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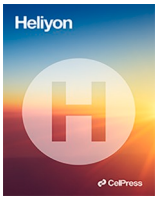

Research article

TRPM4 mRNA stabilization by METTL3-mediated m6A modification promotes calcific aortic valve inflammation

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A R T I C L E I N F O

Keywords:
Transient receptor potential melastatin 4 (TRPM4)
Calcific aortic valve disease(CAVD)
Inflammation
N6-methyladenosine (m6A)
JNK-MAPK signaling pathway

A B S T R A C T

Background: Transient receptor potential melastatin 4 (TRPM4) affects immune responses by regulating calcium homeostasis, but its role in calcific aortic valve inflammation remains unclear. This study aimed to assess the expression and function of TRPM4 in patients with or without calcific aortic valve disease (CAVD).

Methods: The mRNA and protein expression levels of TRPM4 and related factors in calcified and noncalcified tissues were measured using qRT-PCR and Western blot. The proteins interacting with TRPM4 were confirmed by RNA pull-down and RNA immunoprecipitation assays. Dual-Luciferase Reporter Assay was performed to confirm the m6A site of TRPM4.

Results: The mRNA expression levels of TRPM4, TLR4, IL-6, MCP-1, TNF-α, and NF-κB p65 were significantly higher in calcified aortic valve tissues than in noncalcified tissues, and TRPM4 was significantly positively correlated with inflammation-related factors. The protein expression level of TRPM4, TLR4 and NF-κB p65 were significantly higher in calcified aortic valve tissues than in noncalcified tissues. N6-methyladenosine (m6A) modification of TRPM4 mRNA by METTL3-YTHDF1 up-regulated its expression in CAVD. And TRPM4 promoted the level of inflammation via activation of the JNK-MAPK signaling pathway, after knockdown TRPM4, the production of proinflammatory cytokines was significantly suppressed.

Conclusion: The results indicate the pivotal role of TRPM4 in CAVD and highlight METTL3-mediated m6A modification of TRPM4 in promoting inflammation through JNK-MAPK signaling pathway. This work provides potential therapeutic strategy to impede inflammation in CAVD.

1. Introduction

Calcific aortic valve disease (CAVD) is the most common valvular heart disease and the leading cause of aortic stenosis (AS) in the elderly [1]; this illness ultimately leads to heart failure and death. The rate of incident CAVD is 30 % and 40–50 % in people over 65 and over 75 years old, respectively [2,3]. The pathophysiology of CAVD is a complex process that includes endothelial injury, lipid infiltration, chronic inflammation, matrix remodeling, cell differentiation, progressive bone formation, and new angiogenesis [4,5]. Song et al. found that the immune response is involved in the physiological process of CAVD, in which the activation of Toll-like

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receptor 4 (TLR4) is critical [6]. To date, no pharmacological agent can effectively prevent or delay its progression [7]. The mechanism for the initiation and development of the inflammation response of CAVD has not been completely elucidated and require further tests.

Transient receptor potential melastatin 4 (TRPM4) is a Ca2+ activated nonselective cation channel involved in immune response, respiratory reaction, and cerebral vasoconstriction [8]. In astrocytes, the TRPM4 channel plays a vital role in viral protein R-induced proinflammatory response [9]. TRPM4 is also involved in lipopolysaccharide-induced vascular endothelial injury [10]. Aberrant TRPM4 expression significantly influences the phagocytotic activity of macrophages and increases the proinflammatory cytokine secretion in sepsis [11]. However, the role of TRPM4 in CAVD is poorly understood.

N6-methyladenosine (m6A), a critical epigenetic regulator, is the most abundant internal reversible chemical modification in eukaryotic mRNA [12]. m6A modification mainly influences mRNA stability, splicing, and translation [13–15]. Methyltransferase-like 3 (METTL3) is a core m6A methyltransferase enzyme that initiates the m6A modification of target mRNAs [16]. These m6A-modified mRNAs can be recognized by m6A "readers" (IGF2BP1–3, YTHDF1–3, and YTHDC1–3) [17–19]. However, the mechanism by which METTL3 mediates the m6A modification of TRPM4 to affect inflammatory cytokine progression in CAVD has not been reported. Mitogen-activated protein kinases (MAPKs) are signaling components that convert extracellular stimuli into a wide range of cellular responses [20], which include extracellular signal-related kinase (ERK)-1/2, p38, and c-Jun NH2-terminal kinase (JNK). Among these, JNK and p38 are mainly involved in the expression of pro-inflammatory mediators [21].

The mechanisms underlying aortic valve inflammation in in CAVD are rarely explored. In this study, our aim was to investigate the expression of TRPM4 in noncalcified and calcified aortic valve tissues, determine its association with the expression of inflammation cytokines, and find a new target for early CAVD intervention.

In this study, we found that TRPM4 promotes valve calcification and contributes to the progression of CAVD. The objectives of this study were to determine (1) the reasons for the high expression of TRPM4 in CAVD and (2) the mechanisms by which TRPM4 promotes the progression of CAVD.

2. Methods

2.1. Patients and ethics statement

In our study comprised 60 participants including calcified group (calcified valve tissue, n = 30) and noncalcified group (non-calcified valve tissue, n = 30). The patient characteristics and laboratory measurements are summarized in Table 1. All patients were diagnosed by cardiac doppler ultrasonography and treated with aortic valve replacement surgery. The exclusion criteria were as follows: previous treatment with aortic valve replacement surgery, chronic inflammation disease, immune dysfunction, kidney disease and heart failure. Written informed consent was received from all human participants prior to inclusion in the study and in agreement with the Declaration of Helsinki. The consent form and ethical approval were established by the Ethics Committee on Human Subject Research at Central people's Hospital of Zhanjiang (approval number: No. 2022742 B), Guangdong Medical University.

2.2. Collection of aortic valve tissues

Human aortic valve tissues were collected after the cardiopulmonary bypass was performed. Tissues were washed twice with cold PBS and then cut into small pieces, which stored in RNA protection solution and liquid nitrogen until experiment, respectively.

Table 1
Clinical characteristics and laboratory measurements of patients.

Parameters	Noncalcified group (n = 30)	Calcified group (n = 30)	*P* value
Age, y	58 ± 8	62 ± 7	0.0513
Male, n (%)	19(63.3)	14(46.7)	0.2993
BMI (kg/m^2)	24.90 ± 2.85	25.71 ± 1.32	0.1662
Hypertension, n (%)	22(73.3)	26(86.7)	0.3334
Triglycerides (mmol/L)	3.75 ± 1.12	3.86 ± 0.99	0.6857
LDL (mmol/L)	1.87 ± 0.99	1.70 ± 0.94	0.4821
HDL (mmol/L)	1.12 ± 0.56	1.17 ± 0.31	0.6965
Diabetes mellitus, n (%)	5(16.7)	7(23.3)	0.7480
Smoking, n (%)	8(26.7)	11(36.7)	0.5796
Statins, n (%)	20(66.7)	26(86.7)	0.1253
β-Blockers, n (%)	25(83.3)	27(90)	0.7065
ACEi/ARB, n (%)	22(73.3)	26(86.7)	0.3334
Transvalvular pressure gradient (mmHg)	16.48 ± 3.22	87.15 ± 22.72	<0.0001
AVA (cm^2)	3.35 ± 0.41	0.68 ± 0.19	<0.0001

Values are mean ± standard deviation (SD) or %. ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; AVA, aortic valve area; BMI, Body mass index; CAVD, calcific aortic valve disease; CAVs, calcific aortic valves; DCM, dilated cardiomyopathy; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

2.3. RNA isolation and real-time quantitative PCR (QPCR)

Total RNA was isolated from aortic valve tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Quantitation of the mRNA level by qPCR was performed on a real-time PCR system using SYBR Premix Ex Taq (Biorigin, Beijing, China). The cycle thresholds (CT) of the target gene was normalized to the CT of the internal reference β-actin gene and analyzed the results by using the $2-\Delta\Delta Ct$ method. Primer sequences used are listed in Table 2.

2.4. Western blot

The total protein from aortic valve tissues were extracted with RIPA Lysis Buffer (Beyotime, shanghai, China) supplemented with PMSF. Protein concentration was quantified using the BCA Kit (Beyotime). Proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotted to PVDF membranes (Millipore, USA). Membranes were blocked with 5 % non-fat milk and then incubated with antibodies against TRPM4 (1:1,000, A10146, ABclonal, wuhan, China), p38 (1:1,000, A14401, ABclonal, wuhan, China), p-p38 (1:1,000, AP1502, ABclonal, wuhan, China), ERK1/2 (1:1,000, A16686, ABclonal, wuhan, China), *p*-ERK1/2 (1:1,000, #4370, Cell Signal Technology, Danvers, MA, USA), JNK (1:1,000, AP0473, ABclonal, wuhan, China), *p*-JNK (1:1,000, #4668, Cell Signal Technology, Danvers, MA, USA), TLR4 (1:1,000, #38519, Cell Signal Technology, Danvers, MA, USA), Runx2 (1:1,000, #12556, Cell Signal Technology, Danvers, MA, USA), Osterix (1:1,000, #209484, Abcam, Danvers, MA, USA), Osteocalcin (1:1,000, #133612, Abcam, Danvers, MA, USA), N6-methyladenosine (1:5,000, #284130, Abcam, Danvers, MA, USA), METTL3 (1:1,000, #195352, Abcam, Danvers, MA, USA), YTHDF1 (1:1,000, #57530, Cell Signal Technology, Danvers, MA, USA), YTHDF2 (1:1,000, #71283, Cell Signal Technology, Danvers, MA, USA)and NF-κB p65 (1:1,000, #8242, Cell Signal Technology) at 4 °C overnight. Membranes were washed and incubated with corresponding secondary antibody at room temperature. Images of membrane with proteins were taken with chemiluminescence system (Bio-rad).

2.5. RNA N6-methyladenosine (m6A) dot blot assays

The m6A dot blot assay was performed as previously published [19]. Firstly, a 3-fold volume of RNA incubation buffer (65.7 % formamide, 7.77 % formaldehyde, and 1.33 × MOPS) was used to denature poly(A) + RNAs at 65 °C for 5 min. RNA samples (125 ng, 250 ng, 500 ng) mixed with 1 × saline sodium citrate (SSC) on ice for 5 min, and then transferred onto an Amersham Hybond-N+ membrane (Amersham Biosciences) with a Bio-Dot apparatus (Bio-Rad, Hercules, CA, USA). The membrane performed ultraviolet cross-linking for 5 min, followed by staine with 0.02 % methylene blue (Sangon Biotech, Shanghai, China) in 0.3 M sodium acetate (pH 5.2). The membrane was then blocked with 5 % non-fat milk in PBST, and incubated with m6A antibody (1:1000, ab151230, Abcam, UK) overnight at 4 °C. Lastly, after incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, the membrane was visualized using the chemiluminescence method (Bio-Rad, Hercules, CA, USA).

2.6. Dual-luciferase reporter assay

We constructed the wild-type and mutated (A to T at position 14) pmiR-RB-Report-TRPM4 3′-untranslated region (3′-UTR) plasmids. After co-transfection, 293T cells were assayed with a dual-luciferase reporter assay kit (Promega, Madison, WI, USA). The relative luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

2.7. Cell culture and treatment

The human monocytes (THP-1) and human embryonic kidney (HEK) 293T cells were obtained from the Cell Bank of the Chinese

Table 2
Sequences of the primers for qPCR are used in this study.

Gene	Forward (5′-3′)	Reverse (5′-3′)
TRPM1	**TACACGCTATTTCCCCGATGA**	**GCGCGTGATCTTTTGAACTTG**
TRPM2	**GTTACCTTCACCATTGAC**	**GGCTCTTTCCTCATCCTTTCT**
TRPM4	**GCACGACGTTCATAGTTGACT**	**CTTCTCCGTGGTGTGTGCAT**
TRPM7	**GGCCATACTCACTGCACTGCTG**	**AGAGCATCAAGCATAGCCTGTTCC**
Runx2	**GAACCAAGAAGGCACAGACA**	**AACTGCCTGGGGTCTGAAAA**
Osterix	**CTGGGGAAAGGAGGCACAAAGAAG**	**GGGTTAAGGGGAGCAAAGTCAGAT**
TLR4	**AATGCCAGGATGATGCCTCT**	**AGGAAGTACCTCTATGCAGGG**
IL-6	**CTCTCCGCAAGAGACTTCCAG**	**TGTGGGTGGTATCCTCTGTGA**
TNF-α	**GTGACAAGCCTGTAGCCCAT**	**TATCTCTCAGCTCCACGCCA**
NF-κB p65	**CTGAGTCCTGCTCCTTCCA**	**CTTCGGTGTAGCCCATTTGT**
CD68	**CCCAGATTCAGATTCGAGTTCGAGTCAT**	**GTTTTGTTGGGGTTCAGTACAG**
β-actin	**AGGCCGGCTTCGCGGGCGAC**	**CAGGGGAGCACACGCAGCTC**

Academy of Sciences (Shanghai, China). They were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Thermo Fisher Scientific, Rockford, IL, USA) and streptomycin (100 mg/mL) in a humidified atmosphere with 5 % CO2 at 37 °C. For the THP-1 cells differentiation into M1 macrophages, the cells were cultured with 100 ng/mL phorbol-12-myristate 13-acetate (Sigma, St. Louis, MO, USA), followed by incubate with 20 ng/mL interferon (IFN)-γ (Gibco, Waltham, MA, USA) and 10 ng/mL LPS (Sigma) before their use in experiments. Extraction of hAVICs was performed according to a previously reported

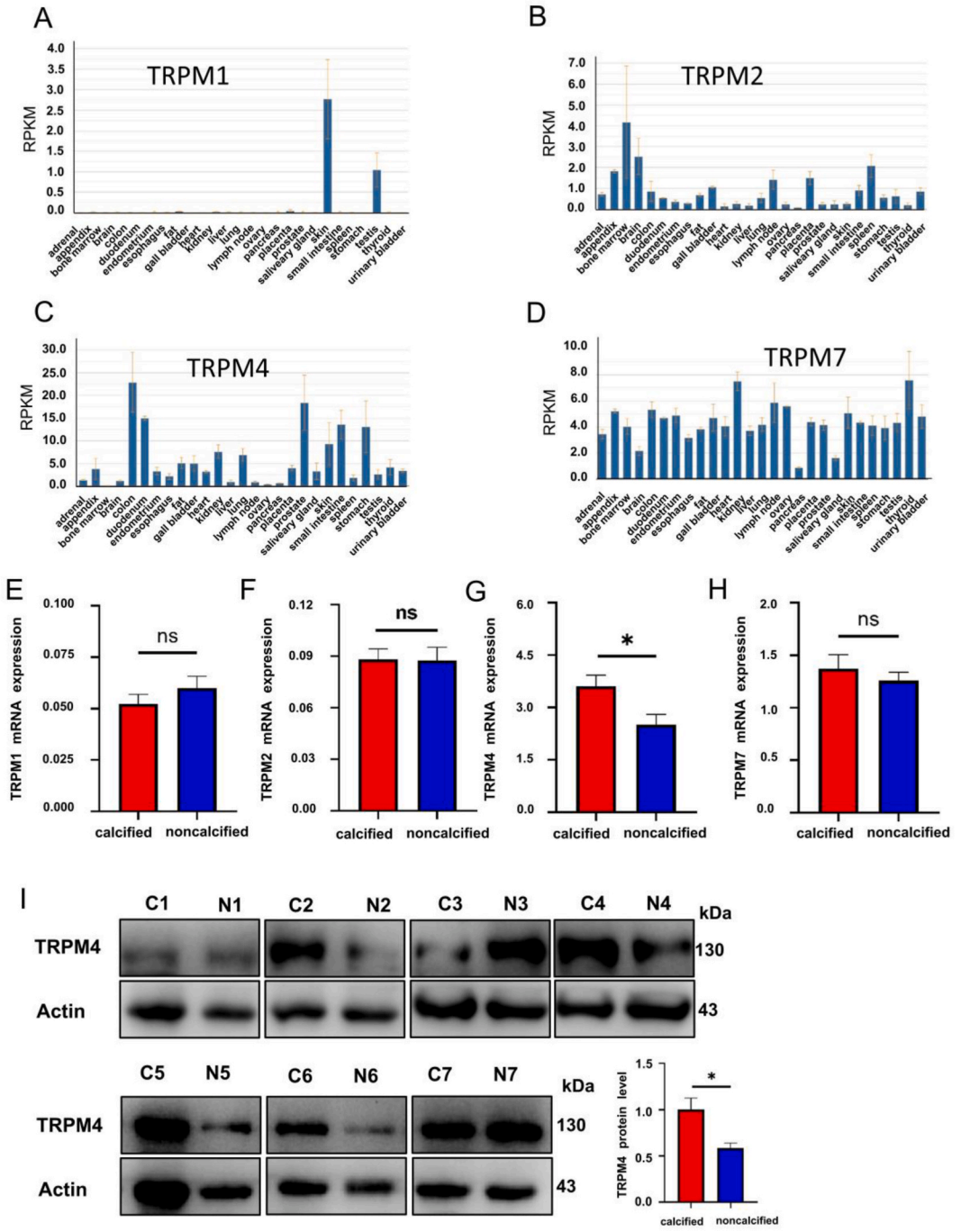

Fig. 1. TRPM4 was up-regulated in human calcified aortic valve tissues. RNA-seq data about TRPM supplied by National Center for Biotechnology Information database. Transcript abundance (FPKM) plotted showed that the expression of TRPM1 (A), TRPM2 (B), TRPM4 (C) and TRPM7 (D) transcript in 27 different tissues from 95 human individuals. (E–H) The RNA levels of TRPM1, TRPM2, TRPM4 and TRPM7 were quantified in 30 pairs of calcified and noncalcified aortic valve tissues using qRT-PCR. (I) TRPM4 protein levels were quantified in 30 pairs of human aortic valves were determined by Western blot. Data are presented as mean ± standard error of the mean (SEM). *P ≤ 0.05. ns, not significant.

protocol [22]. The aortic valve leaflets samples were immersed and the endothelial cell layer was scraped off. The valves cut into pieces and placed in a solution of type II collagenase (Gibco, Carlsbad, California, USA). The precipitate was resuspended with high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, California, USA). Cells at passage 3 were collected for further

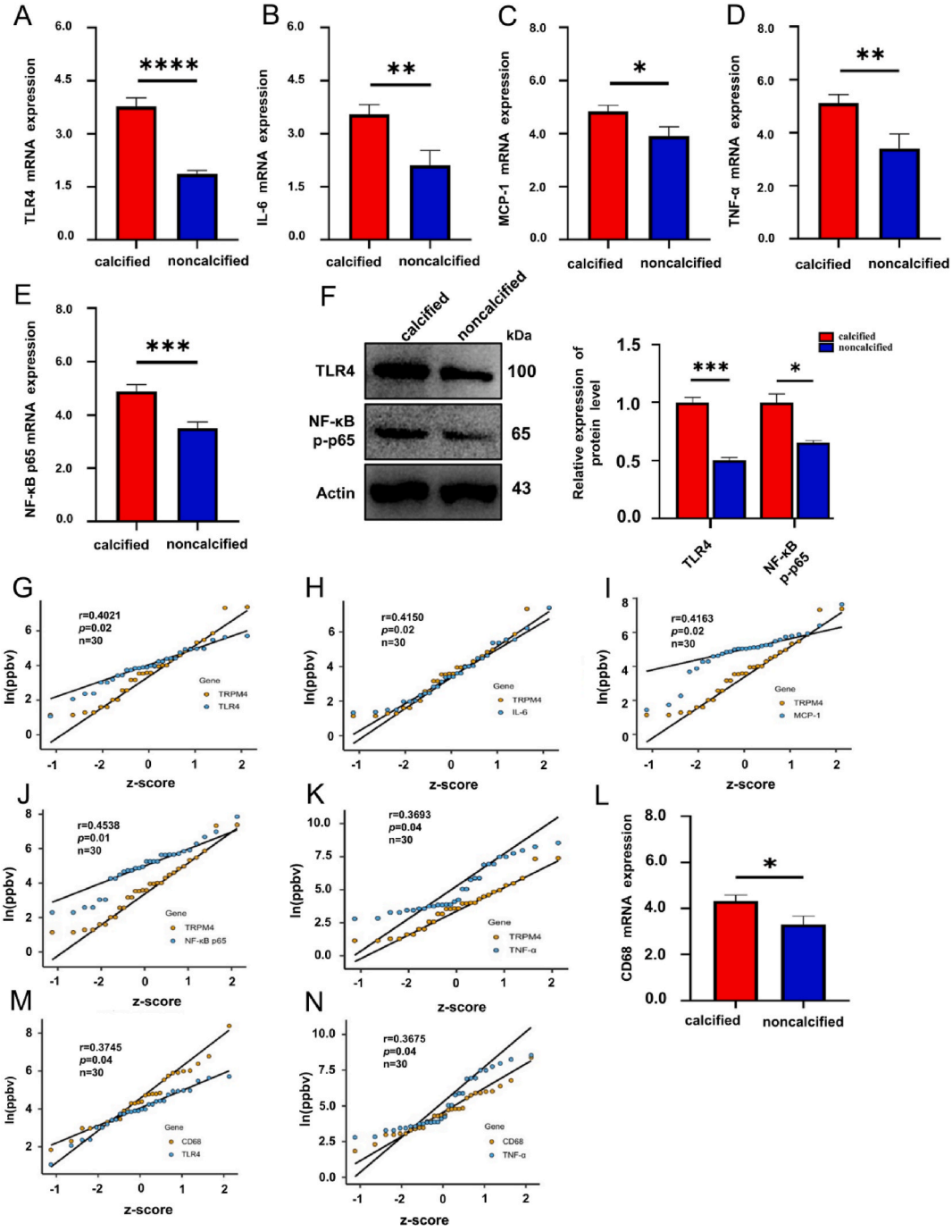

Fig. 2. The correlation of TRPM4 and the key genes of inflammation signal pathway. The expression of mRNA of TLR4 (A), IL-6 (B), MCP-1 (C), TNF-α (D) and NF-κB p-p65 (E) were analyzed by qRT-PCR in calcified and noncalcified aortic valve tissues. (F) Western blot tests were performed to assess the protein levels of TLR4 and NF-κB p65 in aortic valve tissues. Quartile-quartile plots (QQ-plots) showing a relationship between the mRNA expression of TRPM4, TLR4 (G), IL-6 (H), MCP-1 (I), NF-κB p65 (J) and TNF-α (K) taken from calcified aortic valve tissues. (L) The mRNA expression level of CD68 in calcified and noncalcified aortic valve tissues were measured by qRT-PCR. QQ-plots showing a significant positive correlation between the mRNA expression of CD68, TLR4 (M) and TNF-α (N) in calcified aortic valve tissues. Data are presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

experiments.

2.8. RNA stability assays

For the RNA stability assay, the si-METTL3 was transiently transfected into THP-1 cells. After 48 h, the cells were treated with the Actinomycin D (ActD, 5 mg/mL) for the indicated durations before collection. Subsequently, TRPM4 stability in the ActD treatment group was analyzed by qRT-PCR.

2.9. RNA immunoprecipitation assay

TRPM4 could interact potential binding proteins METTL3 and YTHDF1 was performed using the EZMagna RNA immunoprecipitation (RIP) Kit (17–701, Millipore). THP-1 cells were harvested and lysed in complete RIP lysis buffer contains RNase inhibitor. The cell extract was incubated with the anti-METTL3, anti-YTHDF1 or negative control IgG according to the manufacturer's protocol. Finally, coprecipitated RNA was purified and detected the expression of TRPM4 by RT-qPCR.

2.10. RNA pull-down assays

RNA pull-down was performed with the Pierce Magnetic RNA-Protein Pull-Down Kit (20164, Thermo Fisher Scientific, Rockford, IL, USA); biotin-labeled TRPM4 and antisense strands RNA were transcribed and purificated, and incubated with cellular protein extracts from THP-1 cells. RNA-protein complexes were eluted, denaturation, and processed by SDS/PAGE according to the manufacturer's protocol. METTL3 and YTHDF1 levels were detected by western blotting.

2.11. m6A RNA immunoprecipitation followed by quantitative realtime-PCR

The Magna m6A MeRIP Kit (17–10499, Millipore) was used to determine the quantification of m6A-modified TRPM4 levels according to the manufacturer's instructions. Total RNA was isolated from THP-1 cells and performed fragment. Then, fragmented RNA was immunoprecipitated by anti-m6A antibody and purified m6A-containing RNA by RNA purification kit. m6A methylation changes in target gene was analyzed via RT-qPCR analysis.

2.12. Statistical analysis

Data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) and SPSS version 19.01 (IBM, Chicago, IL, USA). The mRNA and protein expression levels of the tissues were shown as mean ± standard error of the mean (SEM). Student's *t*-test and Wilcoxon rank sum test was used to compare between groups. Pearson correlation was performed to determine associations between different variables. For all analyses, $p < 0.05$ was defined as statistically significant.

3. Results

3.1. TRPM4 is highly expressed in calcified aortic valve tissues

TRPM channels are widely distributed in various tissues in human tissues; among the TRPM family members, TRPM1, TRPM2, TRPM4 and TRPM7 are involved in inflammation and immune responses [23,24]. Obtained from the National Center for Biotechnology Information database, the transcript abundances of TRPM1, TRPM2, TRPM4 and TRPM7 transcript in different human tissues are shown in Fig. 1A–D. The mRNA expression levels of these genes were validated by conducting qRT-PCR on 30 pairs of tissue samples. According to the expression level data (Fig. 1E–H), TRPM4 (3.613 ± 0.614 in calcified group vs 2.507 ± 0.2885 in noncalcified group, $P < 0.05$) was an abundant gene and thus selected as the potential candidate gene for further study. Moreover, the protein level of TRPM4 was significantly higher in the calcified aortic valve tissues than in the noncalcified aortic valve tissues (Fig. 1I). These findings demonstrated that TRPM4 is a gene significantly upregulated in calcified aortic valve tissues.

3.2. Expression of inflammation-related factors in calcified and noncalcified aortic valve tissues

To investigate the role of inflammation in CAVD, we evaluated the mRNA expression levels of TLR4 (3.768 ± 0.250 in calcified group vs 1.864 ± 0.094 in noncalcified group, $P < 0.0001$), IL-6 (3.542 ± 0.284 in calcified group vs 2.102 ± 0.423 in noncalcified group, $P < 0.01$), MCP-1 (4.829 ± 0.231 in calcified group vs 3.905 ± 0.356 in noncalcified group, $P < 0.05$), TNF-α (5.109 ± 0.336 in calcified group vs 3.404 ± 0.543 in noncalcified group, $P < 0.01$) and NF-κB p65 (4.886 ± 0.252 in calcified group vs 3.511 ± 0.225 in noncalcified group, $P < 0.001$) in calcified and noncalcified human aortic valve. As shown in Fig. 2A–E, the expression levels of TLR4, IL-6, MCP-1, TNF-α, and NF-κB p65 and the protein levels of TLR4 and NF-κB p65 were upregulated in calcified aortic valve tissues compared with those in the noncalcified tissues (Fig. 2F). To examine the relationship between the inflammation cytokines and TRPM4, we investigated the correlation among TLR4, IL-6, MCP-1, TNF-α, NF-κB p65, and TRPM4 in calcified aortic valve tissues. The results showed that the mRNA expression level of TRPM4 was positively correlated with TLR4, IL-6, MCP-1, TNF-α, and NF-κB p65 (Fig. 2G–K). These findings demonstrated that TRPM4 is involved in the inflammation process of CAVD.

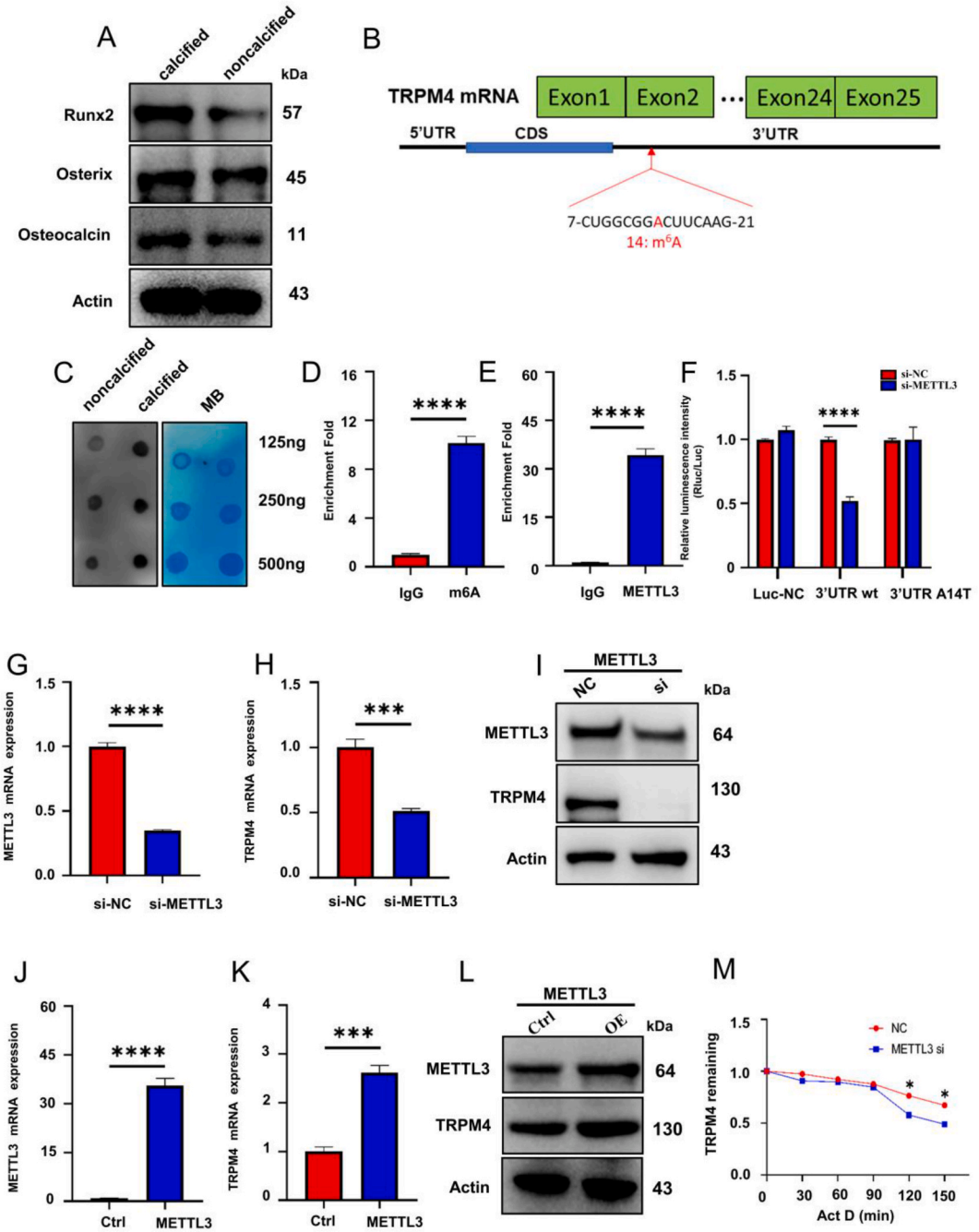

Fig. 3. TRPM4 stability is regulated by METTL3-mediated m6A modification. (A) Results from WB validating the calcification associated genes expression levels in calcified and noncalcified aortic valve tissues. (B) m6A modification sites of TRPM4 mRNA predicted using SRAMP website. (C) mRNA dot blot analysis of m6A levels were performed. (D) Enrichment of m6A-modified TRPM4. (E) The interaction between the METTL3 protein and TRPM4 mRNA was measured by RNA immunoprecipitation (RIP). (F) Dual-luciferase reporter assay of wild-type or site-mutant TRPM4 with or without METTL3 silencing. (G–H) The mRNA expression of METTL3 and TRPM4 in THP-1 cells transfected with the si-METTL3 and Controls by qRT-PCR. (I) The protein levels of METTL3 and TRPM4 in THP-1 cells after transfection with si-METTL3 and Controls by Western blot. (J–K) The mRNA expression of METTL3 and TRPM4 in THP-1 cells transfected with the indicated overexpression plasmids by qRT-PCR. (L) The protein levels of METTL3 and TRPM4 in THP-1 cells after transfection with indicated overexpression plasmids by Western blot. (M) TRPM4 RNA stability in METTL3-knockdown cells treated with Actinomycin D for the indicated periods of time, TRPM4 levels were analyzed by qRT-PCR. Data are presented as mean ± SEM. *$p \leq 0.05$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

3.3. Macrophage infiltration in human aortic valve

inflammation infiltration plays an important role in aortic valve calcification. Thus, we evaluated the expression of macrophages in calcified aortic valve. CD68, a macrophage marker had a higher expression level in the calcified aortic valve than in the noncalcified aortic valve (4.318 ± 0.267 in calcified group vs 3.298 ± 0.374 in noncalcified group, $P < 0.05$) (Fig. 2L). The mRNA expression of CD68 showed a strong positive correlation with TLR4 in the calcified aortic valve (Fig. 2M). In addition, the mRNA expression of CD68

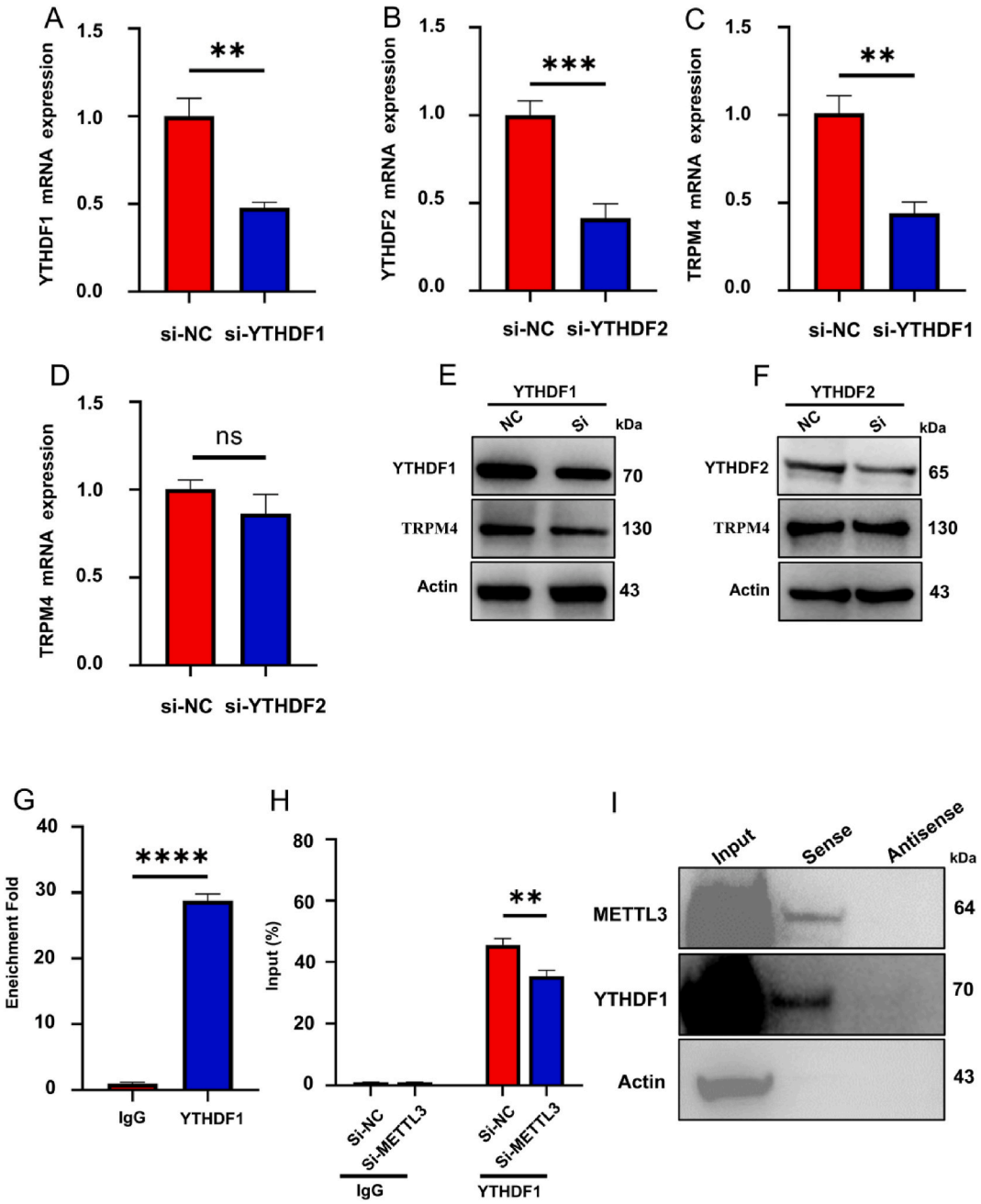

Fig. 4. Increased stability of TRPM4 is mediated by YTHDF1. (A–B) The mRNA expression of YTHDF1 and YTHDF2 in THP-1 cells transfected with the YTHDF1/YTHDF2 knockdown and Controls by qRT-PCR. (C–D) The mRNA expression of TRPM4 in THP-1 cells transfected with the indicated siRNA by qRT-PCR. (E–F) The protein levels of TRPM4 after knockdown of YTHDF1/YTHDF2 in THP-1 cells by Western blot. (G) The interaction between the YTHDF1 protein and TRPM4 mRNA was measured by RIP. (H) The interaction between the YTHDF1 protein and TRPM4 mRNA was measured by RIP in si-METTL3 cells. (I) RNA-pulldown assay showed that METTL3 and YTHDF1 interacted with TRPM4. Data are presented as mean ± SEM. **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

was positively correlated with TNF-α mRNA expression in the calcified aortic valve (Fig. 2N). These findings demonstrated that macrophages may be involved in the development and progression of CAVD.

3.4. TRPM4 is up-regulated in human calcific aortic valves due to m6A modification

The mechanisms leading to aberrant TRPM4 expression in CAVD remain unclear. Some articles reported that m6A plays an important role in CAVD [25]. We examined the three osteogenic markers (Runx2, Osterix, and Osteocalcin) and the level of them were increased in the human calcific aortic valves (Fig. 3A). Online prediction tool SRAMP revealed that consensus m6A site with high confidence was identified in the 3′ untranslated region (3′UTR) of TRPM4 mRNA (Fig. 3B). To assess the relevance of m6A in CAVD, we conducted m6A dot blot analysis and found that the global m6A levels were robustly increased in the human calcific aortic valves (Fig. 3C). Methylated RNA immunoprecipitation (MeRIP) assay confirmed m6A modification in TRPM4 (Fig. 3D). RNA immunoprecipitation (RIP) assay indicated that TRPM4 could bind to METTL3 (Fig. 3E) and RNA pulldown assay revealed that METTL3 interacted with TRPM4 (Fig. 4I). To observe the contribution of the putative m6A site to the regulation of the TRPM4, we introduced a single-site mutations (14A–14T) mut. Luciferase reporter assay indicated that METTL3 silencing decreased the luciferase activity of the wild-type TRPM4 3′UTR reporter (Fig. 3F). Moreover, METTL3 depletion significantly reduced the mRNA and protein levels of TRPM4 (Fig. 3G, H, 3I) and its mRNA half-life (Fig. 3M). Conversely, METTL3 overexpression increased TRPM4 mRNA and protein levels (Fig. 3J, K, 3L). These results showed that METTL3-mediated m6A modification maintains the mRNA stability of TRPM4 in CAVD.

3.5. Increased stability of TRPM4 is mediated by YTHDF1

YTH domain family members mediate the stability of m6A-modified mRNAs. Thus, we assessed the involvement of YTHDF1/2 in

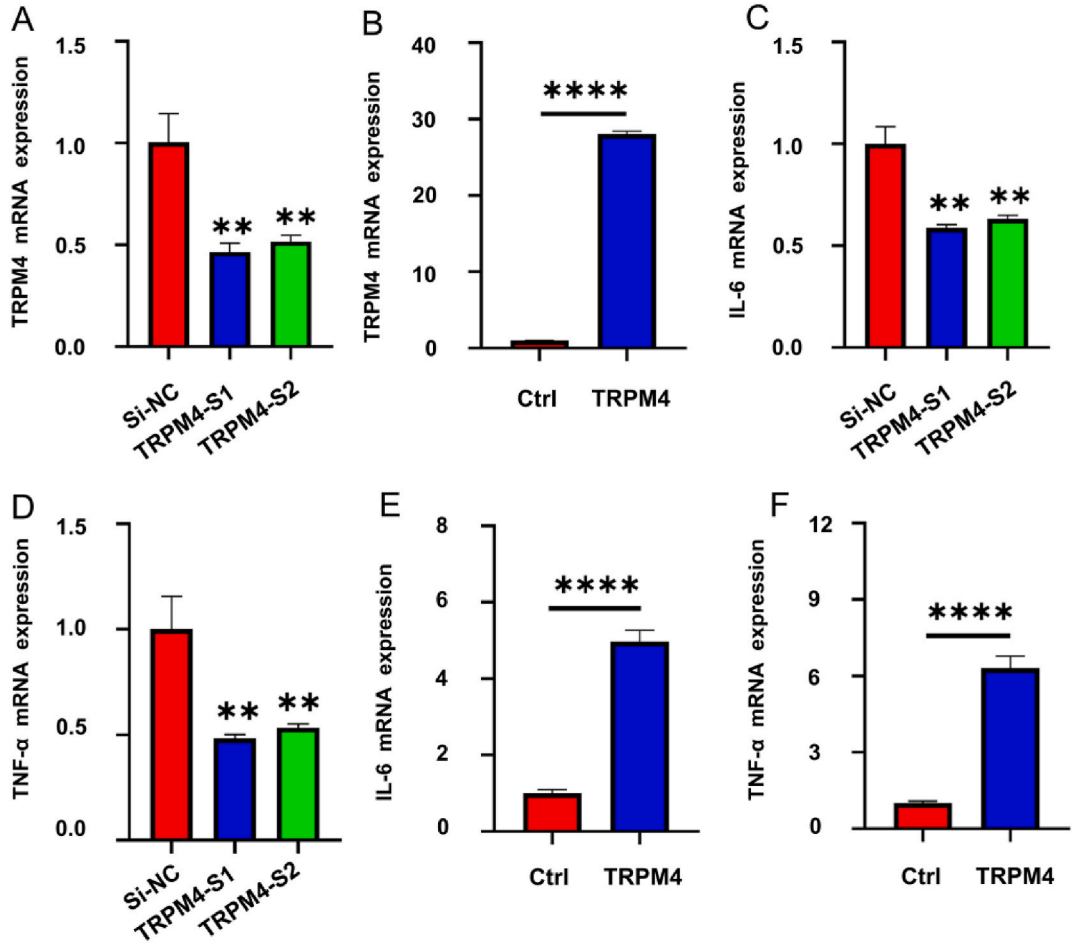

Fig. 5. TRPM4 provokes cellular inflammatory responses. (A–B) The relative mRNA expression of TRPM4 in THP-1 cells transfected with the TRPM4 knockdown or overexpression by qRT-PCR. (C–D) The mRNA expression of IL-6 and TNF-α in THP-1 cells transfected with the indicated siRNA by qRT-PCR. (E–F) The mRNA expression of IL-6 and TNF-α in THP-1 cells transfected with the indicated overexpression plasmids by qRT-PCR. Data are presented as mean ± SEM. **$p \leq 0.01$ and ****$p \leq 0.0001$.

TRPM4 mRNA stabilization and confirmed the knockdown efficiency of YTHDF1 and YTHDF2 (Fig. 4A–B). We also found that YTHDF1 knockdown markedly suppressed TRPM4 mRNA expression, but YTHDF2 knockdown had a limited effect (Fig. 4C–D). Western blot analysis indicated that instead of YTHDF2, YTHDF1 knockdown inhibited TRPM4 protein expression (Fig. 4E–F). RIP assay showed that YTHDF1 directly interacted with TRPM4 mRNA (Fig. 4G) and this interaction was inhibited by METTL3 silencing (Fig. 4H). In addition, RNA-pulldown assay indicated that TRPM4 could bind to YTHDF1 (Fig. 4I). The above data showed that the increased stability of TRPM4 induced by m6A modification is mediated by YTHDF1.

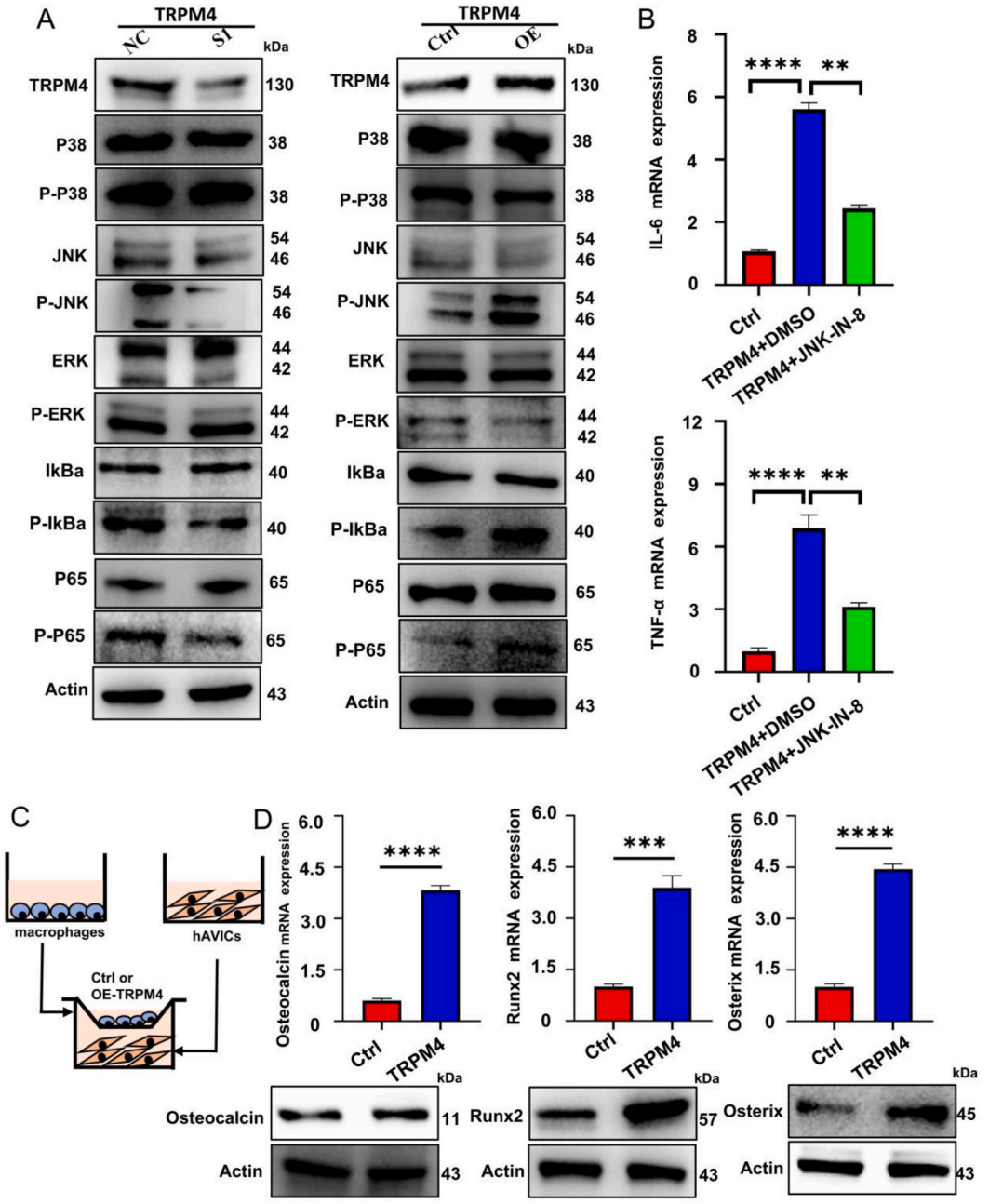

Fig. 6. Effects of TRPM4 on MAPKs–NF–κB signaling pathway in THP-1 cells. (A). The protein levels of members of the MAPKs–NF–κB signaling pathway was examined in transfected TRPM4 siRNA or TRPM4 overexpression plasmids. (B). The effect of TRPM4 upregulation on IL-6 and TNF-α expression was supressed by treatment with JNK–IN–8 (10 μM) were determined by qRT-PCR. (C). Schematic diagram of THP-1 cells transfected with TRPM4 overexpression plasmids and co-cultured with hAVICs. (D). The mRNA and protein levels of Runx2, Osterix, and Osteocalcin were determined by qRT-PCR and WB. Data are presented as mean ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

3.6. Knockdown of TRPM4 inhibits inflammation responses

To further determine the proinflammatory properties of TRPM4 in THP-1 cells, we used two independent TRPM4-specific small interfering RNAs (siRNAs) to knockdown the RNA level of TRPM4 and applied overexpression plasmids for TRPM4 to increase the RNA of TRPM4 (Fig. 5A–B). TRPM4 knockdown significantly decreased the levels of IL-6 and TNF-α (Fig. 5C–D) and TRPM4 overexpression significantly promoted the levels of IL-6 and TNF-α (Fig. 5E–F). All these findings suggested that TRPM4 acts as an proinflammatory driver in the development and progression of CAVD.

3.7. TRPM4 activates the inflammation through JNK-MAPK signaling pathway in THP-1 cells

Studies have reported that the MAPK–NF–κB inflammation signaling pathway is involved in aortic valve calcification [26]. To explore the effects of TRPM4 on the MAPK–NF–κB signaling pathway, TRPM4 knockdown significantly decreased the phosphorylation levels of JNK, IκBα and p65 (Fig. 6A). However, TRPM4 overexpression significantly promoted the phosphorylation levels of JNK, IκBα and p65. Besides, the increased level of IL-6 and TNF-α induced by the TRPM4 was diminished by treatment with JNK–IN–8, an inhibitor of the JNK-MAPK signaling pathway (Fig. 6B). Next, we assessed procalcification effects of overexpressing TRPM4 macrophages on human aortic valve interstitial cells (hAVICs) in a co-culture system using the transwell approach (Fig. 6C). As shown in Fig. 6D, co-culture of hAVICs with overexpressing TRPM4 macrophages increased expression of Runx2, Osterix, and Osteocalcin. These results indicate that TRPM4 promoted the level of inflammation via activition of the JNK-MAPK signaling pathway, and overexpressing TRPM4 macrophages promote a procalcification phenotype in hAVICs.

4. Discussion

CAVD is a multifactorial disease, and its pathogenesis is complex and closely related to lipoprotein deposition, oxidation, inflammation, and osteogenic transformation of heart valve interstitial cells [27]. Some risk factors have been identified, but the molecular mechanisms of aortic valve calcification remain unclear. Currently, pharmacotherapy is ineffective in slowing the progression of CAVD [28]. In this study, we showed that TRPM4 was upregulated in calcified aortic valve tissues and increased TRPM4 levels in human calcified aortic valves were associated with inflammation-related factors. Targeting METTL3 increases m6A-modified TRPM4 mRNA in a YTHDF1-dependent manner leading to TRPM4 upregulation. Moreover, TRPM4 knockdown significantly reduced the expression of inflammatory cytokines TNF-α and IL-6. These findings suggested that TRPM4 acts as an proinflammatory driver contributing to CAVD.

The Na+, K+, and Ca2+ channels have been extensively studied in using electrophysiological, biochemical, and molecular biology methods, and their channel modulators have been used as antiarrhythmic or antihypertensive drugs. Recently, a new participant regulating Ca^{2+} channel has been discovered in the expanding family of transient receptor potential (TRP) cation channels. TRPM4 is a $Ca^{(2+)}$-activated non-selective cation channel, which is activated by an increase in intracellular $Ca^{(2+)}$ [29]. Currently, with the increasing characterization of TRPM4 knockout mouse models and the use of patch-clamp techniques, there is clear evidence indicating that TRPM4 plays a crucial role in cardiac conduction and vascular function. Our research has found high expression of TRPM4 in calcified aortic valve tissues. However, the reason behind the elevated expression of TRPM4 in calcified aortic valve tissues is the first research point in this study.

Rencetly, epigenetics has been demonstrated to be closely related to the development of CVADs, and RNA modification, which is an emerging field of epigenetics, has been gradually studied [30]. Hundreds of modifications of RNA were reported in regulating its decay, stability, translation and splicing, among them, m6A is recognized as the most abundant post-transcriptonal modification in eukaryocytes. The methyltransferase complex (METTL3, METTL14 and WTAP). demethylases (ALKBH5 and FTO) as well as “readers” (YTHDF1-3, YTHDC1-2) dynamically mediate the RNA m6A modification. In this study, we confirmed the m6A modification in TRPM4, and through RIP, RNA pull-down, and dual-luciferase assays, we demonstrated that METTL3 regulates the m6A modification of TRPM4. As is well known, in the m6A methyltransferase complex, METTL3 specifically recognizes the RRACH sequence in RNA and catalyzes the conversion of adenosine to m6A, with METTL3 being a key enzyme in this complex. Currently, a retrospective study by Li et al. has summarized the role of METTL3 in CAVD by mediating m6A modification of key RNAs implicated in CAVD, promoting their stability and upregulation of expression, such as NLRP1 and STAT1 [31].

After METTL3 mediates the m6A modification of TRPM4 mRNA, exerting the m6A effect requires the involvement of m6A-binding proteins, known as “readers”. m6A RNA-binding proteins recognize and bind to m6A methylation sites, with the most prominent m6A RNA-binding proteins being members of the YTH family. Our study found that the increased stability induced by m6A modification of TRPM4 is mediated by YTHDF1, which has been reported to increase ribosome occupancy and enhance translation efficiency by directly interacting with translation initiation factors [32]. Similarly, YTHDF1 plays a crucial role in CAVD. For instance, Han et al. reported that ALKBH5 enhances YTHDF1 mRNA stability in an m6A-dependent manner, promoting YAP translation, thereby enabling cardiomyocytes to re-enter the cell cycle and proliferate [33]. Wang et al. also documented YTHDF1-mediated m6A methylation regulating cardiovascular cell differentiation [34]. These studies further corroborate our findings that YTHDF1 promotes TRMP4 translation in an m6A-dependent manner, resulting in its upregulation in CAVD tissues.

CAVD is the result of a chronic interaction among inflammation, fibrosis, and calcification [27]. In our study, we showed that the cytokine levels of IL-6, TNF-α and MCP-1 increased in aortic valve tissue samples. As an M1 marker, the mRNA expression level of CD68 was higher in calcified aortic valve than in noncalcified aortic valve, suggesting the presence of inflammatory infiltrates in the aortic valve. These findings also determined that the mRNA expression levels of TLR-4, and TNF-α were significantly positively

correlated with CD68 mRNA in calcified aortic valve tissues, implicating the macrophage as the protagonist in this increased inflammatory activation. Increasing evidence suggests that the vital role of inflammation in the initiation and propagation phases of the CAVD [35], especially in the function of Toll-like receptors (TLRs). Thus, we evaluated the expression levels of TLR4, NF-κB p65, and critical pro-inflammatory cytokines. Our results demonstrated that the expression levels of TLR4, IL-6, MCP-1, TNF-α, and NF-κB p65 were elevated in calcified aortic valve tissues. These results suggest that the aortic valve of patients with CAVD is subjected to the infiltration of inflammatory factors, and inflammation plays an important role in the pathology of CAVD.

TRPM4 is highly expressed in some organs and is involved multiple diseases [36–39]. It participates in the physiological responses of immune cells, such as T cells, mast cells, and dendritic cells, when concert with the Ca2+ release activates Ca2+ channels [40–42]. The lack of TRPM4 can affected macrophage population and subsequently increased the production of monocytes and proinflammatory cytokines [11]. In addition, TRPM4 is a novel pharmacological target to treat oxidative stress-mediated inflammatory diseases [43]. In this study, we revealed that TRPM4 was significantly highly expressed in calcified aortic valve tissues and was significantly positively correlated with TLR4, IL-6, MCP-1, TNF-α, and NF-κB p65. We also confirmed by RNA pull-down and RIP-qPCR that TRPM4 combined with METTL3/YTHDF1 to increase stability. TRPM4 knockdown markedly reduced the production of inflammatory factors in THP-1 cells. How TRPM4 participates in the regulation of inflammatory cytokines is what we next concern.

With regard to the above results, macrophage was regarded as the protagonist in the increased inflammatory activation in CAVD. Also, MAPK–NF–κB inflammation signaling pathway is involved in aortic valve calcification [26]. The NF-κB and MAPK signaling pathways are crucial pathways in anti-inflammatory effects. It has been reported that various genes can activate the p38 and JNK signaling pathways through the MAPK cascade, leading to the production of inflammatory and apoptotic factors [44]. Our results showed that TRPM4 promoted the phosphorylation levels of JNK, IκBα and p65, which was reversed by the inhibitor of the JNK-MAPK signaling pathway, JNK–IN–8. Meanwhile, the transwell assay of co-culture system of hAVICs with overexpressing TRPM4 macrophages increased expression of Runx2, Osterix, and Osteocalcin revealed the macrophages promote a procalcification phenotype in hAVICs. Macrophages are the primary responders to inflammation, and induce immune responses to endogenous and exogenous stimuli, playing a central role in host defense and immune regulation.However, the specific mechanism underlying between the TRPM4 and procalcification phenotype in hAVICs is still need to be explored.

Also, there're some limitations in this study. On the one hand, the in vivo study for further proving the METTL3/TRPM4/JNK/MAPK/NF-κB signaling pathway axis to regulate inflammation involving calcific aortic valve disease in order to avoid the communicate or cross-talk in regulating the cellular response to TRPM4. Also, the mechanism of TRPM4 promoting the hAVICs' calcification development is also needed to be investigated.

5. Future perspectives

Over the past two decades, there has been significant progress in our understanding of CAVD. The pathological cells and molecular mechanisms involved in CAVD include extracellular matrix degradation, abnormal matrix deposition, fibrosis, mineralization, inflammation, lipid accumulation, and neovascularization. However, transferring these findings into targeted therapies for CAVD remains challenging. Thanks to new multi-omics approaches, we will be able to greatly enhance our understanding of the molecular and cellular pathways involved in this multifactorial disease, enabling us to identify new targets for drug therapy and appropriate follow-up in CAVD. Currently, targeting CAVD mainly involves (1) lipid-lowering approaches, (2) treatment targets for aortic maladaptive remodeling, and (3) multi-omics searching for new treatment targets for CAVD progression [45]. In this study, we explored the expression and function of TRPM4 in patients with CAVD through various methods, finding that TRPM4 is highly expressed in CAVD and emphasizing the METTL3-mediated TRPM4 m6A modification, which promotes inflammation by regulating the JNK/MAPK pathway, leading to aortic valve calcification. This discovery supplements research on treatment targets for aortic maladaptive remodeling, and future validation of TRPM4 as a target to promote CAVD progression is still required.

6. Conclusion

TRPM4 expression was significantly upregulated in CAVD due to METTL3-mediated m6A modification. A significantly positive correlation was observed between TRPM4 expression and critical pro-inflammatory related cytokines in calcified aortic valve tissues. TRPM4 activates the inflammation through JNK-MAPK signaling pathway in THP-1 cells. Therefore, novel TRPM4 possibly regulates aortic valve inflammation and may be become a potential new biomarker and therapeutic target in CAVD.

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CRediT authorship contribution statement

Jianguo Wu: Writing – review & editing, Writing – original draft, Project administration, Conceptualization. **Haozong Huang:** Methodology, Investigation, Conceptualization. **Wenkai Yang:** Validation, Resources. **Tufeng Xue:** Funding acquisition, Formal analysis. **Wenjuan Wang:** Software. **Guang-Di Zheng:** Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31871.

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