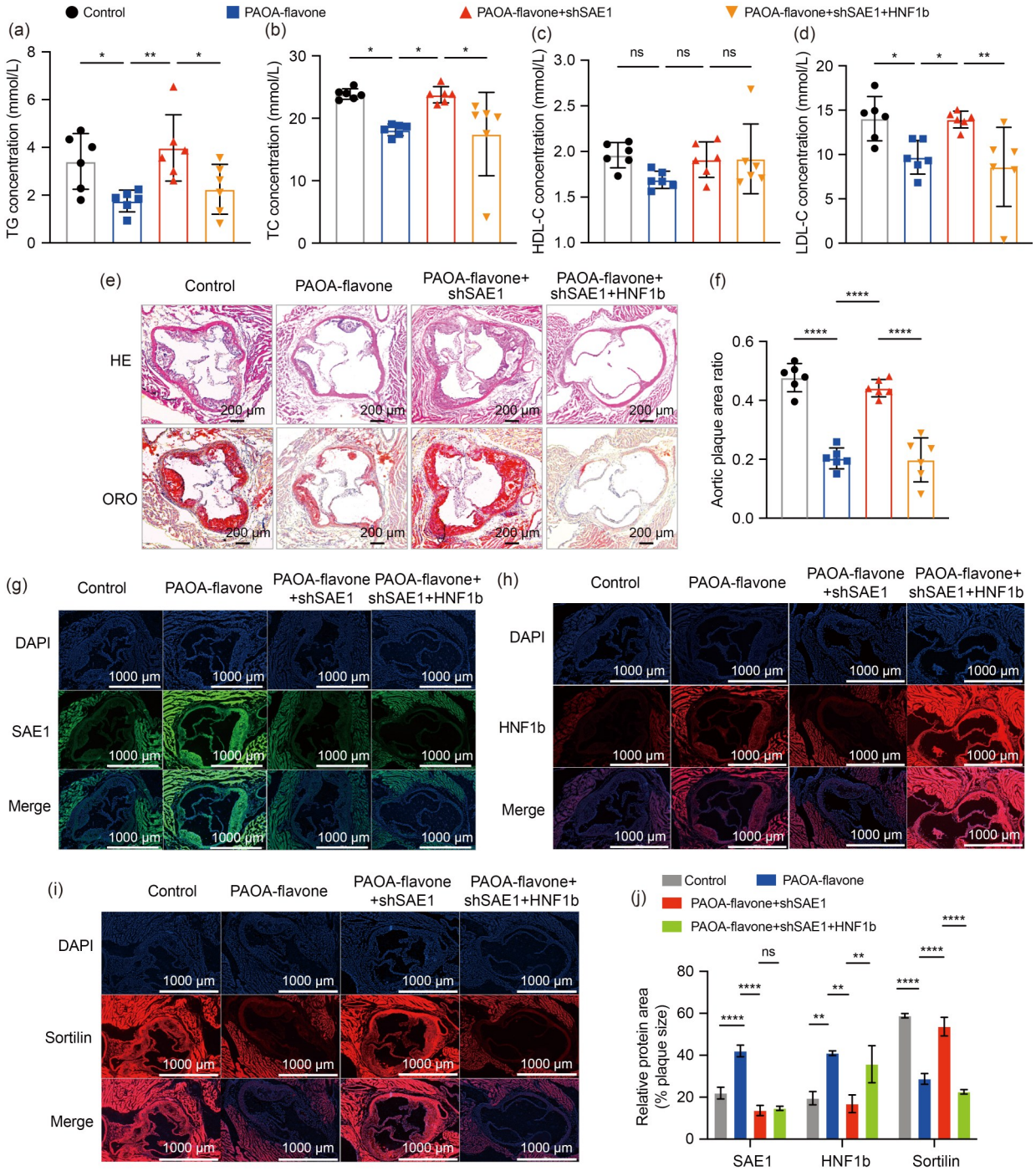


**Fig. 6 Flavone of *Polygonatum odoratum* (PAOA-flavone) upregulated the expression of small ubiquitin-related modifier (SUMO)-activating enzyme subunit 1 (SAE1) and hepatocyte nuclear factor 1β (HNF1b) to resist aortic lipid deposition and atherosclerosis (AS) in vivo. (a–d) The levels of plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) were determined using the enzymatic oxidation method in PAOA-flavone-treated apolipoprotein E-deficient ($ApoE^{-/-}$) mice infected with recombinant shSAE1 adeno-associated virus (AAV) alone or in combination with HNF1b AAV. (e, f) The atherosclerosis lesion and lipid deposition were stained by hematoxylin eosin (HE) and Oil Red O (ORO) in the frozen sections of the aortic sinus, and the aortic plaque area was quantified in the ORO-stained frozen sections. (g–j) Aortic SAE1, HNF1b, and sortilin expression levels and quantitative analysis were measured by immunofluorescence detection in PAOA-flavone-treated $ApoE^{-/-}$ mice infected with shSAE1 AAV alone or co-transfected with HNF1b AAV. The results are expressed as mean±standard deviation (SD), with $n$=6 (a–d, f) or $n$=3 (j). $^{*}$ $P$<0.05; $^{**}$ $P$<0.01; $^{****}$ $P$<0.0001; ns, non-significant. DAPI: 4',6-diamidino-2-phenylindole; shSAE1: SAE1 short hairpin RNA.**

SUMOylation of HNF1b protein to reduce macrophage sortilin expression, cellular lipid content, and atherosclerotic lesions in the aortic wall. These results demonstrated that HNF1b transcriptionally suppressed sortilin expression and macrophage lipid accumulation to inhibit aortic lipid deposition and the development of atherosclerosis. This anti-atherosclerotic effect was enhanced by PAOA-flavone-facilitated, SAE1-catalyzed SUMOylation of the HNF1b protein (Fig. 7).

HNF1b is an essential transcriptional regulator of lipid metabolism and has antiatherosclerosis efficacy (Wang et al., 2017; Chan et al., 2018). Due to the lack of ADI and ADIII in the C-terminal amino acids, HNF1b targets and inhibits the transcription of related lipid genes to manipulate the processes of lipid metabolism, such as lipid anabolism, including synthesis, absorption, and storage, and lipid degradation (Mendel et al., 1991; Long et al., 2017; Wu et al., 2017; Wang W et al., 2020; Watanabe et al., 2020; Liu et al., 2022). HNF1b has been shown to antagonize peroxisome proliferator-activated receptor γ (PPARγ)-induced lipid synthesis in 3T3-L1 preadipocytes and HFD-fed mice. HNF1b also downregulates the expression levels of sterol regulatory element-binding protein-1 (SREBP-1) and acetyl-CoA carboxylase (ACC), which hinders the synthesis and absorption of lipids in AML-12 hepatocytes (Patitucci et al., 2017; Wang et al., 2017; Su et al., 2018). Based on the reported literature and our previous research showing that sortilin accelerates macrophage lipid accumulation and aortic atherogenesis (Zhong et al., 2016; Lv et al., 2019, 2020), we endeavored to explore whether HNF1b exerts anti-atherosclerotic action by suppressing sortilin in macrophages. Consistently, our study showed that HNF1b bound to a region of the sortilin promoter containing the TATA box, the core transcription unit (approximately 10 bp), and transcriptionally inhibited sortilin expression and sortilin-mediated lipid accumulation in macrophages. Furthermore, HNF1b oe significantly reduced aortic lipid deposition and atherosclerotic plaques, ameliorating the occurrence of AS in HFD-fed LDLR$^{-/-}$ mice, and the atheroprotective effect of HNF1b was offset by sortilin overexpression concurrently. Despite the beneficial effects of HNF1b on lipid metabolism and atherogenesis, which processes of lipid metabolism (such as uptake, transport, storage, or efflux) are controlled by HNF1b in macrophages needs to be determined. Alternatively, mechanisms of HNF1b-mediated transcriptional repression may be more complex. For

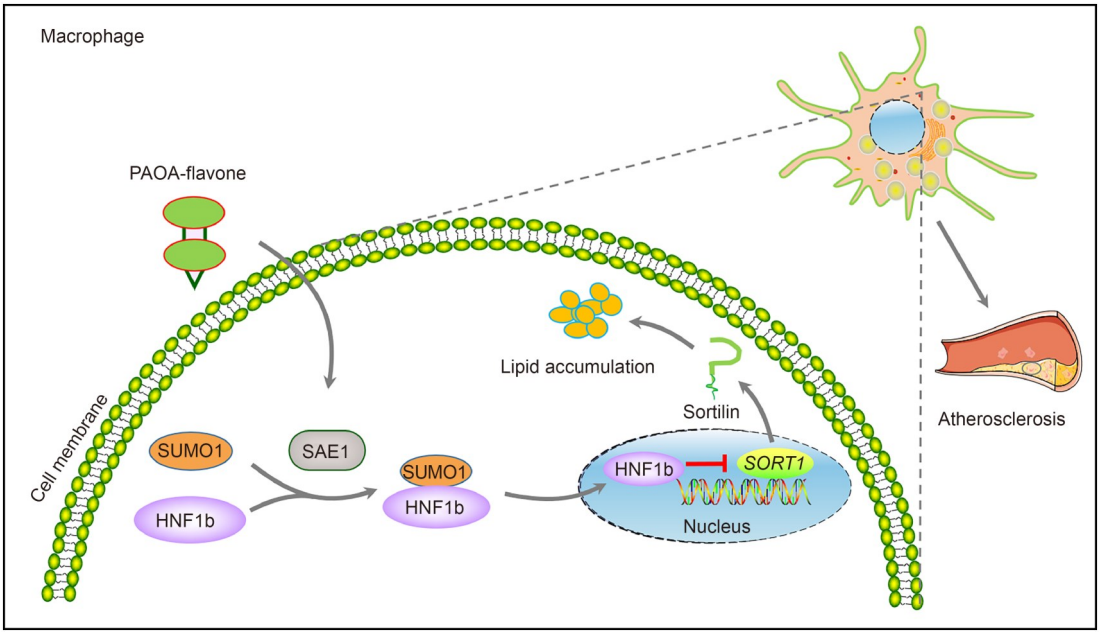


**Fig. 7 Schematic representation of the effect of the flavone of *Polygonatum odoratum* (PAOA-flavone) on small ubiquitin-related modifier (SUMO)-activating enzyme subunit 1 (SAE1)-facilitated hepatocyte nuclear factor 1β (HNF1b) SUMOylation and sortilin-mediated lipid accumulation in macrophages and aortic atherogenesis. PAOA-flavone promotes SAE1-catalyzed SUMOylation of HNF1b and elevates cellular levels of HNF1b protein, suppressing sortilin expression and sortilin-mediated lipid accumulation in macrophages, and alleviating aortic lipid deposition and atherosclerosis development.**

instance, HNF1b inhibits Slug repression by interacting with retinoblastoma-binding protein 7 (RBBP7)/retinoblastoma suppressor-associated protein 46 (RbAP46) in human prostate cancer cells. Some cofactors likely participate in the HNF1b-mediated transcriptional repression of macrophage sortilin and need further investigation (Lu et al., 2020; Wang JQ et al., 2020).

SUMOylation, recently emerging as an important post-translational mechanism, modulates the expression and distribution of some transcription factors in participating in lipid metabolism processes (Boggio et al., 2004; Gill, 2004; Johnson, 2004; Yang et al., 2020; Salas-Lloret and González-Prieto, 2022). Similar to ubiquitination, SUMO modification undergoes a multistep enzymatic cascade reaction to bring SUMO to the lysine residues of substrate proteins with E1 (SAE1/SAE2), E2, and E3 enzymes. SUMOylation has been further established to cooperate with the heat shock proteins heat shock factor 1 (HSF1) and HSF2 and the β-catenin activation factor transcription factor 4 (Tcf-4) to positively regulate the biological function of transcription factors (Watanabe et al., 2020). It has been confirmed that SUMOylation controls the expression of SREBPs to maintain the homeostasis of lipid metabolism in Hela cells (Hirano et al., 2003). Consistent with the report by Hirano et al. (2003), results from our laboratory also revealed that the SUMO1 modification did not heighten *HNF1b* mRNA level but did increase its protein level in macrophages, which reduced the cellular lipid content and foam cell formation. The SUMOplot™ Analysis Program website further predicted that the K42 residue of the HNF1b protein serves as a potential site for accepting the SUMO1 donor. It is speculated that SUMOylation can prevent HNF1b degradation in a ubiquitination-dependent manner, maintain its stability, and enable more HNF1b to enter the nucleus, thus leading to a stronger suppression of sortilin expression and a reduction in macrophage lipid accumulation and aortic AS lesions, and this requires detailed investigation in the future. In addition, there are other SUMO proteins encoded by the human genome, including SUMO2 and SUMO3, and their effects on HNF1b expression and biological function warrant further exploration.

Many surveys have shown that flavones improve lipid deregulation and inhibit the progression of atherosclerotic cardiovascular diseases, and one of their atheroprotective mechanisms is probably related to SUMO modification (Hishikawa et al., 2005; Mulvihill et al., 2010; Chanet et al., 2012; Pawellek et al., 2017; Meng et al., 2022). Naringenin was observed to reduce cholesterol synthesis and accelerate fatty acid oxidation, resulting in a decrease in hepatic TG content and nascent very LDL (VLDL) secretion in $ApoE^{-/-}$ mice (Mulvihill et al., 2010; Chanet et al., 2012). Saponins, soybean flavonoids, enhance the activity of triacylglycerol lipase, and greatly mobilize the serum triglycerides and fatty acids to liver, muscle, and adipose tissues, thereby reducing plasma levels of cholesterol and LDL in male Wistar rats (da Silva et al., 2001; Matsuura, 2001; Rodrigues et al., 2005). In line with the abovementioned findings, PAOA-flavone also exhibited a therapeutic efficacy on lipid dysmetabolism, obviously lessening macrophage lipid accumulation and vascular plaque formation (Kumar and Pandey, 2013; Pawellek et al., 2017). Our study further revealed that PAOA-flavone was able to increase the expression of the SAE1 enzyme to accelerate the SUMO1 modification of the HNF1b protein in macrophages. PAOA-flavone-activated SUMO1 modification was abrogated by the silencing of SAE1 and reduced SUMO1 modification of the HNF1b protein, indicating that SAE1-catalyzed SUMOylation is indispensable for PAOA-flavone-elevated HNF1b level and its anti-atherosclerotic property. This finding is consistent with published data, showing that hinokiflavone causes a significant increase in SUMO1 modification in multiple human cell lines (Pawellek et al., 2017). However, the regulatory mechanism underlying PAOA-flavone-modulated SAE1 expression in macrophages remains unknown. The mRNA level of *HNF1b* was unaltered at 1000 μg/mL (the commonly used dose in reported studies) of PAOA-flavones but inexplicably increased at 2000 μg/mL PAOA-flavone, suggesting that there are some transcriptional mechanisms independent of SUMOylation that control HNF1b expression in the presence of high doses of PAOA-flavone.

In summary, we revealed a novel downstream pathway for a factor of great interest, HNF1b, in lipid metabolism regulation, which significantly reduced macrophage lipid accumulation and vessel plaque formation via transcriptional suppression of sortilin expression in vitro and in vivo. HNF1b displays promising prospects for application in the diagnosis and therapy of lipid dysmetabolism-derived atherosclerotic

cardiovascular diseases. The mechanism upstream of HNF1b was also further investigated, revealing that PAOA-flavone-promoted, SAE1-catalyzed SUMOylation enhances the atheroprotective efficacy of HNF1b and relieves foam cell formation and the development of aortic AS lesions. Further study is required to comprehensively evaluate the role of SUMOylation in lipid metabolism processes and atherogenesis under pro-atherosclerotic conditions. An anti-atherosclerotic diet enriched with flavone, especially including fruit and vegetables, should be strongly advocated in daily life, with the use of food as medicine to achieve the early prevention of hypolipidemia and AS, instead of clinical treatment in a hospital (Li XX et al., 2020; Xue et al., 2021).

## Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary materials.

## Acknowledgments

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## Author contributions

Fang LIU performed the experimental research and data analysis, wrote and edited the manuscript. Shirui CHEN and Xinyue MING performed the establishment of animal models. Huijuan LI performed the experiments and analyzed the data. Zhaoming ZENG providied us the samples of PAOA-flavone and gave some advice for its use. Yuncheng LV contributed to the research design and writing and editing of the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

## Compliance with ethics guidelines

Fang LIU, Shirui CHEN, Xinyue MING, Huijuan LI, Zhaoming ZENG, and Yuncheng LV declare that they have no conflict of interest.

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 (No. GLMC-IACUC-2022011).

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**Supplementary information**

Figs. S1 and S2; Table S1; Materials and methods