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Lysine methyltransferase Smyd2 suppresses p53-dependent cardiomyocyte apoptosis

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Abstract

Apoptosis, or programmed cell death, is an essential physiological process for proper embryogenesis as well as for homeostasis during aging. In addition, apoptosis is one of the major mechanisms causing cell loss in pathophysiological conditions such as heart failure. Thus, inhibition of apoptosis is an important approach for preventive and therapeutic strategies. Here we show that the histone 3 lysine 4- and lysine 36-specific methyltransferase Smyd2 acts as an endogenous antagonistic player of p53-dependent cardiomyocyte apoptosis. Smyd2 protein levels were significantly decreased in cardiomyocytes upon cobalt chloride-induced apoptosis or myocardial infarction, while p53 expression was enhanced. siRNA-mediated knockdown of Smyd2 in cultured cardiomyocytes further enhanced cobalt chloride-induced cardiomyocyte apoptosis. In contrast, Smyd2 overexpression resulted in marked methylation of p53 and prevented its accumulation as well as apoptotic cell death in an Hsp90-independent manner. Moreover, overexpression, of Smyd2, but not Smyd2Y240F lacking a methyl transferase activity, significantly rescued $CoCl_2$-induced apoptosis in H9c2 cardioblasts. Finally, *Smyd2* cardiomyocyte-specific deletion *in vivo* promoted apoptotic cell death upon myocardial infarction,

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which correlated with enhanced expression of p53 and pro-apoptotic Bax. Collectively, our data indicate Smyd2 as a cardioprotective protein by methylating p53.

Keywords

Smyd2; cardiomyocyte; apoptosis; p53; heart failure

1. Introduction

Apoptosis plays an important role in a variety of diseases including ischemic heart disease, which often leads to heart failure, a major cause of morbidity and mortality worldwide [1]. Thus, it is important to identify new regulators of apoptosis. Smyd2 is a histone 3 lysine 4- and lysine 36-specific methyltransferase, which has been shown to repress p53-mediated apoptosis in response to various types of DNA damage by methylation of the transcription factor p53 at lysine 370 [2,3]. This methylation causes dissociation of p53 from the promoters of its target genes such as p21 (a cyclin-dependent protein kinase important for cell-cycle control) [3].

Smyd2 belongs to the class of histone lysine methyltransferases (HKMTs) that methylate lysine residues on histones and other proteins to contribute to gene regulation. With the exception of the DOT1-like histone H3 methyltransferase, all HKMTs contain a SET (Suppressor of variegation, Enhancer of zeste, Trithorax) domain, required for substrate binding and catalysis [4]. Based on the sequences surrounding the SET domain, HKMTs have been divided into related subfamilies. One of these subfamilies consists of five members (Smyd1-5) and is characterized by inclusion of a MYND (Myeloid, Nervy and DEAF-1) domain containing a zinc-finger motif, which contributes to protein-protein interactions [5]. This zinc finger motif plays an important role for the transcriptional control of cell cycle regulation, differentiation, proliferation and muscle formation [6,7].

Members of the Smyd family exhibit several functions by not only methylating histones but also a number of non-histone proteins. For example, Smyd2 methylates p53 [3], retinoblastoma tumor suppressor (RB) [8], heat shock protein 90 (Hsp90) [9] and estrogen receptor alpha (ERα) [10]. In the heart, the Smyd protein family appears to play an important role. Targeted deletion of the founding member *Smyd1*/*Bop* in mice disturbed maturation of ventricular cardiomyocytes and affected proper right ventricular formation [11]. Subsequently, it has been shown that Smyd1 and Smyd2 play an important role for myofibril organization and contraction of skeletal and cardiac muscle in zebrafish [9,12,13]. Smyd2 is transiently expressed during mouse heart development. However, cardiomyocyte-specific deletion of *Smyd2* has suggested that *Smyd2* is dispensable for proper mouse heart development [14]. Whether Smyd2 plays a role in the pathophysiology of the heart remains unclear. Given that Smyd2 regulates p53-mediated apoptosis and the clear implication of apoptotic regulation in heart disease [15], the aim of this study was to analyze the role of Smyd2 in cardiomyocyte apoptosis. We provide evidence for an endogenous anti-apoptotic role of Smyd2 in cardiomyocytes *in vitro* and *in vivo* identifying Smyd2 as a cardioprotective factor.

2. Material and methods

2.1. Animal model

All investigations conform with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and were approved by the local Animal Ethics Committee in accordance to governmental and international guidelines on animal experimentation (Regierungspräsidium Darmstadt, Hessen, Germany, Gen. Nr. B 2/231). Conditional *Smyd2* knockout (cKO) mice harboring cardiomyocyte specific deletion of *Smyd2* were generated by crossing *Smyd2* floxed mice with mice expressing Cre recombinase under the control of the *Nkx-2.5* promoter as described previously [14]. Mice were subjected to myocardial infarction (MI) by coronary artery occlusion. Sham-operated mice served as controls (SHAM). Mice were euthanized at indicated time points after MI for isolation of total RNA or immunohistochemistry. All surgical procedures were performed as described recently [16]. In brief, mice were anesthetized intraperitoneally by injection of ketamine (100 mg/kg body weight) and xylazine (6 mg/kg body weight). Mice were intubated endotracheally and ventilated with a rodent ventilator (Hugo Sachs Electronics, Mach, Germany). A thoracotomy was performed at the fourth intercostal space. All muscles overlying the intercostal space were laid open and retracted with 5-0 silk threads; the intercostal muscles were transsected. A ligature with a 7-0 prolene thread (Ethicon, Norderstedt, Germany) was placed around the left anterior descending artery just below the atrioventricular border. Discoloration of the ventricle and ECG-changes provided evidence of ischemia. The lung was reinflated and muscle and skin layers were closed separately. The animals were weaned by the respirator and extubated. Sham-operated animals were subjected to similar surgery, except that the ligature was not tied tightly.

2.2. Cardiomyocyte cell culture and induction of apoptosis

Neonatal ventricular cardiomyocytes of Sprague Dawley rats were isolated from either postnatal day 1 or 3 and cultured as described previously [17]. Neonatal cardiomyocytes were cultured for 48 h in the presence of 5% horse serum and 20 μM of cytosine β-D-arabinofuranoside (AraC) (Sigma-Aldrich) before stimulation or adenovirus administration to prevent proliferation of non-myocytes (> 90% cardiomyocytes). Subsequently, cells were washed, serum starved for 12 h for synchronization, and then infected with adenovirus for 48 h. To induce apoptosis, cardiomyocytes were then exposed to hypoxic stress by culturing the cells in medium containing 750 μM Cobalt Chloride ($CoCl_2$) for 24 h (control: diluent DMSO). For inhibition of Hsp90, cardiomyocytes were treated for 1 h with 17-AAG (100 nM) (Alexis) prior to the addition of $CoCl_2$. To analyze protein degradation P1 cardiomyocytes were treated for 24 h with MG-132 (10 μM) (Sigma), which was added one hour before the addition of $CoCl_2$ (750 μM).

2.3. H9c2 cells

H9C2 cells were grown at 37°C in DMEM medium supplemented with 10% fetal bovine serum, 2 mM Glutamax, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere. Cells were transiently transfected using Lipofectamine® LTX reagent

according to the manufacturer's instructions. After 24 h of transfection, cells were treated with 1 mM $CoCl_2$ for 24 h.

2.4. Adenoviruses

Adenoviruses expressing β-Gal and Smyd2 (Applied Biological Materials Inc.) under the control of a CMV promoter were utilized for cardiomyocyte transfection (50 MOI per cell).

2.5. Evaluation of apoptosis

The prevalence of cardiomyocyte apoptosis was assessed utilizing several assays: the CaspACE™ Assay System, Colorimetric (Promega), the Annexin V-Cy3 apoptosis detection kit (BioVision) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining (Calbiochem). All assays were performed according to the instructions of the manufacturers. Counting positive apoptotic cardiomyocyte nuclei was performed in 7 independent fields of the infarct border zone in the left ventricle at 3 distinct levels. To determine the rate of apoptosis in H9c2 cells Annexin V & Dead Cell Assay was performed utilizing Muse™ Cell Analyzer from Millipore following manufacturer's instruction. Briefly, after the indicated treatments, the cells were incubated with Annexin V and Dead Cell Reagent (7-AAD) and the events for dead, late apoptotic, early apoptotic, and live cells were counted.

2.6. RNA isolation and RT-qPCR analyses

Total RNA from paraffin-embedded tissues was isolated from the infarcted portions using the Recover All™ Total Nucleic Acid Isolation Kit according to manufacturer's instructions (Ambion). RNA from neonatal cardiomyocytes or H9c2 cells was extracted using the RNeasy Mini Kit (Qiagen). For Real-Time PCR analysis, cDNA was amplified with IQTM SYBR® Green SuperMix (Biorad) and Bio-Rad iCYCLER iQ5. Following primers were utilized: rat p53 forward: 5′-GACGCTGCCCCCACCATGAG-3′, reverse: 5′-ACCACCACGCTGTGCCGAAA-3′; mouse p53 forward: 5′-AGCAGGGCTCACTCCAGCTACC-3′, reverse: 5′-AGTCAGGCCCCACTTTCTTGACC-3′; rat Smyd2 forward: 5′-CTACCCCGTGTACTCCCTCA-3′, reverse: 5′-CTGGGGTGACTGTGAGGTTT-3′; rat Hif1 forward: 5′-GCCACACTGCGGCTGGTTAC- 3′, reverse: 5′-GCCACACTGCGGCTGGTTAC-3′; 18S RNA forward: 5′-ACCGCAGCTAGGAATAATGGA-3′, reverse: 5′-GCCTCAGTTCCGAAAACCA-3′; rat gapdh forward: 5- CAGAAGACTGTGGATGGCCC-3, reverse: 5-AGTGTAGCCCAGGATGCCCT-3. Real-Time PCR was performed in triplicates and relative gene expression was calculated on the basis of ΔCt values to 18S RNA or *gapdh.*

2.7. Protein analysis

Whole cell lysates (50–100 μg) were separated by SDS-PAGE. Protein concentrations were determined using Biorad DC protein assay (Biorad). Equal amounts of proteins were resolved using NuPAGE Novex Bis-Tris Gels (Invitrogen) blotted onto nitrocellulose membranes and incubated with primary antibodies. Following primary antibodies were utilized: rabbit anti-Smyd2 (1:800), mouse anti-p53 (1C12) (1:1000), rabbit anti-

p53K370me1 (1:1000) [3], rabbit anti-PARP (1:1000), rabbit anti-Hsp90 (1:800), rabbit anti-Bcl2 (1:800) and rabbit anti-Pan-actin (1:2000) (all from Cell Signaling). Antigen-antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibodies (Amersham) and SuperSignal ® ECL detection system (BioRad).

2.8. Immunohistochemistry

H9c2 cells were fixed with 3.7% formaldehyde (Sigma) for 15 min, permeabilized with 0.5% Triton X-100/PBS for 10 min, blocked with blocking buffer (5% goat serum, 0.2% Tween-20 in PBS) for 20 min and incubated for 1 h with anti Myc-Tag (9B11) antibody (Cell Signaling) diluted 1:2000 in blocking buffer. All steps were carried out at room temperature. Either ALEXA 488- or ALEXA 594-conjugated antibodies (1:200, Molecular Probes) were used to detect immune complexes. 4′,6′-diamidino-2-phenylindole (DAPI, Sigma) (0.5 mg/mL PBS) was used to visualize DNA. For immunohistochemical analysis, the slides were deparaffinized and rehydrated. Endogenous peroxidase was blocked by immersing tissue sections in methanol containing 3% H_2O_2, followed by washing in PBS. Non-specific antigens were blocked by incubation of the tissue sections with 2.5% (v/v) horse serum for 20 min. The sections were then incubated for overnight at 4°C with rabbit polyclonal anti-Bax antibody (Santa Cruz). After extended washing in PBS, primary antibody staining was visualized using the VECTASTAIN ABC Kit with Nova RED (Vector Laboratories). Tissue sections were counterstained with haematoxylin, dehydrated and mounted.

2.9. siRNA interference

siRNA-mediated gene knockdown was performed by using 50 nM of siRNAs against Smyd2 and p53. As negative control the AllStars Negative Control siRNA (Qiagen) was utilized. siRNAs were transfected into freshly isolated neonatal cardiomyocytes by electroporation (Amaxa) [18]. siRNAs were designed and synthesized by Qiagen against Smyd2 mRNA (Smyd2: 5′-CAG GAA CGA CCG GTT AAG AGA -3′) and p53 mRNA (Tp53: 5′-CAC GTA CTC AAT TTC CCT CAA-3′).

2.10. Statistical analyses

For immunofluorescence analyses, 200 to 300 cardiomyocyte nuclei per experiment were counted. Data are expressed as mean ± SEM of at least three independent experiments. The data were analyzed using GraphPad Prism (version 4.00, GraphPad Software, Inc.). The statistical significance of differences was evaluated by either a Student’s t-test or one-way ANOVA for multiple comparisons followed by Bonferroni’s post hoc test. A difference of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Smyd2 is downregulated upon apoptosis induction

Smyd2 is expressed in postnatal cardiomyocytes [14] and is known to inhibit p53-mediated apoptosis by methylation upon DNA damage [3]. To test the hypothesis that Smyd2 regulates p53-mediated apoptosis in cardiomyocytes, apoptosis was induced by $CoCl_2$ [15,19]. $CoCl_2$ treatment resulted in significant downregulation of Smyd2 protein expression

(Fig. 1A, B). Consistent with the induction of apoptosis, $CoCl_2$ resulted in a marked accumulation of p53 and downregulation of poly ADP ribose polymerase (PARP), a DNA-binding enzyme involved in DNA damage processing and apoptosis [20,21] (Fig. 1A, B). In addition, Hsp90, which can promote pro- as well as anti-apoptotic effects [19,22], was downregulated. To better understand how Smyd2 expression is regulated upon apoptosis induction we measured RNA expression levels (Fig. 1C) and utilized the proteasome inhibitor MG-132 (Fig. 1D). Our data suggest that Smyd2 expression upon $CoCl_2$-induced apoptosis is regulated at the transcriptional level but also through a proteasome-dependent pathway.

To determine if downregulation of Smyd2 before induction of apoptosis enhanced apoptosis, we targeted Smyd2, and as a control p53, with siRNA. Western Blot analyses confirmed high efficiencies of both knockdowns in cardiomyocytes (Fig. 2A). As p53 knockdown did not affect Smyd2 expression, p53 appears not to be an upstream regulator of Smyd2 expression (Fig. 2A). Apoptosis was induced 48 h after siRNA transfection (Fig. 2B) and quantified 24 h later by staining with Ca^{2+}-dependent phospholipid-binding Annexin V. As expected, p53 knockdown protected cardiomyocytes from $CoCl_2$-induced apoptosis (Fig. 2C). In contrast, Smyd2 knockdown significantly increased the number of Annexin V-positive cells (Fig. 2C). Taken together, these data suggest that Smyd2 might have an anti-apoptotic function in cardiomyocytes.

3.2. Smyd2 overexpression protects cardiomyocytes from apoptosis

To further strengthen the hypothesis that Smyd2 is an endogenous anti-apoptotic factor in cardiomyocytes we tested whether ectopic overexpression of Smyd2 would affect cardiomyocyte survival upon $CoCl_2$ treatment. Smyd2 overexpression in neonatal cardiomyocytes was achieved by adenovirus infection [23] with a transduction efficiency of > 90% (Fig. 3A). $CoCl_2$ treatment in control-infected cardiomyocytes (Ad-β-Gal) marked increased expression of p53 and reduced expression of the anti-apoptotic protein Bcl2 (Fig. 3B–D). Ectopic expression of Smyd2 significantly reduced the accumulation of p53 protein and partially restored the expression levels of Bcl2 (Fig. 3B–D). Furthermore, Smyd2 reduced caspase-3 activity in $CoCl_2$-treated cardiomyocytes (Fig. 3E). Finally, overexpression of Smyd2 resulted in a significant lower number of Annexin V-positive cardiomyocytes compared to the Ad-β-Gal control virus (Fig. 3F). Taken together, these data demonstrate that Smyd2 acts as an anti-apoptotic factor in cardiomyocytes.

As Smyd2 is known to inhibit p53-mediated apoptosis upon DNA damage by methylation [3] we tested whether p53 can also be methylated in cardiomyocytes. Overexpression of Smyd2 resulted in a marked increase in p53 methylation, as demonstrated by Western Blot analysis using an anti-p53K370me1 antibody (Fig. 3G). To determine if the methylation activity of Smyd2 is required for its anti-apoptotic effect we utilized the rat cardioblast cell line H9c2. As in primary rat cardiomyocytes, $CoCl_2$-induced apoptosis resulted in H9c2 cells in downregulation of Smyd2 (Supplemental Fig. 1A). Overexpression of Myc-Smyd2 in H9c2 cells significantly rescued $CoCl_2$-induced apoptosis (Fig. 3H). In contrast, overexpression of Myc-Smyd2Y240F, a Smyd2 mutant lacking methyl transferase activity [6], had no anti-apoptotic effect (Fig. 3H). The transfection efficiency (60% for both

plasmids) was tested in parallel based on Myc expression. Collectively, these data suggest that Smyd2 also inhibits $CoCl_2$-induced apoptosis by methylation of p53.

3.3. The anti-apoptotic effect of Smyd2 is independent of Hsp90

Previously, it has been demonstrated that Hsp90 can promote pro- as well as anti-apoptotic effects [19,22]. Hsp90 is methylated by Smyd2 [5], which has been shown to be important for the stability of sarcomeric titin and muscle function [9,13]. However, it was unknown whether Hsp90 played a role as mediator of the anti-apoptotic effect of Smyd2. To determine the role of Hsp90, the Hsp90 inhibitor 17-allylamino-17-demethoxy geldanamycin (17-AAG) was utilized. As previously shown 17-AAG (100 nM) treatment markedly decreased $CoCl_2$-induced apoptosis in neonatal cardiomyocytes as determined by Annexin V staining [19] but could not enhance the protective effect of p53 knockdown (Fig. 4A). In addition, 17-AAG abolished the pro-apoptotic effect of Smyd2 knockdown (Fig. 4A). To determine whether Smyd2 mediates its anti-apoptotic effect via Hsp90 we performed Smyd2 overexpression experiments in the presence and absence of 17-AAG. Ectopic overexpression of Smyd2, but not β-Gal control protected cardiomyocytes from $CoCl_2$-induced apoptosis as determined by Annexin V-staining (Fig. 4B, C). While 17-AAG treatment significantly reduced the number of Annexin V-positive cardiomyocytes infected with the control adenovirus Ad-β-Gal, it had no significant effect on the anti-apoptotic effect of ectopic Smyd2 (Fig. 4B, C). These data indicate that the anti-apoptotic function of Smyd2 is independent of active Hsp90.

3.4. Smyd2 is required to limit p53-mediated apoptosis in the heart after injury

Cardiomyocyte apoptosis plays a major role in heart failure [1]. To assess whether Smyd2 is involved in the progression of heart failure, the expression levels of Smyd2 were determined after MI in mice, induced by ligation of the left anterior descending artery (LAD). Smyd2 protein expression levels dropped at day one after MI and remained at lower levels compared to SHAM-operated animals (Fig. 5A and Supplemental Fig. 1B, C). Concomitantly, the levels of methylated p53 were downregulated while the total level of p53 was markedly increased at day one after MI as previously shown [19]. In addition, PARP levels were decreased (Fig. 5A and Supplemental Fig. 1). These data suggest that Smyd2 methylates p53 also *in vivo*.

Previously, we observed that Smyd2 is not required for heart development and cardiac physiology [14]. To determine the effect of cardiomyocyte-specific elimination of Smyd2 in cardiac pathophysiology we performed MI studies in conditional *Smyd2* knockout mice (*Smyd2* cKO) harboring a cardiomyocyte-specific *Smyd2* deletion. *Smyd2* cKO displayed significantly elevated numbers of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)-positive cardiomyocytes at the infarct border zone (Fig. 5B, C). RT-qPCR analyses of RNA isolated from infarcted regions revealed an approximate 2-fold induction in the expression of *p53* in *Smyd2* cKO in comparison with $Smyd2^{fl/fl}$ control littermates (Fig. 5D). Note, that p53 mRNA levels were not altered in Sham-operated *Smyd2* cKO mice (Fig. 5D). In addition, immunohistochemistry analyses showed that the expression of the pro-apoptotic protein Bax was markedly elevated in regions surrounding the infarct areas of the LVs as compared to splenic control sections (Fig. 5E).

These data indicate that Smyd2 is required to limit the damage to the heart in cardiac pathophysiology. Taken together, our data suggest that Smyd2 overexpression might be a therapeutic strategy to prevent or reduce p53-dependent cardiomyocyte apoptosis in cardiac disease.

4. Discussion

Our study identified a previously unrecognized endogenous function of Smyd2 in limiting p53-mediated apoptosis in the heart after injury. Several lines of evidence support this conclusion. Smyd2 is downregulated during cardiomyocyte apoptosis *in vitro* and Smyd2 knockdown enhances $CoCl_2$-induced apoptosis. In contrast, overexpression of Smyd2 but not Smyd2Y240F abolishes $CoCl_2$-induced apoptosis. Finally, cardiomyocyte-specific ablation of Smyd2 enhances *in vivo* cardiomyocyte apoptosis rates upon MI.

Previously, we reported that *Smyd2* cKO exhibit under physiological conditions no obvious phenotype [14]. However, it remained unclear whether Smyd2 plays a role under pathophysiological conditions. Recently, it was demonstrated that cytoplasmic Smyd2 is important for sarcomeric organization and thus the maintenance of cardiac function in zebrafish by methylating Hsp90 and promoting the interaction of a Smyd2-methyl-Hsp90 complex with titin [13]. In the present study, we observed that Smyd2 expression, both in apoptotic cardiomyocytes *in vitro* and in hearts after MI is downregulated and inversely correlated with the expression levels of p53. The maximum activation of p53 expression was detected between 1 to 4 days after LAD closure, as reported earlier [15]. Furthermore, Smyd2 was reported to represses the apoptotic activity of p53 through methylation of lysine 370 [3]. Here we show that p53 is also methylated in the heart. Thus, we hypothesized that Smyd2 might have a critical function in limiting p53-mediated apoptosis in heart disease. This is important as accumulating evidence points to a crucial role of p53 in stress-induced apoptosis in the heart. Levels of p53 were shown to be upregulated after ischemia-reperfusion [19,24] as well as in oxidative [25] and mechanical stress [15,26]. In addition, it has been demonstrated in disease models that inhibition of p53 function is beneficial for heart function [15,19,27,28,29]. For example, targeted deletion of p53 as well as overexpression of cardiomyocyte-restricted dominant-interference p53 resulted in reduced levels of cardiomyocyte apoptosis and restored cardiac function in acute doxorubicin cardiotoxicity [27,30].

Sham-operated Smyd2 cKO mice do not express altered levels of p53 (Fig. 5D). siRNA-mediated knockdown of p53 did not alter Smyd2 expression in primary rat cardiomyocytes (Fig. 2A). Neither inhibition of Smyd2 degradation (Fig 1C) nor overexpression of Smyd2 (resulting in p53 methylation, Fig. 3G) had an obvious effect on p53 expression. These data suggest that Smyd2-mediated methylation of p53 does not effect the stability of p53 but rather its function. The observed correlation of increased p53 levels to decreased levels of methylated p53 after MI thus might be due to secondary effects (Fig. 5A).

Here we show by overexpression and knockdown experiments that Smyd2 is a cardioprotective factor in p53-mediated apoptosis and thus has an additional function in the heart besides sarcomeric organization. This is also supported by the observation that the

cardioprotective effect of Smyd2 seems to be independent of Hsp90 in contrast to its role in sarcomere function in cardiac and skeletal muscle [9,13]. The chaperone Hsp90 can interact with Smyd2 enhancing its methyltranferase activity as well as its specificity in regard to H3K4 methylation, which is essential for genes of cell cycle and transcription regulation [31]. Smyd2 interacts with the C-terminal dimerization domain of HSP90 methylating it at two distinct regions: K615 and K209 [5]. In addition Smyd2 mediates H3K36 dimethylation *in vitro*, which in the absence of Hsp90 is relatively weak [6], suggesting that H3K4 may be the predominant site of methylation *in vivo*. However, we observed that inhibition of Hsp90 with 17-AAG had no effect on the cardioprotective effect of Smyd2, even though 17-AAG significantly inhibited apoptosis in cardiomyocytes as shown previously [19]. These data indicate that the anti-apoptotic activity of Smyd2 is independent of Hsp90 and suggest that the observed anti-apoptotic function of Smyd2 is exerted through its nuclear activity in contrast to its cytoplasmic requirement in sarcomeric organization.

Our data suggest that Smyd2 is not regulated by p53 and that Smyd2 does not affect p53 expression on a transcriptional level. However, we confirm here that Smyd2 can methylate p53 [3] and provide evidence that the methylation activity of Smyd2 is required for its anti-apoptotic effect in cardioblasts. In the future it will be interesting to determine if also other targets of Smyd2 such as RB (lysine 860) [8] play a role in cardiomyocyte apoptosis.

4.1. Conclusion

In the present study we have identified lysine methyltransferase Smyd2 as an endogenous p53 antagonist in cardiomyocytes. Our data indicate that Smyd2 is an anti-apoptotic factor acting independently of Hsp90. Given our findings, inducing or maintaining Smyd2 expression or activating its downstream targets may be a useful therapy for protecting patients from apoptosis-mediated cardiac disease and therefore, warrants further preclinical investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

$CoCl_2$	cobalt chloride
MI	myocardial infarction
PARP	poly ADP ribose polymerase
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling

RB	retinoblastoma tumor suppressor
Hsp90	Heat Schock Protein 90
pNA	chromophore p-nitroaniline
17-AAG	17-allylamino-17-demethoxy geldanamycin
LAD	left anterior descending artery

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Highlights

- Heart failure triggering p53-dependent apoptosis warrants an anti-apoptotic approach.
- Lysine methyltransferase Smyd2 is an endogenous p53 antagonist predominantly expressed in cardiomyocytes.
- Smyd2 methylates p53 in primary cardiomyocytes
- Modulation of Smyd2 expression affects cardiomyocyte apoptosis.
- *Smyd2* cardiomyocyte-specific deletion leads to elevated p53 expression levels and apoptosis after myocardial infarction.

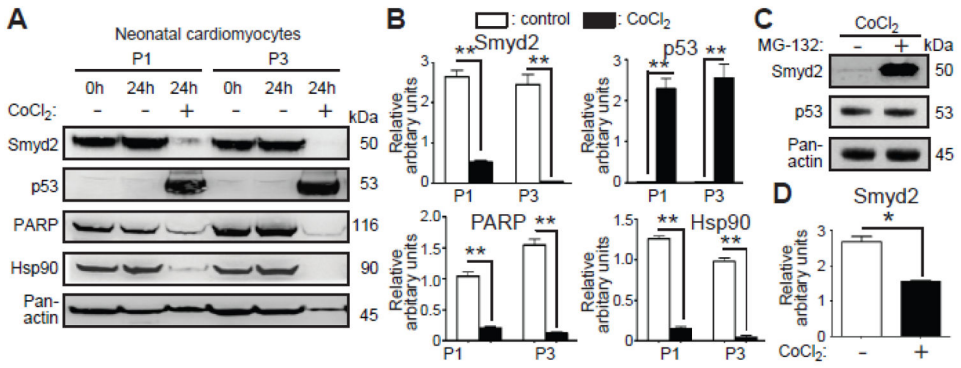

Fig. 1.

Smyd2 is downregulated under apoptotic conditions in neonatal rat ventricular cardiomyocytes. (A) Western blot analysis of protein extracts (50 μg) isolated from rat ventricular cardiomyocytes from postnatal days 1 (P1) and 3 (P3) show a marked decrease of Smyd2 protein 24 h after $CoCl_2$ treatment (750 μM). $CoCl_2$ mimics the hypoxic pathway resulting in p53 accumulation and a decrease of total amount of PARP and Hsp90. Membranes were re-probed with anti-Pan-actin antibodies, as a loading control. (B) Optical density of the protein bands in (A) was determined and normalized to the corresponding expression levels of Pan-actin. **: $p < 0.001$. Data are mean ± SEM, n = 3. (C) MG-132 (10 μM) treatment abolished downregulation of Smyd2 protein expression upon $CoCl_2$ (750 μM)–induced apoptosis (n=3). (D) Quantitation of *smyd2* expression via RT-qPCR of RNA isolated from cardiomyocytes before and after the treatment with $CoCl_2$ (750μM) showing reduced expression of *smyd2* after $CoCl_2$-induced apoptosis. Expression levels were normalized to 18S RNA. *: $p < 0.01$. Data are mean ± SEM, n = 3.

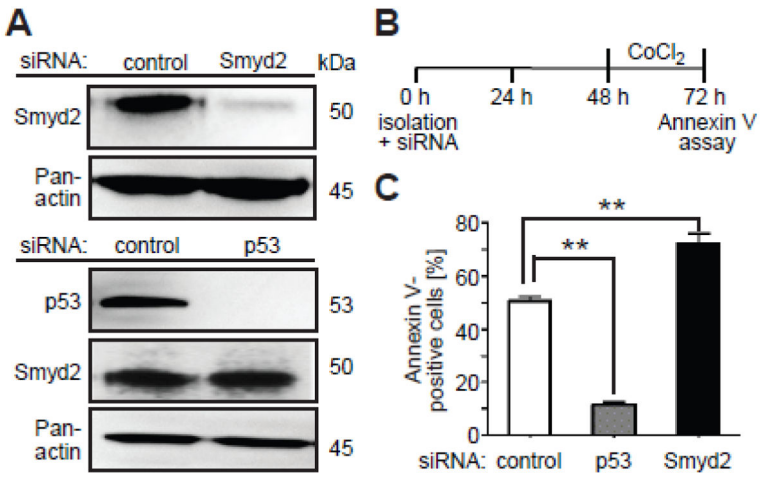

Fig. 2.

Smyd2 knockdown enhances cardiomyocyte apoptosis. (A) Western blot analysis of protein extracts (50 μg) from P1 cardiomyocytes 48 h after transfection with siRNAs targeting Smyd2 and p53 employing anti-Smyd2 and anti-p53 antibodies. Loading control: Pan-actin. (B) Scheme of the experiments utilized to determine the effect of siRNA-mediated knockdown of p53 and Smyd2 on $CoCl_2$-induced apoptosis. (C) Quantification of cardiomyocyte apoptosis after siRNA-mediated knockdown of p53 and Smyd2. $CoCl_2$ treatment resulted in a significant number of Annexin V-positive cells (50.40 ± 2.0%). Smyd2 knockdown significantly increased (72.33 ± 6.8%) while p53 knockdown significantly decreased (11.67 ± 0.9%) the percentage of Annexin V-positive cells. Data are mean ± SEM, **: $p < 0.001$, $n = 3$.

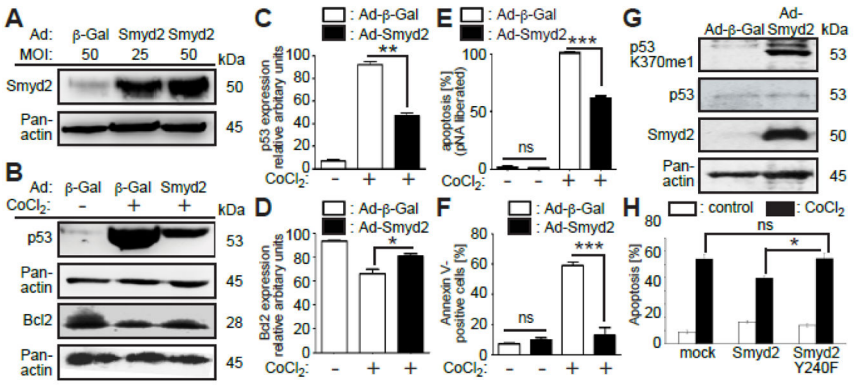

Fig. 3.

Smyd2 overexpression promotes cardiomyocyte survival. (A) Western blot analysis of protein extracts (70 μg) from P1 cardiomyocytes 48 h after infection with adenoviruses expressing β-Gal or Smyd2 employing anti-Smyd2 antibodies. Loading control: Pan-actin. (B) Western blot analysis of protein extracts (50 μg) from P1 cardiomyocytes 72 h after infection with adenoviruses expressing β-Gal or Smyd2 in the presence or absence of $CoCl_2$ during the last 24 hours. Blots were analyzed for p53 and Bcl2 expression. Loading control: Pan-actin. Note: Smyd2 overexpression in $CoCl_2$-treated cultures led to a decrease in p53 accumulation and increased Bcl2 expression levels. (C, D) Quantification of p53 and Bcl2 expression levels in (B). Data are mean ± SEM, **: $p < 0.001$, $n = 3$. (E) Caspase-3 assay performed on proteins isolated from P1 cardiomyocytes after Smyd2 or β-Gal overexpression in the presence or absence of $CoCl_2$. Enforced Smyd2 overexpression reduced Caspase-3 activity as measured by liberation of pNA (chromophore p-nitroaniline), a marker for DEVDase activity in the sample. Data are mean ± SEM, ***: $p < 0.0001$, $n = 3$. (F) Quantification of apoptosis measured via Annexin V staining of P1 cardiomyocytes after Smyd2 or β-Gal overexpression in the presence or absence of $CoCl_2$. Smyd2 overexpression significantly decreased $CoCl_2$-induced apoptosis. Data is shown as a mean ± SEM. ***: $p < 0.0001$, $n = 3$. (G) Western Blot analysis of protein extracts after adenoviruses expressing β-Gal or Smyd2. Blots were probed with the p53K370me1 antibody and re-probed afterwards with the anti-p53, Smyd2 and Pan-actin antibodies (n=2). (H) Quantitative analysis of apoptotic H9c2 cells with the Annexin V & Dead Cell Assay after transfection with Smyd2 and Smyd2 mutant Myc-Smyd2Y240F lacking methyl transferase activity. Data is shown as a mean ± SEM. *: $p < 0.01$, $n = 4$.

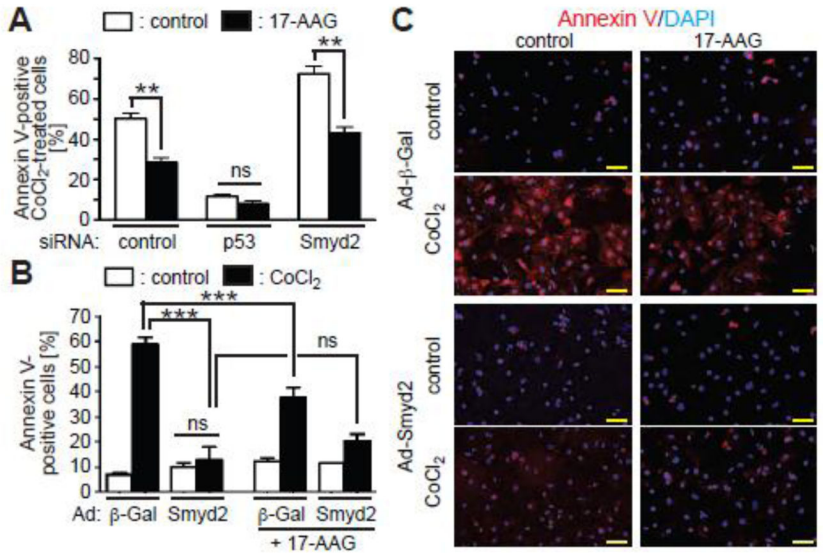

Fig. 4.

The cardioprotective effect of Smyd2 is Hsp90-independent. (A) Quantitative analysis of Annexin V staining of P1 cardiomyocytes transfected with siRNAs targeting Smyd2 or p53 or with a non-silencing negative control and subsequent $CoCl_2$ treatment after 48 h in the presence or absence of the Hsp90 inhibitor 17-AAG for 24 h. Scale bars: 50 μm. Note: knockdown of p53 significantly decreased while knockdown of Smyd2 increased the level of apoptosis, as measured by Annexin V after $CoCl_2$ treatment. Data are mean ± SEM, **: $p < 0.001$, $n = 3$. (B) Quantitation of Annexin V staining of P1 cardiomyocytes 72 hours after infection with adenoviruses expressing β-Gal or Smyd2 and $CoCl_2$ treatment during the last 24 h in the presence or absence of the Hsp90 inhibitor 17-AAG. Note: Smyd2 overexpression significantly decreased $CoCl_2$-induced apoptosis as measured by Annexin V staining. This cardioprotective effect was not disrupted upon 17-AAG treatment indicating that Smyd2 mediates its protective effect independent of Hsp90. Data are mean ± SEM, ***: $p < 0.0001$, $n = 3$. (C) Representative pictures for Annexin V staining utilized for the quantification in (B). Scale bars: 50 μm.

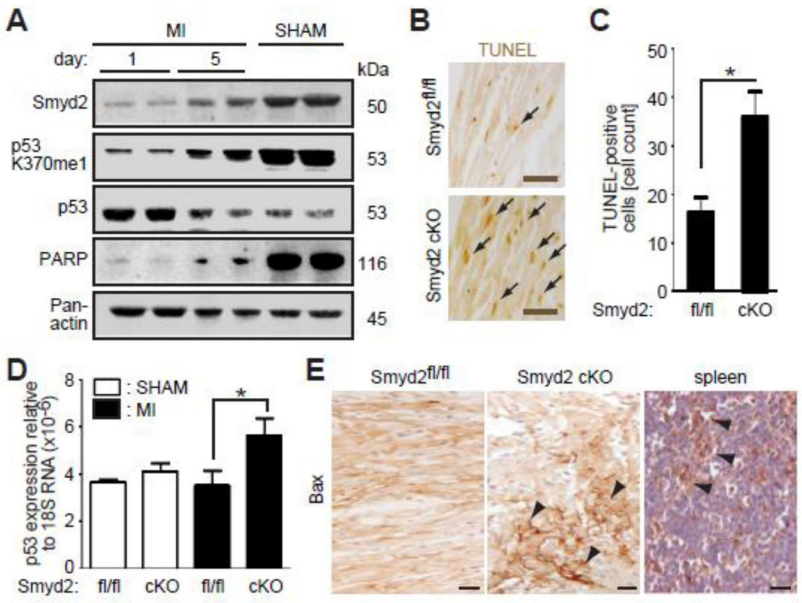

Fig. 5.

Smyd2 is required to limit p53-mediated apoptosis in the heart after injury. (A) Western blot analysis of protein extracts (50 μg) from adult hearts of mice after SHAM-operation or LAD ligation employing anti-Smyd2, p53K370me1, anti-p53 and anti-PARP antibodies. Loading control: Pan-actin. Note: Smyd2 expression was concomitantly decreased to the decline of methylated p53, while the total p53 levels were markedly elevated. (B) Representative pictures of TUNEL staining of sections of infarcted hearts from Smyd2$^{fl/fl}$ or Smyd2 cKO mice revealing a significant increase of apoptosis in Smyd2 cKO hearts. Scale bar: 20 μm. Arrows indicate examples of TUNEL-positive cardiomyocytes. (C) Quantification of (B) summarizing the number of TUNEL-positive cardiomyocyte nuclei at 2 and 4 days after LAD ligation. *: $p < 0.01$. Data are mean ± SEM, n = 8. (D) Quantitation of *p53* expression via RT-qPCR of RNA isolated from Smyd2$^{fl/fl}$ and Smyd2 cKO LV tissues after SHAM operation or LAD ligation showing enhanced expression of *p53* in Smyd2 cKO mice after MI. *: $p < 0.01$. Data are mean ± SEM, n = 8. (E) Representative images of Bax staining of Smyd2$^{fl/fl}$ and Smyd2 cKO LV tissues after LAD ligation showing enhanced expression of Bax2 in Smyd2 cKO mice after MI. As a control sections of spleen were utilized. Arrowheads indicate Bax2-positive cells.