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SMYD1 modulates the proliferation of multipotent cardiac progenitor cells derived from human pluripotent stem cells during myocardial differentiation through GSK3β/β-catenin&ERK signaling

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Abstract

Background The histone-lysine *N*-methyltransferase SMYD1, which is specific to striated muscle, plays a crucial role in regulating early heart development. Its deficiency has been linked to the occurrence of congenital heart disease. Nevertheless, the precise mechanism by which SMYD1 deficiency contributes to congenital heart disease remains unclear.

Methods We established a SMYD1 knockout pluripotent stem cell line and a doxycycline-inducible SMYD1 expression pluripotent stem cell line to investigate the functions of SMYD1 utilizing an in vitro-directed myocardial differentiation model.

Results Cardiomyocytes lacking SMYD1 displayed drastically diminished differentiation efficiency, concomitant with heightened proliferation capacity of cardiac progenitor cells during the early cardiac differentiation stage. These cellular phenotypes were confirmed through experiments inducing the re-expression of SMYD1. Transcriptome sequencing and small molecule inhibitor intervention suggested that the GSK3β/β-catenin&ERK signaling pathway was involved in the proliferation of cardiac progenitor cells. Chromatin immunoprecipitation demonstrated that SMYD1 acted as a transcriptional activator of GSK3β through histone H3 lysine 4 trimethylation. Additionally, dual-luciferase analyses indicated that SMYD1 could interact with the promoter region of GSK3β, thereby augmenting its transcriptional activity. Moreover, administering insulin and Insulin-like growth factor 1 can enhance the efficacy of myocardial differentiation in SMYD1 knockout cells.

Conclusions Our research indicated that the participation of SMYD1 in the GSK3β/β-catenin&ERK signaling cascade modulated the proliferation of cardiac progenitor cells during myocardial differentiation. This process was partly reliant on the transcription of GSK3β. Our research provided a novel insight into the genetic modification effect

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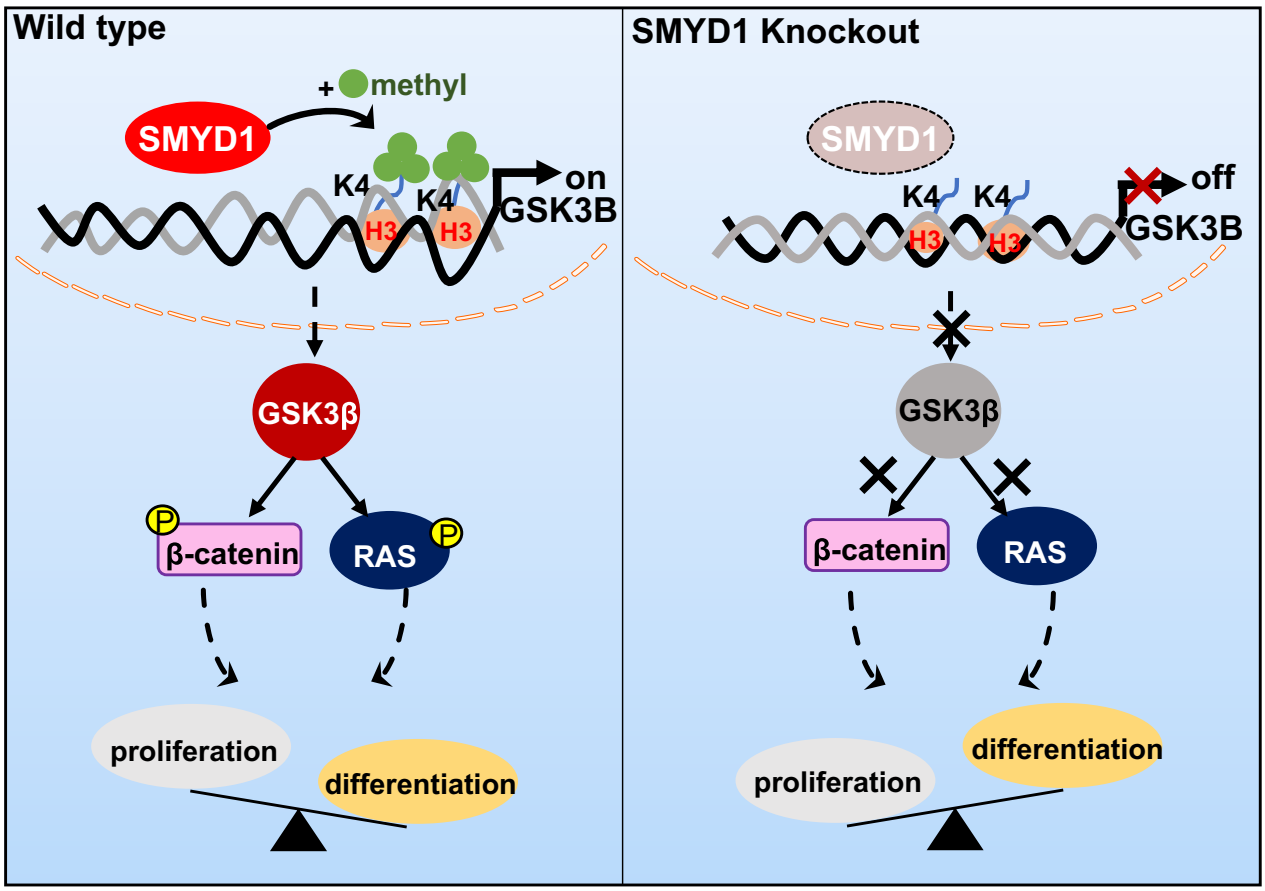
of SMYD1 during early myocardial differentiation. The findings were essential to the molecular mechanism and potential interventions for congenital heart disease.

Keywords SMYD1, Human pluripotent stem cells, Myocardial differentiation, Histone modification, GSK3β

Graphical abstract



Background

Congenital heart disease (CHD) is the most common birth defect, affecting approximately 1.24% of newborns and accounting for 28% of major congenital anomalies [1, 2]. Growing evidence suggests that epigenetic modification plays a vital role in CHD [3, 4]. Chromatin remodeling and gene activation or silencing are crucial in controlling cardiac gene expression [5, 6]. For example, histone H3 lysine 4 (H3K4) methylation links to actively transcribed genes and is highly enriched at the promoters of genes related to cardiac development [7]. However, little is known about the association between histone modification abnormalities and CHD.

SET and MYND domain containing 1 (SMYD1) is a histone-lysine *N*-methyltransferase specific to striated muscle, playing a crucial role in the development of the heart [8]. Mice lacking *Smyd1* gene expression perish at embryonic day 9.5 due to insufficient development of the right ventricle [9]. A patient harboring a homozygous mutation in the *SMYD1* gene presented with a coronary left ventricular fistula and patent foramen ovale [10]. Prior research has demonstrated that Smyd1 orchestrates early heart development by activating the Isl1 promoter through trimethylation of H3K4 (H3K4me3) and suppressing Anf expression via histone deacetylase [11]. In addition, SMYD1 can modulate heart energy metabolism and myocardial contractility [12–14]. Furthermore, overexpression of SMYD1 protects the heart from ischemic injury [15]. However, myocardial differentiation is a prerequisite for cardiac development and

function. The molecular mechanism underlying SMYD1 function for the regulation of myocardial differentiation remains unclear. As gastrulation-stage human embryos are not obtainable and there are differences between human and mouse species [16], cardiomyocytes derived from human pluripotent stem cells (hPSCs) are a better option for studying SMYD1 functions in early cardiac differentiation.

The cardiomyocytes derived from hPSCs can be produced massively and manipulated easily in vitro, and they have been extensively used in studying cardiac diseases [17, 18]. The differentiation of hPSCs into cardiac cells mimics the early embryonic stages of cellular lineage specification [19, 20]. It is thus considered a suitable model for studying the molecular mechanism of early cardiogenesis. During hPSCs differentiation from primitive mesoderm into cardiac lineages, cardiac progenitor cells (CPCs) are the primary source of cardiomyocytes. Canonical Wnt/β-catenin signaling is an essential regulator of CPCs, which is responsible for their proliferation and differentiation in vivo and in vitro [21]. GSK3β plays a vital role in the Wnt/β-catenin signaling pathway, facilitating myocardial differentiation [22, 23]. Inhibition of GSK3β in vitro enhances the proliferation of cardiovascular progenitor cells derived from hPSCs [24].

In this study, we established a SMYD1-deficient cardiomyocyte model derived from hPSCs to investigate the specific role of SMYD1 during early myocardial differentiation. The absence of SMYD1 disrupted the expression of CPCs stage markers NKX2-5 and ISL1 and promoted excessive CPCs proliferation, significantly reducing myocardial differentiation efficiency. Furthermore, the doxycycline-inducible SMYD1 re-expression balanced the proliferation of CPCs and improved the efficiency of myocardial differentiation. Transcriptome sequencing and chromatin immunoprecipitation (ChIP) demonstrated that SMYD1 activated the transcription of GSK3β through H3K4me3 to regulate GSK3β/β-catenin&ERK signaling involved in myocardial differentiation. Dual-luciferase analyses further showed that SMYD1 interacted with the promoter region of GSK3β and enhanced the transcription of GSK3β. Insulin and Insulin-like growth factor 1 (IGF-1) restored myocardial differentiation efficiency in the SMYD1-deficient cell line. We believe that elucidating the molecular mechanisms that underlie SMYD1 regulation of myocardial differentiation will contribute to our understanding of the mechanisms of CHD.

Methods

Cell culture and myocardial differentiation

This study was approved by the Ethics Committee of Beijing Anzhen Hospital (2016015). Healthy human urine cells were isolated and then reprogrammed into human induced pluripotent stem cells using the Reproeasy hiPSC reprogramming kit (Cellapy, Beijing, China), following the acquisition of informed consent. The hPSCs, including human induced pluripotent stem cells and human embryonic stem cells H9 (WiCell Research Institute: WAe009-A-94, Wisconsin, USA) were maintained in PSCeasy medium (Cellapy) daily. Chemically defined small molecules were utilized to induce the differentiation of hPSCs into cardiomyocytes through modulation of the Wnt/β-catenin pathway [25]. Cardiomyocyte differentiation was carried out using the human cardiomyocyte differentiation kit (Cellapy). CardioEasy medium, abbreviated as CDM3, contains RPMI 1640 medium, O. sativa-derived recombinant human albumin, and l-ascorbic acid 2-phosphate. hPSCs reach 80–90% confluence before differentiating into cardiomyocytes. At day 0 of myocardial differentiation, hPSCs were cultured in CDM3 medium with 6 μM CHIR99021 for 48 h. On day 2, the medium was changed to CDM3 with 2 μM Wnt-C59 for another 48 h. On day 4, the medium was switched to CDM3. Subsequently, replace CDM3 every 2 days to keep cultivating cardiomyocytes. Typically, contracting cardiomyocytes were observed starting from day 7. HEK-293 T cells (Shanghai Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM containing 10% FBS.

Generation of SMYD1 knockout and doxycycline-inducible SMYD1 expression cell lines

Single-guide RNA (TGAGCGGGCTTATTCCGCAG) was designed using an online tool (http://crispr.mit.edu/). We ligated the sgRNA into the epiCRISPR vector and then electroporated the plasmid mixture into the hPSCs using the nuclear receptor system (Lonza, Germany). The electrotransformed cells were cultured in PSCeasy medium containing 10 μM of Rho kinase inhibitor Y-27632 (MCE, USA) on the first day, followed by 0.3 μg/ml puromycin (Cellapy) selection for 7 days. The positive clones were collected for Sanger sequencing verification.

SMYD1 knockout (KO) cells were plated into 24-well plates at 150,000 cells/well in 0.5 ml of PSCeasy medium. After 24 h, at 70% confluency, they were infected with a mixture of tTS/rtTA lentivirus and Tet-on SMYD1 lentivirus (Vectorbuilder, Guangzhou, China) according to the manufacturer's protocol. The infected cells were sieved with 2 μg/ml puromycin and 200 μg/ml hygromycin (Beyotime, Jiangsu, China) for 14 days. Positive clones were picked out for subsequent experiments. The expression of SMYD1 was induced by adding 2 μg/ml doxycycline (MCE).

RNA extraction and quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA was extracted with TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA was reverse-transcribed into cDNA using the Prime-ScriptTM reverse transcription system (Takara, Japan). qPCR was performed using 2×SYBR Master Mix (Takara) on the iCycler iQ5 (Bio-Rad). The relative quantification was calculated using the $\triangle\triangle C_T$ method. All primer sequences are listed in Table 1.

Western blot

The cells were washed with PBS and harvested on ice using RIPA lysis buffer (Beyotime) containing protease phosphatase inhibitor mixture (Beyotime). Cell lysates were placed on ice for 30 min and then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was quantified using the BCA kit (Beyotime). Heat-denatured proteins are separated by SDS-PAGE and transferred to a PVDF membrane (EMD Millipore, Germany). After blocking with 5% skim milk for 1 h at room temperature, the membrane was incubated with the primary and secondary antibodies. All the antibodies used are shown in Table 2.

Immunofluorescence

Cell slides were fixed with 4% paraformaldehyde (Solarbio, Beijing, China) for 15 min, permeabilized with 0.1% Triton X-100 (Sigma, USA) for 15 min, and blocked with 3% BSA (Sigma, USA) for 30 min. The primary antibody was incubated at 4 °C overnight, and the secondary antibody at room temperature in the dark for 2 h. Data were collected using a confocal microscope (Leica DMI 4000B). All the antibodies used for immunofluorescence are shown in Table 2.

Flow cytometry

Dissociated cell suspension was rinsed in PBS, fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.1% Triton X-100 for 15 min, and blocked with 3% BSA at room temperature for 30 min. Cells were incubated with the primary and secondary antibodies for 30 min each. After washing and resuspending with PBS, the samples were assessed using a flow cytometer (EPICS XL, Beckman).

RNA sequencing

Cells were lysed with TRIzol Reagent. The total RNA samples were qualified for library preparation and sequenced on the Illumina platform (Illumina, USA) by Annoroad Gene Technology Corporation (Beijing, China). The differential gene screening of two samples was based on the difference fold (fold change value) and the q value (P value after correction). Data analysis and graphing were performed using R-package.

ChIP-qPCR

ChIP assays were carried out using the ChIP-IT High Sensitivity® kit (Proteintech, USA) as per the manufacturer's description. Briefly, at day 5 of differentiation, wild-type (WT) and SMYD1 KO cells were cross-linked with 1% formaldehyde and sonicated to fragment DNA. DNA–Protein complexes were immunoprecipitated using anti-H3K4me3 (ab8580, Abcam). qPCR was performed using 2×SYBR Master Mix on the iCycler iQ5 Systems (for primer sequences, see Table 1). Results were calculated using normalized to input measurements, and enrichment was calculated according to the $\triangle\triangle C_T$ method.

Luciferase assay

GSK3B and GSK3B-mu (L1–L6) promoter fragments were subcloned into the pGL4.10 reporter vector (Vectorbuilder). HEK293T cells were cultured in 96-well plates to approximately 70% confluency. Subsequently, cells were transfected with Lipo3000 (Invitrogen, USA), SMYD1 plasmid (Vectorbuilder), and GSK3B/GSK3B-mu (L1–L6) plasmid. Luciferase activity was measured after 72 h of transfection using the dual-Luciferase reporter assay system (Promega, USA) with a microwell plate luminescent detector LB960 (Berthold, Germany).

Data analysis and statistics

All experiments were conducted at least three times. All data are presented as the mean ± standard error of

Table 1 Primer sequences used for qPCR

Gene	Forward 5′-3′	Reverse 3′-5′
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
DPPA4	GACCTCCACAGAGAAGTCGAG	TGCCTTTTTCTTAGGGCAGAG
REX1	CGCGGTAACAGGGACAAATGTA	AGCAAACACCTGCTGGACTG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
GSK3B	ATTCCCAGACGCCTGTTACG	TGAGGAGCGCTGTCTGC
ChIP-qPCR	attcccagacgcctgttacg	tgaggagcgctgtctgc

Table 2 Primary and secondary antibodies

Type	Antibody	Manufacturer and catalog number	Species	Application	Dilution
Primary	Anti-OCT4	Santa Cruz sc-9081	Mouse	Immunofluorescence	1:100
	Anti-SSEA4	Abcam ab16287	Rabbit	Immunofluorescence	1:100
	Anti-SMDY1	Abcam ab181372	Rabbit	Western blot	1:1000
	Anti- cTnT	Santa Cruz sc-20025	Mouse	Immunofluorescence Flow cytometry	1:200
	Anti-NKX2-5	Abcam ab205263	Rabbit	Western blot	1:1000
	Anti-ISL1	Abcam ab86501	Mouse	Immunofluorescence Western blot	1:200 1:1000
	Anti-Ki67	Santa Cruz sc-23900	Mouse	Immunofluorescence	1:100
	Anti-β-catenin	Santa Cruz sc-7963	Mouse	Immunofluorescence Western blot	1:100 1:500
	Anti-Ras	Cell Signaling Technology #3965	Rabbit	Western blot	1:1000
	Anti- p-c-Raf (Ser338)	Cell Signaling Technology #9427	Rabbit	Western blot	1:1000
	Anti-p-ERK1/2	Cell Signaling Technology #4370	Rabbit	Immunofluorescence	1:1000
	Anti- ERK1/2	Cell Signaling Technology #4695	Rabbit	Western blot	1:1000
	Anti-GSK3β	Santa Cruz sc-81462	Mouse	Western blot	1:200
	Anti-p-GSK3α/β (Tyr216/Tyr279)	Abcam ab68476	Rabbit	Western blot	1:1000
	Anti-GSK3β (Ser 9)	Abcam ab75814	Rabbit	Western blot	1:1000
	Anti-Caspase 3	Cell Signaling Technology #9662	Rabbit	Western blot	1:1000
	Anti-Cleaved Caspase 3	Cell Signaling Technology #9661	Rabbit	Western blot	1:1000
	Anti-Bax	Cell Signaling Technology #2774	Rabbit	Western blot	1:1000
	Anti-Bcl2	Cell Signaling Technology #15071	Mouse	Western blot	1:1000
	Anti-β-tublin	Santa Cruz sc-166729	Mouse	Western blot	1:500
	Anti-p-JNK	Cell Signaling Technology #4668	Rabbit	Western blot	1:1000
	Anti-JNK	Cell Signaling Technology #9251	Rabbit	Western blot	1:1000
	Anti-p-P38	Cell Signaling Technology #9211	Rabbit	Western blot	1:1000
	Anti-P38	Cell Signaling Technology #9212	Rabbit	Western blot	1:1000
	Anti-p-AKT	Proteintech 66444-1-Ig	Mouse	Western blot	1:1000
	Anti-AKT	Proteintech 10176-2-AP	Rabbit	Western blot	1:1000
	Anti-H3K4me3	Abcam ab8580	Rabbit	ChIP	2ug
	Anti-IgG	Proteintech 30000-0-AP	Rabbit	ChIP	2ug
	Anti-GAPDH	Proteintech 60004-1-Ig	Mouse	Western blot	1:10000
Secondary	IRDye 800CW Goat anti-Rabbit IgG	LI-COR 926-32211	Goat anti- Rabbit IgG	Western blot	1:20000
	IRDye 800CW Goat anti- Mouse IgG	LI-COR 926-	Goat anti- Mouse IgG	Western blot	1:20000
	Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	Abcam ab150077	Goat anti- Rabbit IgG	Immunofluorescence Flow cytometry	1:500 1:3000
	Goat Anti-Mouse IgG H&L (Alexa Fluor® 488)	Abcam ab150113	Goat anti- Mouse IgG	Immunofluorescence	1:500
	Goat Anti-Mouse IgG H&L (Alexa Fluor® 647)	Abcam ab150115	Goat anti- Mouse IgG	Immunofluorescence	1:500
	Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594)	Abcam ab150080	Goat anti- Rabbit IgG	Immunofluorescence	1:500

the mean. A student's t-test or one-way ANOVA was used to determine statistical significance. A value of $P < 0.05$ was considered statistically significant (*), with $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Results

SMYD1 deficiency drastically reduces the myocardial differentiation efficiency of hPSCs

To investigate the impact of SMYD1 on myocardial

differentiation, we employed the CRISPR/Cas9 system to generate SMYD1 KO hPSC lines. The sgRNA designed for targeting the first exon of the *SMYD1* gene is as follows: TGAGCGGGCTTATTCCGCAGTGG (Fig. 1A). Sanger sequencing showed that a total of seven edited SMYD1 homozygous knockout cells were acquired, including three distinct editing methods. One method of editing involved the insertion of two TC bases, leading to the termination of coding at the 43rd codon and potentially resulting in the formation of a truncated glycine protein (Fig. 1B). In cells with 16 deleted bases, a termination codon was observed at the 42nd codon post-editing, predicting the potential formation of a truncated glycine protein (Fig. S1A). Another editing approach involved the deletion of two GC bases, causing coding termination at the fourth codon thereafter and potentially resulting in the formation of a truncated phenylalanine protein (Fig. S1B). These editing methods produced frameshift mutations that resulted in the premature appearance of termination codons, leading to the loss of typical protein function. Among the seven SMYD1 knockout cell lines, one hPSC line (with inserted TC bases) was chosen for further experimentation.

We performed qPCR and immunofluorescence experiments using the WT and SMYD1 KO cell lines for the pluripotency markers (SOX2, DPP4, REX1, SSEA4, and OCT4), and the results showed that there was no significant difference in pluripotency (Fig. S1C, D). Similar to WT, SMYD1 KO cells showed a normal karyotype (Fig. S1E) along with the ability to differentiate into three germ layers (Fig. S1F). As SMYD1 expression gradually increases during myocardial differentiation, we used chemically defined small-molecule differentiation kits to generate cardiomyocytes (Fig. 1C). Western blot results showed that SMYD1 was successfully eliminated at the protein level (Fig. 1D, E). However, compared with WT, the myocardial differentiation efficiency of SMYD1 KO cells decreased significantly at day 15. The immunofluorescence results of myocardial marker TNNT2 supported the phenotype (Fig. 1F, G). Furthermore, flow cytometry results showed that WT's differentiation efficiency was approximately 70%, while SMYD1 KO cells was only 30% (Fig. 1H, I). The above results indicated that SMYD1 deficiency had no significant effect on stem cell pluripotency but drastically decreased cardiomyocyte differentiation efficiency.

SMYD1 deficiency leads to excessive cell proliferation capacity at the early myocardial differentiation stage

Breaking cell specialization stages causes defects in cardiac development [26, 27]. To ascertain the temporal involvement of SMYD1 in myocardial differentiation, protein expression levels of SMYD1 were assessed at four distinct differentiation stages (mesoderm progenitor day 1, mesoderm day 3, cardiac progenitor cell day 5, and immature cardiomyocyte stages day 7). Notably, we observed that SMYD1 begins to express at the CPCs stage (day 5) in WT cells and increases at cardiomyocyte stage, while SMYD1 expression is absent in the KO cells (Fig. 2A, B). During myocardial differentiation, a specific population of CPCs gets enriched in the expression of genes such as NKX2-5 and ISL1 [21]. NKX2-5 is a cardiac transcription factor activated in CPCs during early cardiogenesis [28]. Compared with the WT, the expression of NKX2-5 in the SMYD1 KO cells was delayed and insufficient. We also found that ISL1 had dysregulated expression in SMYD1 KO cells (Fig. 2A, B). These results indicated that the deletion of SMYD1 disturbed the regular cardiomyocyte specialization of CPCs during myocardial differentiation.

Given that cell proliferation and spontaneous apoptosis play essential roles in the differentiation of hPSCs into cardiomyocytes [29]. We subsequently performed EDU labeling and cell apoptosis assay to examine CPCs proliferation and apoptosis at day 5. Interestingly, SMYD1 KO cells exhibited increased proliferation capacity compared to WT cells (Fig. 2C, D). This result was further confirmed by the double immunofluorescence staining of ISL1 and Ki67 (Fig. 2E, F). When compared with WT cells, apoptosis of SMYD1 KO cells increased slightly, as shown by flow cytometry (Fig. S2A, B), but the major apoptotic molecular makers showed no significant difference between WT and SMYD1 KO cells (Fig. S2C, D). The above results suggested that the knockout of SMYD1 reduced cardiomyocyte differentiation, potentially due to heightened proliferation of CPCs rather than CPCs apoptosis.

Restoration of SMYD1 expression rescues myocardial differentiation phenotypes

Subsequently, to confirm whether SMYD1 deficiency increased the proliferation of CPCs and reduced the efficiency of myocardial differentiation, we established doxycycline-inducible SMYD1 expression hPSCs based on SMYD1 knockout line (Fig. 3A). Doxycycline was added or not at 48 h before CPCs stage (day 5). Western blot results showed that the doxycycline-inducible SMYD1 cell line was successfully generated (Fig. 3B, C). Additionally, the immunofluorescence and flow cytometry results showed that the restoration of SMYD1 expression improved the efficiency of myocardial differentiation in SMYD1 KO cells (Fig. 3D–G). Furthermore, correction of SMYD1 deficiency reversed the excessive proliferation of CPCs compared with SMYD1 KO cells (Fig. 3H, I). Immunofluorescence staining of ISL1 and Ki67 also supported this phenomenon (Fig. 3J, K). The

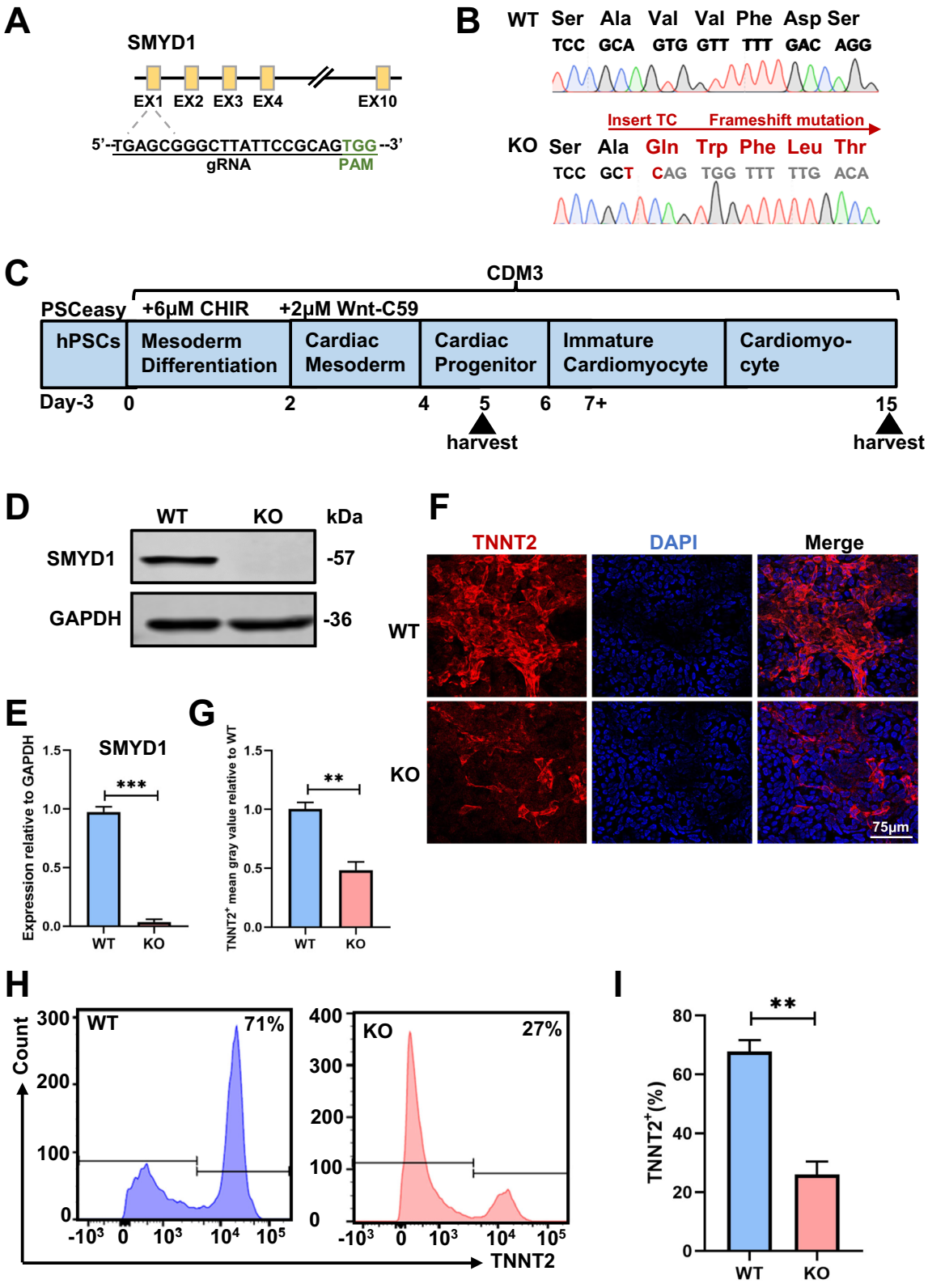


Fig. 1 SMYD1 deficiency drastically reduces the myocardial differentiation efficiency of hPSCs **A** Diagram of the sgRNA positions targeting SMYD1. **B** TC bases insertion resulting in frameshift mutation. **C** Schematic representation of hPSCs myocardial differentiation stages **D**, **E** Western blot of SMYD1 protein at day 15, quantification of protein expression was normalized by GAPDH. **F**, **G** Immunostaining of TNNT2 in WT and SMYD1 KO cardiomyocytes at day 15. Scalebar, 75 μm. **H**, I Flow cytometry analysis for TNNT2 in WT and SMYD1 KO cardiomyocytes at day 15

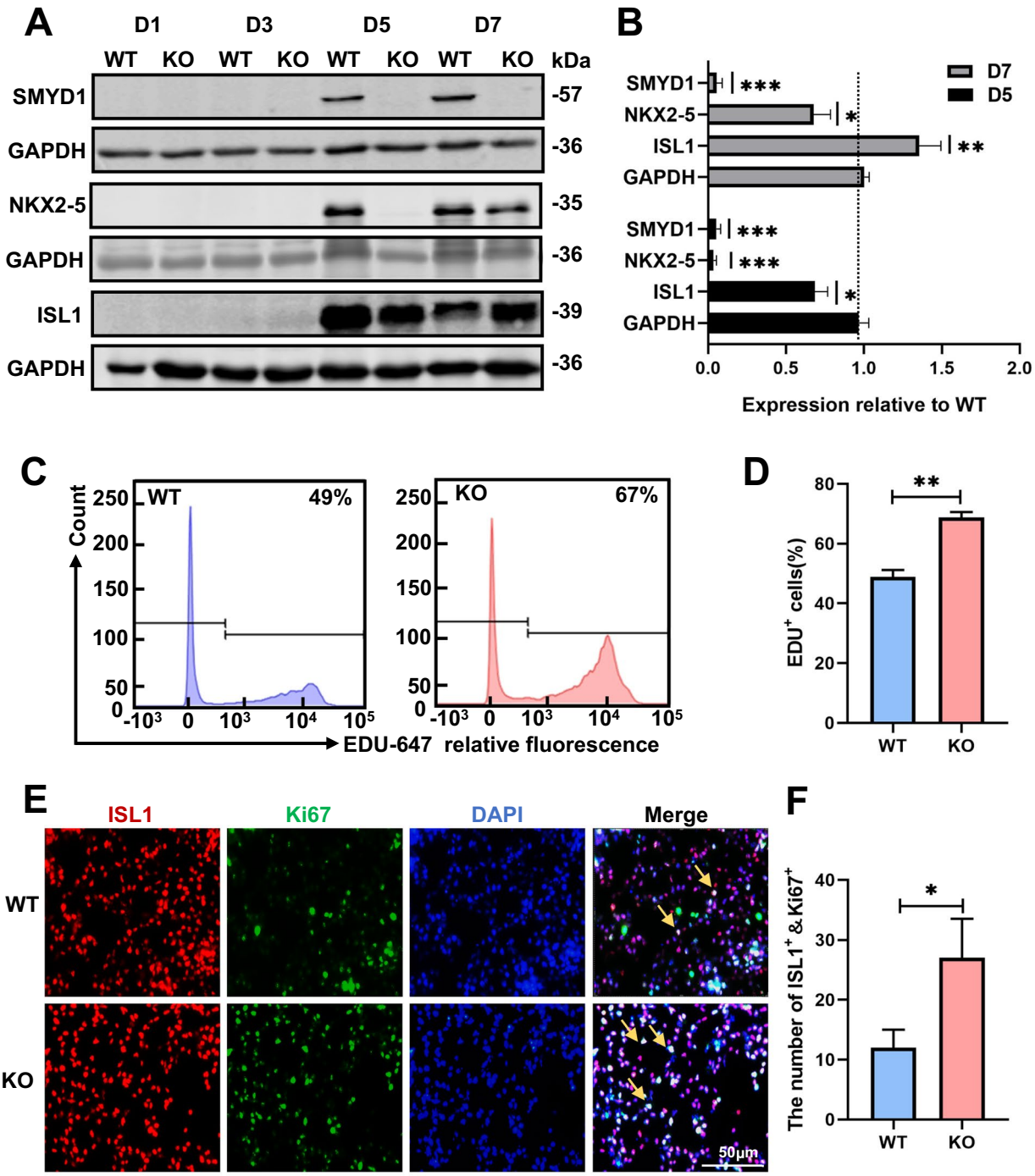


Fig. 2 SMYD1 deficiency leads to excessive cell proliferation capacity during the early myocardial differentiation stage. **A**, **B** Western blot showing SMYD1, NKX2-5 and ISL1 in WT and SMYD1 KO cells at day 5, quantification of protein expression was normalized by WT. **C**, **D** Flow cytometry analysis for EDU^+ in WT and SMYD1 KO cells at day 5. **E**, **F** Representative immunostaining for ISL1 and Ki67 expression in WT and SMYD1 KO cells at day 5. Scalebar, 50 μm

results demonstrated that doxycycline-inducible SMYD1 expression confirmed that the deficient myocardial differentiation and abnormally increased early-phase proliferation capacity in SMYD1 KO cells were not artifacts of genome editing or cellular subcloning.

SMYD1 participates in myocardial differentiation regulation by suppressing the Wnt/β-catenin&ERK signaling pathways

To investigate the regulatory effects of SMYD1 on myocardial differentiation, we performed a global transcriptomic analysis of WT and SMYD1 KO cells during the CPCs stage (day 5). Compared with WT, SMYD1 KO cells have a total of 3,766 differentially expressed genes, including 1,931 upregulated genes and 1,835 downregulated genes (Fig. 4A). Further, KEGG pathway analysis revealed multiple proliferation–differentiation-related signaling pathway enrichments, such as Wnt/β-catenin, MAPK, PI3K/AKT, and RAS-ERK signaling pathways (Fig. 4B). These results implied that SMYD1 may be involved in myocardial differentiation via a specific signaling pathway. Subsequently, we examined certain key molecules in several signaling pathways. Western blot results showed that the expression of p-AKT/AKT, p-JNK/JNK, and p-P38/P38 was comparable to that of the WT (data not shown). Previous studies demonstrated that the RAS-ERK pathway mediates various biological processes, including cell proliferation, differentiation, survival, and motility [30, 31]. Western blotting analysis further demonstrated that the RAS-ERK signaling pathway is involved in early myocardial differentiation. The levels of RAS, p–c-Raf, and its downstream protein p-ERK/ERK were noticeably increased in SMYD1 KO cells (Fig. 4C, D). In addition, the Wnt/β-catenin signaling pathway was also upregulated in KO cells with increased β-catenin protein (Fig. 4C, D). As expected, doxycycline-inducible SMYD1 expression reversed the abnormal activation of the Wnt/β-catenin and RAS-ERK signaling pathways (Fig. 4C, D). Immunofluorescence data further supported the above results (Fig. 4E). The above results suggested that SMYD1 participates in early myocardial differentiation by regulating the Wnt/β-catenin and RAS-ERK signaling pathways.

We considered whether the SMYD1 KO phenotype could be rescued via pharmaceutical interventions in the Wnt/β-catenin and RAS-ERK signaling pathways. At day four of differentiation, 5 μM CHIR-99021 (MCE, HY-10182), the Wnt/β-catenin signaling activator, and 5 μM IWP2 (MCE, HY-13912), the inhibitor of Wnt/β-catenin signaling, were added to cells, respectively. After 24 h of incubation, the culture medium was replaced with fresh, drug-free media. The subsequent flow cytometry results showed that CHIR-99021 suppressed the myocardial differentiation efficiency of WT cells, with no notable impact on SMYD1 KO cells, whereas IWP2 promoted the myocardial differentiation efficiency of SMYD1 KO cells (Fig. 4F, G). Similarly, 10 μM Ro-677476 (MCE, HY-100403), the RAS-ERK signaling activator, and 5 μM LY3214996 (MCE, HY-101494), the inhibitor of RAS-ERK signaling, were added to cells. Flow cytometry results showed that LY3214996 enhanced myocardial differentiation efficiency in SMYD1 KO cells (Fig. 4H, I). Protein expression further confirmed the effective intervention of the drugs (Fig. 4J-M). Thus, SMYD1 regulated early myocardial differentiation by suppressing the Wnt/β-catenin and RAS-ERK signaling pathways.

SMYD1 acts as a transcriptional activator of GSK3β via Histone H3 Lysine 4 Trimethylation

Previous studies have reported that SMYD family members exert their regulatory functions by methylating H3K4 [32]. It has been reported that Smyd1 regulates cardiac energy via H3K4me3 at the Pgc-1α promoter [14]. GSK3β kinase-mediated phosphorylation of β-catenin and RAS critically influences the cross-talk between Wnt/β-catenin and RAS-ERK pathways [33, 34]. We observed reduced proteins and mRNA in GSK3β in SMYD1 KO cells, whereas re-expression of SMYD1 rescued GSK3β expression (Fig. 5A-C). Many ChIP sequencing studies have shown enrichment of H3K4me3 within the GSK3β locus (WashU EpiGenome database) (Fig. 5D). Thus, we performed a ChIP experiment with H3K4me3-specific antibodies on WT and SMYD1 KO cells at day 5 of differentiation. The results showed that H3K4me3 enrichment within the GSK3β locus was significantly reduced in SMYD1 KO cells (Fig. 5E). These results suggested that SMYD1 regulated GSK3β expression by modulating chromatin accessibility at the promoter.

Additionally, it has already been reported that SMYD1 binds directly to the promoter region of Isl1

(See figure on next page.)

Fig. 3 Restoration of SMYD1 expression rescues myocardial differentiation phenotypes. **A** Pattern of doxycycline-inducible SMYD1 expression. **B**, **C** Western blot of SMYD1 at day 5 of differentiation, quantification of protein expression was normalized by GAPDH. **D**, **E** Immunostaining for TNNT2 in WT, SMYD1 KO and doxycycline-inducible SMYD1 at day 15. Scalebar, 50 μm. **F**, **G** Flow cytometry analysis for TNNT2 in WT, SMYD1 KO and doxycycline-inducible SMYD1 at day 15. **H**, **I** Flow cytometry analysis for EDU^+ in WT, SMYD1 KO and doxycycline-inducible SMYD1 cells at day 5. **J**, **K** Proliferation marker Ki67 and cardiac progenitor cells marker ISL1 of cell immunofluorescent staining at day 5. Scalebar, 50 μm.

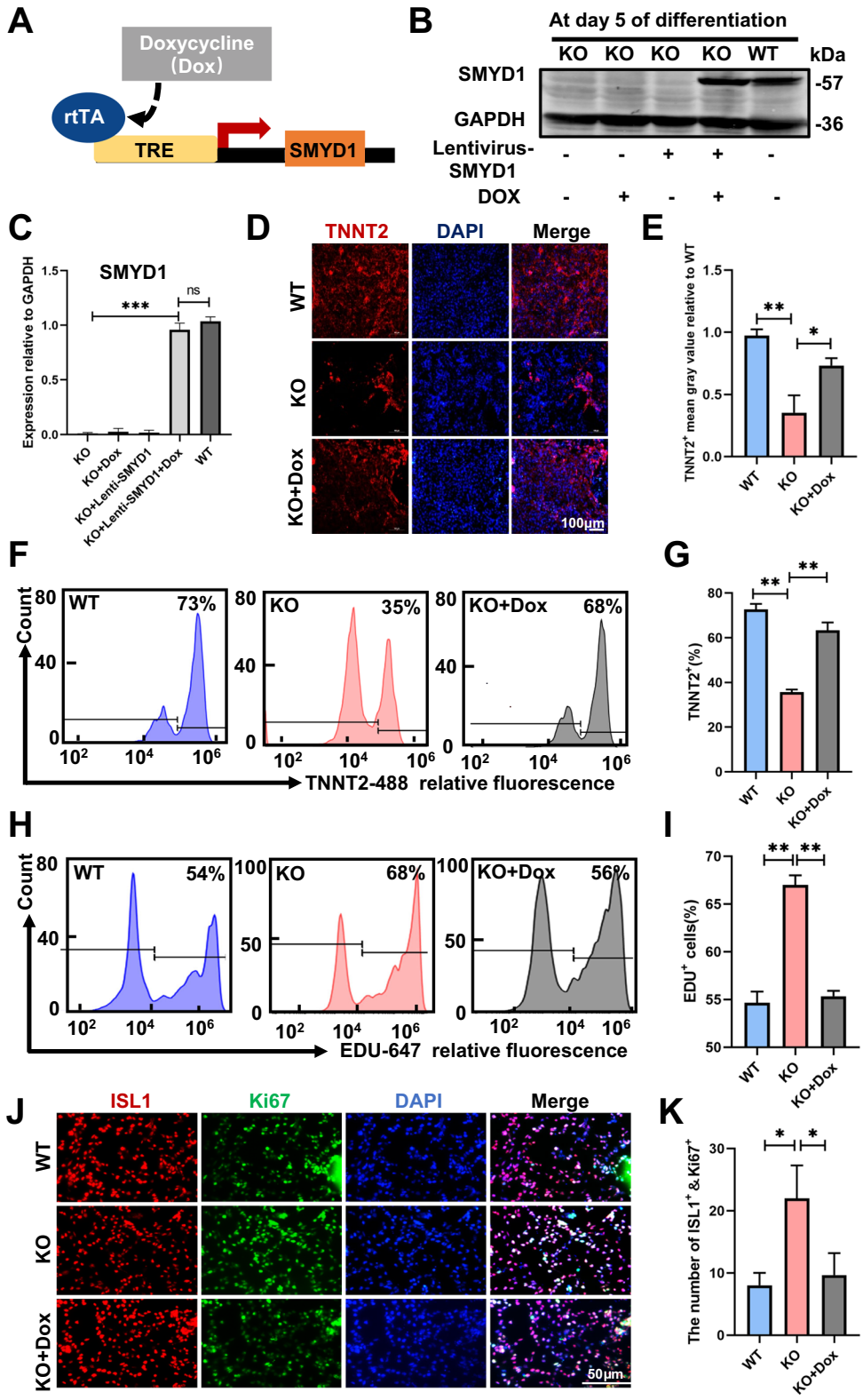


Fig. 3 (See legend on previous page.)

and Pgc-1α in the heart. To identify potential binding motifs for SMYD1, we analyzed the 1-kb GSK3β promoter sequence and identified two "CCCTCC" motifs and four "GGAGGG" motifs [11, 35]. To further study these potential binding sites, we mutated the six motifs and named them mu-L1 to mu-L6, respectively (Fig. 5F). Dual-luciferase analyses confirmed that SMYD1 interacted with the GSK3β promoter region and enhanced GSK3β transcription. Of these sites, we observed the most potent inhibition at mu-L5, suggesting that mu-L5 is essential for SMYD1-dependent transcriptional activity of GSK3β (Fig. 5G). Overall, these findings demonstrated that SMYD1 acted as a transcriptional activator of GSK3β through H3K4me3, thereby promoting myocardial differentiation.

Insulin/IGF-1 improves myocardial differentiation of SMYD1-deficient cells

Generally, administering insulin during the early stages of myocardial differentiation may lead to a decrease in myocardial differentiation[36]. Nonetheless, it has been observed that insulin can enhance myocardial differentiation in SMYD1 knockout cells. At day 4 of differentiation, 10 μg/ml insulin or 100 ng/ml IGF-1 were added to cells, respectively. Following incubation for 24 h, the culture media was replaced with a fresh, drug-free media. The immunofluorescence results revealed that insulin/IGF-1 strikingly improved the myocardial differentiation efficiency of SMYD1 KO cells at day 15 (Fig. 6A, B). Consistent results were confirmed using flow cytometry (Fig. 6 C, D). Subsequent protein assay results revealed that the intervention of insulin/IGF-1 reduced the expression of p-ERK in SMYD1 knockout cells, similar to WT (Fig. 6E, F). The above results showed that insulin and IGF-1 could reduce downstream molecule p-ERK expression in SMYD1 KO cells, rescuing the myocardial differentiation deficiency in SMYD1 KO cells.

Discussion

In this study, we generated SMYD1-deficient human myocardial differentiation cell models to explore the reason for SMYD1 mutation in causing CHD. The results showed that SMYD1 deficiency led to cardiac dysplasia-associated phenotypes, including dysregulated CPCs proliferation at the early differentiation stage and decreased cardiomyocytes at the late differentiation stage. Further transcriptome sequencing and small molecule inhibitor experiments proved that the GSK3β/β-catenin&ERK signaling pathway was involved in myocardial differentiation in SMYD1 deletion. The ChIP and luciferase analyses showed that SMYD1 regulated the activation of the GSK3β promotor region via H3K4me3. Additionally, insulin/IGF-1 improved myocardial differentiation efficiency in SMYD1 knockout cells. Overall, our studies have clarified the initial evidence regarding the biological significance of SMYD1 in the myocardial differentiation process, particularly in the activation of GSK3β. These results revealed the molecular mechanism involved in CHD caused by SMYD1 dysfunction.

The role of SMYD1 in regulating cardiac development has been previously described in animal models [37]. However, the mechanism by which myocardial differentiation, a pre-condition for cardiac development, is regulated by SMYD1, remains unknown. Due to the difficulties in detecting myocardial differentiation in vivo, hPSCs provide a better platform for further research on myocardial differentiation in vitro. To understand the underlying mechanisms of SMYD1 dysfunction on myocardial differentiation, we established SMYD1- knockout and doxycycline-inducible SMYD1 human cardiomyocyte models. Compared with WT, SMYD1-deficient cells showed low efficiency in myocardial differentiation, a result that is consistent with the ventricle hypoplasia observed in SMYD1 deletion mice. The correct development of cell populations into specific lineages is a sure indicator for differentiating hPSCs into cardiomyocytes. NKX2-5 and ISL1, as CPCs markers, directly control cardiomyocyte identity and CPCs differentiation [38]. In general, the duration of the cardiac progenitor cell phase is brief yet crucial. Deletion of SMYD1 caused a delay in the expression of NKX2-5 and ISL1, suggesting that SMYD1 knockout may prolong the cardiac progenitor cell phase, as evidenced by heightened proliferation of cardiac progenitor cells. Although previous work has revealed that SMYD1 conditioned KO mice embryonic heart exhibited a decrease in proliferation, this reduction mainly occurred in the outflow tract [39]. It is posited that the observed phenotypic variance could potentially be attributed to disparities in the temporal detection of cardiac progenitor cell proliferation in vivo versus

(See figure on next page.)

Fig. 4 SMYD1 participates in myocardial differentiation regulation by suppressing GSK3β/β-catenin&ERK signaling **A** The volcano plot shows up- and down-regulated genes in SMYD1 KO compared with WT at day 5. Red denotes upregulated and blue denotes down-regulated genes, $p < 0.05$. **B** The top 10 significantly enriched KEGG pathways. **C**, **D** Western blot of Wnt/β-catenin and RAS-ERK signaling pathway-related proteins at day 5, quantification of protein expression was normalized by GAPDH. **E** Immunostaining for β-catenin and p-ERK in WT, SMYD1 KO cells and doxycycline-inducible SMYD1 cells at day 5. Scalebar, 100 μm. **F**–**I** Flow cytometry for comparisons of $TNNT2^{+}$ after drug intervention at day 15. **J**–**M** Western blot of β-catenin and p-ERK/ERK proteins after drug intervention, quantification of protein expression was normalized by GAPDH

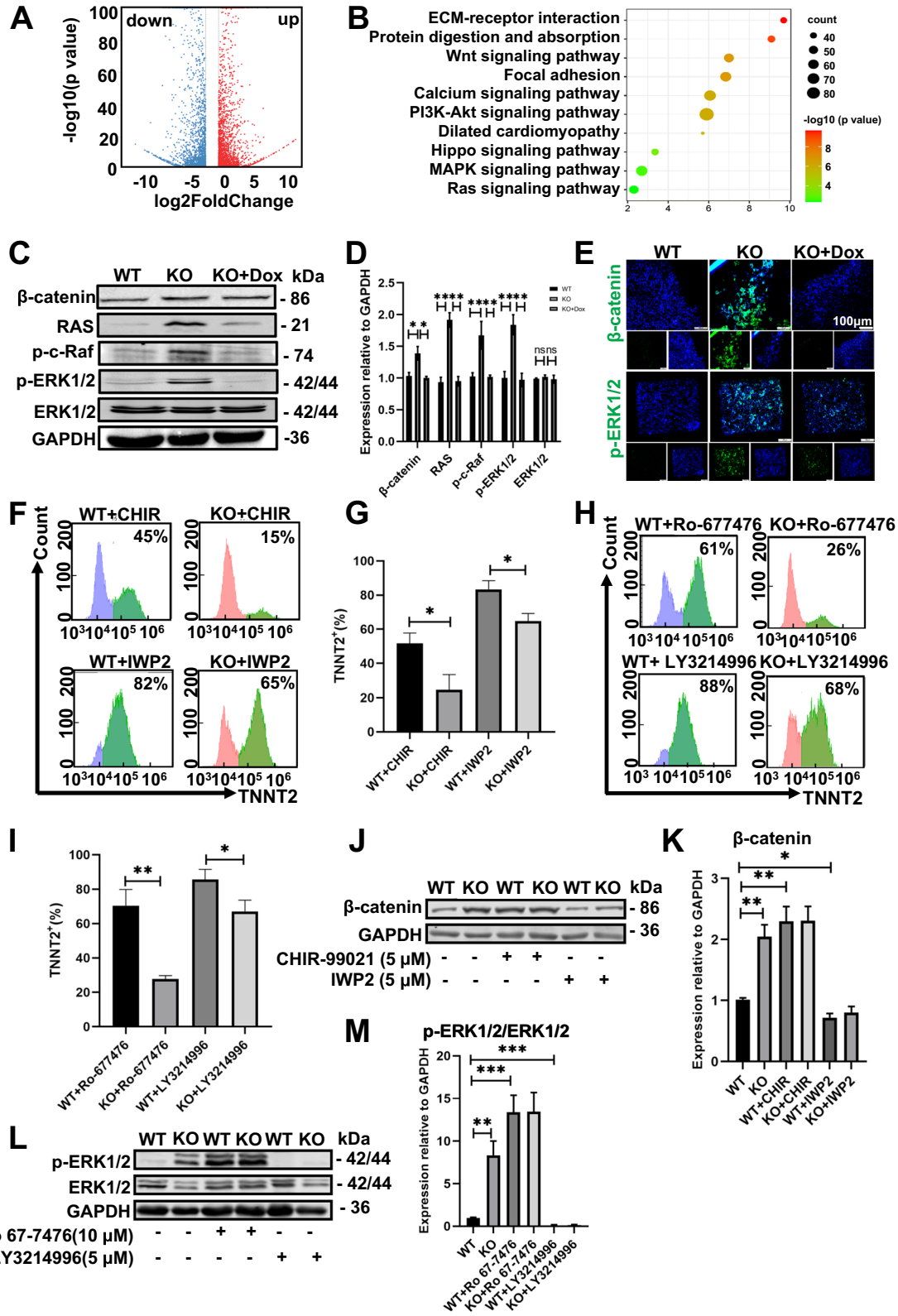


Fig. 4 (See legend on previous page.)

in vitro. Specifically, cardiac progenitor cells are observed to migrate laterally towards the cephalic fold on embryonic day 7.25, subsequently coalescing to form a crescent-shaped heart by embryonic day 7.5. SMYD1 expression initiates on embryonic day 7.75, coinciding with forming a crescent-shaped population of cardiac progenitor cells. In vivo detection of cardiac progenitor cell proliferation occurs on embryonic day 9.5, concurrent with the development of cardiac tubules and the initiation of cardiac chamber formation. Our observation of increased proliferation capacity in SMYD1 knockout cardiac progenitor cells corresponds to the developmental stage of embryonic day 7.75, as opposed to embryonic day 9.5. At day 5 of differentiation, the double immunofluorescence staining of ISL1 and Ki67 supported the results.

Wnt/β-catenin signaling regulates the proliferation and differentiation of CPCs in myocardial differentiation [21]. Our transcriptome analysis suggested that differentially expressed genes were enriched in multiple proliferation-differentiation-related pathways. Notably, the Wnt/β-catenin and RAS-ERK signaling pathways played roles in cardiomyocyte differentiation in SMYD1 KO cells. Our data indicated that activated Wnt/β-catenin signaling blocked myocardial differentiation in SMYD1 deficiency at day 5. Using small molecule inhibitors to control canonical Wnt signaling at certain times during cardiac differentiation reveals that excessive Wnt signaling on day 5 leads to decreased cardiomyocyte production on day 15 [40]. The RAS-ERK signaling is a conserved signaling cascade that transmits signals to promote differentiation and proliferation of cells. Our results demonstrated that RAS, p–c-Raf, and p-ERK/ERK expression were increased, and inhibition of the signaling improved the efficiency of myocardial differentiation, suggesting that the RAS-ERK signaling pathway was abnormally activated in SMYD1 KO cells.

Collectively, these data indicated that the GSK3β/β-catenin&ERK signaling is aberrantly activated in SMYD1 KO cells. The abnormal activation of these two pathways is known to occur in various cancer types [41, 42]. Research has shown that Wnt/β-catenin signaling promotes stabilization of RAS by inhibiting RAS phosphorylation on threonine (Thr)-144 and Thr-148 by GSK3β [43]. Similarly, aberrant Wnt/β-catenin signaling in colorectal tumors inhibits GSK3β and prevents RAS degradation [33, 44]. Therefore, we hypothesized that GSK3β, an upstream regulator of the Wnt/β-catenin and RAS-ERK signaling pathways, is controlled by SMYD1 during cardiac cell differentiation. Our results showed that GSK3β was downregulated in SMYD1 KO cells, while SMYD1 compensation rescued the expression of GSK3β and improved cardiac differentiation efficiency. Recent research reveals that SMYD1 can specifically bind to 5'-CCCTCC-3' or 5'-GGAGGG-3' motifs on the promoter region of Isl1 to direct cardiogenesis [11]. Our ChIP and luciferase analyses revealed that SMYD1 activated GSK3β promotor region through the H3K4me3 and L5 site, which was important for SMYD1-dependent transcriptional activity of the GSK3β. These data strongly suggested that SMYD1 was a transcriptional activator of GSK3β.

Insulin is a frequently utilized element in mammalian cell culture. The insulin/IGF-1 signaling pathway is crucial for regulating human stem cell self-renewal [45] and endoderm differentiation [46]. Additionally, various differentiation protocols have demonstrated that insulin signaling negatively impacts the cardiac differentiation of hPSCs [36]. However, small molecule myocardial differentiation protocol based on Wnt signaling showed that insulin does not strongly inhibit human cardiomyocyte generation [40]. Unexpectedly, the presence of insulin/IGF-1 improved the efficiency of myocardial differentiation in SMYD1 knockout cells. In wild-type cells, insulin/IGF-1 stimulation triggered the phosphorylation of ERK, whereas, in SMYD1 knockout cells, insulin/IGF-1 suppressed the upregulation of p-ERK and facilitated myocardial differentiation. This implied that insulin may be involved in the myocardial differentiation of SMYD1 knockout cells via non-traditional signaling mechanisms.

Conclusions

In conclusion, we established SMYD1-deficient and doxycycline-inducible SMYD1 cardiomyocyte models derived from hPSCs to explore the molecular mechanisms of SMYD1-related human CHD. Our work provided a novel insight into the initial evidence regarding the biological significance of SMYD1 in the early myocardial differentiation process.

(See figure on next page.)

Fig. 5 SMYD1 acts as a transcriptional activator of GSK3β through Histone H3 Lysine 4 trimethylation **A**, **B** Western blot of GSK3β and p-GSK3β protein in WT, SMYD1 KO cells and doxycycline-inducible SMYD1 cells at day 5, quantification of protein expression was normalized by GAPDH. **C** Quantified analysis of GSK3B mRNA. **D** H3K4me3 enrichment within promoter regions of GSK3B is predicted in the heart by the WashU EpiGenome Database. **E** ChIP-qPCR revealed that H3K4me3 enrichment was found in the promoter regions of GSK3β in WT cells. **F** Schematic diagram of SMYD1 binding sites during the GSK3B -1 kb promoter region. **G** Luciferase reporter assays using SMYD1 plasmid and GSK3B-wt or mutant promoters

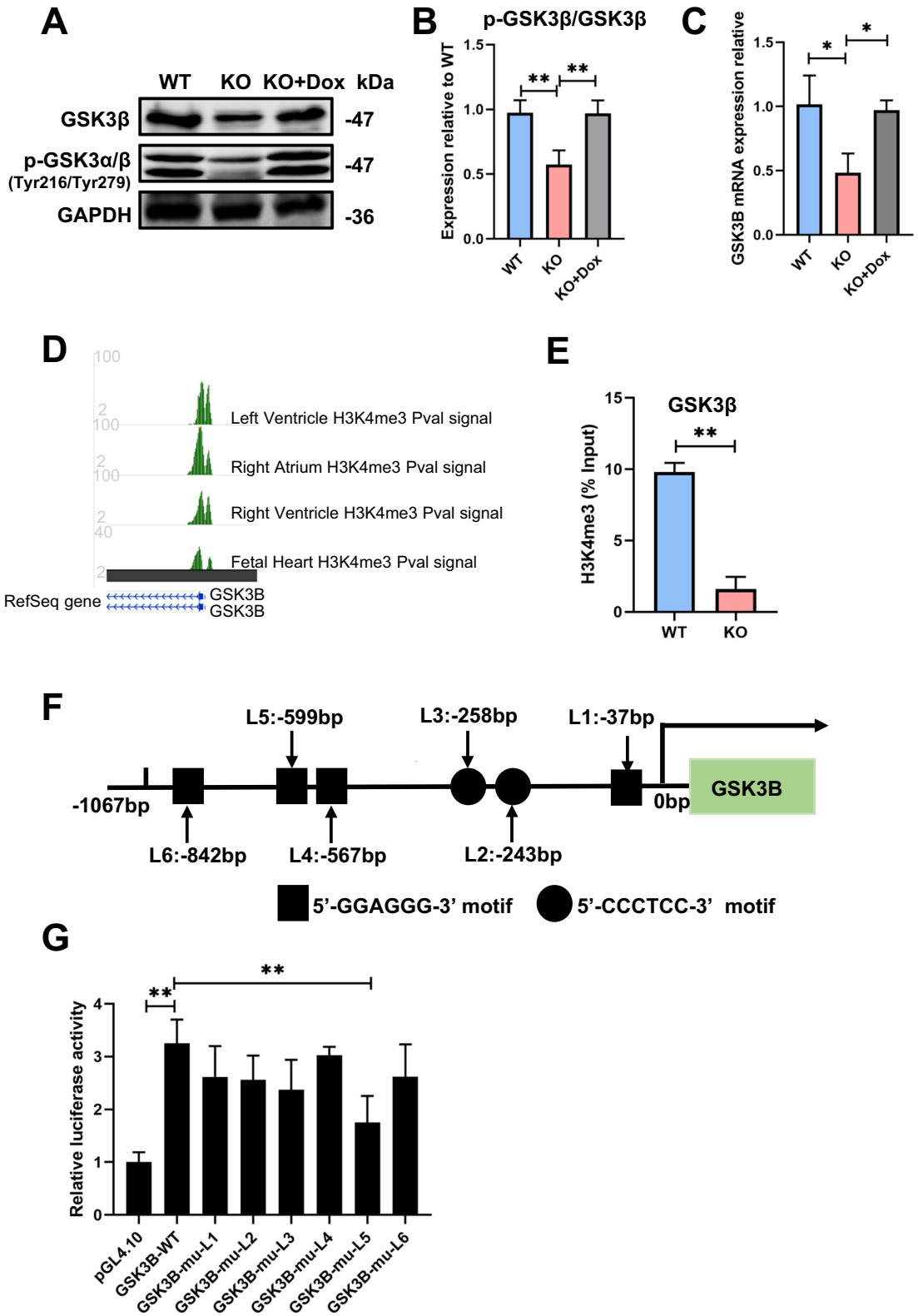


Fig. 5 (See legend on previous page.)

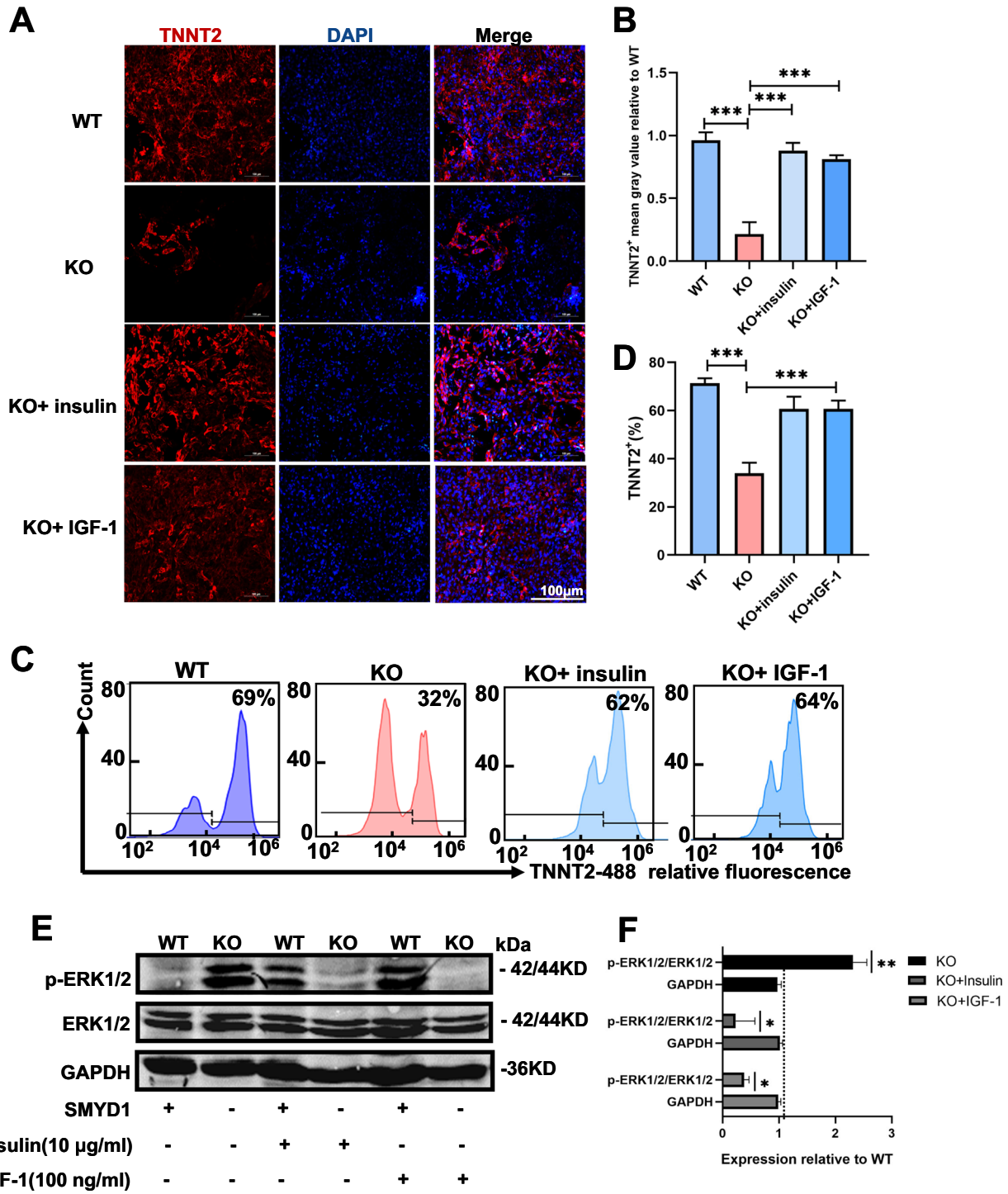


Fig. 6 Insulin/IGF-1 improves myocardial differentiation of SMYD1-deficient cells **A**, **B** Immunostaining for TNNT2 in WT, KO, KO + insulin, or IGF-1 cardiomyocytes at day 15. Scalebar, 100 μm. **C**, **D** Flow cytometry analysis for TNNT2 in WT, KO, KO + insulin, or IGF-1 cardiomyocytes at day 15. **E**, **F** Western blot of p-ERK and ERK protein at day 5, quantification of protein expression was normalized by WT

Abbreviations

CHD	Congenital heart disease
H3K4	Histone H3 Lysine 4
H3K4me3	Trimethylating H3K4
hPSCs	Human pluripotent stem cells
CPCs	Cardiac progenitor cells
WT	Wild type
KO	Knockout

ChIP	Chromatin immunoprecipitation
qPCR	Quantitative real-time polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-024-03899-7.

Additional file 1: Figure 1 SMYD1 knockout does not affect pluripotency of hPSCs a, b Pattern diagram of SMYD1 knockout demonstrating the genic positions of their editing sites. c Quantified analysis of pluripotency markers. d Pluripotency markers SSEA4 and OCT4 of cell lines immunofluorescent staining. Scale bar = 25μm. e Karyotype analysis. f In vitro differentiation of SMYD1 KO into three germ layers. Figure 2 SMYD1 knockout has little effect on apoptosis during myocardial differentiation a, b Flow cytometry analysis for Annexin V-APC/7-AAD in WT and SMYD1 KO at day 5. c, d Western Blot analysis for apoptotic molecules in WT and SMYD1 KO at day 5, quantification of protein expression was normalized by β-tubulin. Figure 3 Full-length blots are presented.

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Not applicable.

Artificial Intelligence
The authors declare that artificial intelligence is not used in this study.

Author contributions
LF and JYX designed this study; CY and BR performed the majority of cell experiments and data analysis. CY provided the manuscript preparation. MSH and ZYS conducted the molecular experiments. ZM contributed to the function analysis. ZYS and LWJ have been helping with revisions. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated during this study are included in this published article [and its supplementary information files]. The datasets used during the current study are available from the corresponding author on reasonable request. The raw data of RNA-Seq data involved in this manuscript have been uploaded to the GEO database, and the accession number is GSE267824.

Declarations

Ethical approval and consent to participate
Human induced pluripotent stem cells were derived from human urine cells with written consent from the donors, and the study was approved by the Ethics Committee of Beijing Anzhen Hospital ("Clinical genetic testing program for hereditary cardiovascular disease" on July 14, 2016, with Registration Number 2016015). Human embryonic stem cells H9 was obtained from the WiCell Research Institute, with ethical approval available at hpscreg (https://hpscreg.eu/cell-line/WAe009-A).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interests.

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