

Septin4 Aggravates Hypoxia-Induced Cardiomyocytes Injury by Promoting HIF-1 α Ubiquitination and Degradation through VHL

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Research

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Abstract

Backgrounds: Myocardial ischemia, characterized by insufficient nutrients and oxygen supply, is the most common cause of ischemic heart disease. Hypoxia-induced cardiomyocytes injury is the pathogenic feature of myocardial ischemia. Although previous studies have reported that the proapoptotic protein Septin4 contributes to prevent some cancers by promoting tumor cells apoptosis mainly through X-linked inhibitors of apoptosis (XIAPs), little is known its role in hypoxia-induced cardiomyocytes injury and its other new interacting partner.

Results: In the current study, Septin4 is found to be involved in cardiomyocytes injury to hypoxia. The overexpression of Septin4 significantly aggravated hypoxic cardiomyocytes apoptosis assessed by cell viability assay and flow cytometry analysis. Mechanistically, hypoxia-inducible factor 1 alpha (HIF-1 α) is confirmed as a novel protein mainly binding with GTPase domain of Septin4 by co-immunoprecipitation. Additionally, we found that the protective factor HIF-1 α is down-regulated by Septin4 and the underlying mechanism is the von Hippel-Lindau protein (VHL)-mediated ubiquitin-proteasome degradation.

Conclusions: These findings suggest that Septin4 aggravates hypoxia-induced cardiomyocytes injury by promoting HIF-1 α ubiquitination and degradation by targeting to VHL, which may be beneficial to provide effective strategies for clinical treatment of myocardial ischemia and ischemic heart disease.

Background

Ischemic heart disease (IHD) causes approximately 10 million deaths worldwide each year and the IHD patients' number is expected to increase by 46% by 2030[1]. The most common cause of IHD is myocardial ischemia, a pathological state that can't supply adequate blood, which leads to the ischemic and hypoxic injury of myocardium [2-4]. Hypoxia, that is, the reduction of available oxygen, has a huge impact on the metabolism of the heart and then changes the normal heart function[5].

Hypoxia-inducible factor 1 (HIF-1) is a major transcription factor mediating cellular adaptation to hypoxia by regulating both the distribution and utilization of oxygen[6]. Actually, it is HIF-1 α , the oxygen-regulated subunit of HIF-1, that activates HIF-1 under hypoxia[6]. Willam C et al. reported an elevated expression levels of HIF-1 α protein in the rat heart after ligation of the coronary artery [7]. Kido M et al. found that the persistent overexpression of HIF-1 α resulted in decreased myocardial infarct size and improved murine heart function after four weeks' myocardial infarction [8]. Pre-treatment with a HIF-1 α activator (dimethyloxalylglycine, OMDG) in animal modelled after ischemic-reperfusion showed an improved heart function [9, 10]. These reveal that HIF-1 α plays a role in cardioprotection.

Septin4, a protein localized at mitochondrion, is shown to be a proapoptotic protein[11]. XIAP (X-linked IAP), a member of inhibitors of apoptosis (IAPs) family regulating cell apoptosis, inhibit apoptosis by the direct inhibition of caspases [12, 13]. Up to date, it has been well studied that Septin4 promotes cell apoptosis mainly by binding XIAP [14-16] via the XIAP/BIR3 domain [17, 18]. Recently, BAX [19] and Bcl-2[20, 21] are also found to be the substrate of Septin4 to promote apoptosis in some tumor cells.

However, it is still unclear whether Septin4 participates in cardiomyocytes injury. In addition, whether there is another completely new substrate for Septin4 in cardiomyocytes also needs further research.

For the first time, our study confirmed that the expression levels of Septin4 were increased from hypoxia-induced cardiomyocytes. Knockdown of Septin4 alleviated cardiomyocytes apoptosis, but overexpression of Septin4 on the basis of Septin4 silencing aggravated it. Mechanistically, we first confirmed that HIF-1 α was a novel protein binding with Septin4 mainly via the GTPase domain of the latter. In addition, HIF-1 α was down-regulated through the VHL-E3 ubiquitin ligase complex-proteasome pathway mediated by Septin4. These findings could be beneficial to provide effective strategies for clinical treatment of myocardial ischemia and IHD.

Results

Septin4 participates in hypoxia-induced cardiomyocytes injury

Given that no study has evaluated the effect of hypoxia-induced cardiomyocytes injury on Septin4 expression so far, we subjected H9c2 cells to hypoxia treatment for 0, 6, 12 and 24 hours to establish hypoxia-induced injury. Successful establishment of the hypoxic model was confirmed by the observation of an obviously decreased cells viability (Additional file 1 Supplemental Fig. 1c) and a significantly increased cells apoptosis rate (Additional file 1 Supplemental Fig. 1d, e). By western blot analysis, we found obviously increased expression levels of Septin4, HIF-1 α and cleaved caspase3 with the prolonging of hypoxic time (Additional file 1 Supplemental Fig. 1a, b). Thus, Septin4 may play a role in the hypoxia-induced injury model, which was discussed in the following experiments on overexpression and knockdown of Septin4 in H9c2 cells.

Cell viability assay and flow cytometry analysis showed that overexpression of Septin4 significantly aggravated hypoxia-induced H9c2 apoptosis (Fig. 1c and Fig. 1d, e), while knockdown of Septin4 by using the identified most efficacious Septin4 siRNA sequence (Fig. 1f) significantly alleviated hypoxia-induced H9c2 apoptosis (Fig. 1h and Fig. 1i, j). In addition, overexpression of Septin4 significantly increased hypoxia-induced H9c2 cells apoptosis maker cleaved caspase3 protein expression (Fig. 1a, b), while knockdown of Septin4 significantly decreased cleaved caspase3 (Fig. 1f, g), and HIF-1 α showed an opposite trend.

Taken as a whole, these results demonstrated that Septin4 played a negative role in cardiomyocytes survival under hypoxia.

Septin4 participates in hypoxia-induced cardiomyocytes injury by down-regulating expression levels of HIF-1 α

The increase in HIF-1 α expression levels by hypoxia induces HIF-1 activity, which is quite important in protecting cardiomyocytes from ischemic heart disease [22-24]. We next explored the role of Septin4 in the expression changes of HIF-1 α in H9c2 cells under hypoxia. First, we found that the expression levels

of both Septin4 and HIF-1 α increased with the prolonging hypoxic time (Fig. 1a, b). Second, we evaluated the effect of Septin4 on HIF-1 α in hypoxic H9c2 cells and found that the expression levels of HIF-1 α significantly increased from hypoxic H9c2 cells with stably silenced of Septin4, while markedly decreased after the overexpression of Septin4 in H9c2 cells on the basis of stably silenced of Septin4 (Fig. 2a, b).

At the same time, we observed an opposite expression trend of the apoptosis maker cleaved caspase3 compared to that of HIF-1 α in each group (Fig. 2a, b). In addition, cell viability assay (Fig. 2c) and flow cytometry analysis (Fig. 2d, e) showed that the hypoxia-induced apoptosis was relieved with stably silenced of Septin4, while was aggravated with overexpression of Septin4 on the basis of stably silenced of Septin4.

These findings suggested that Septin4 was involved in hypoxia-induced cardiomyocytes injury by down-regulating HIF-1 α .

Septin4 interacts with HIF-1 α mainly through its GTPase domain

To explore the mechanism of Septin4 in cardiomyocytes injury, we investigated whether Septin4 and HIF-1 α actually interact, which then affected the injury to H9c2 cells. First, results of endogenous co-immunoprecipitation demonstrated that Septin4 is a novel protein interacting with HIF-1 α (Fig. 3a, b). Next, we found that under hypoxic stimulation, the binding effect of Septin4 and HIF-1 α was enhanced (Fig. 3c, d). These findings suggested that Septin4 participated in hypoxia-induced cardiomyocytes injury by interacting with HIF-1 α .

Furthermore, to explore which domain of Septin4 bound with HIF-1 α , four truncated plasmids of Septin4 were produced according to their functional domains (Fig. 3e). We then overexpressed full-length Septin4 or various truncated mutants of it in 293T cells for co-immunoprecipitation, the results demonstrated that Septin4 mainly interacted with HIF-1 α via its GTPase domain (Fig. 3f).

Septin4 mediates the proteasome degradation of HIF-1 α

To further to confirm whether Septin4 affected the expression of HIF-1 α , Flag-Septin4 was transfected in H9c2 cells with an increasing amount. We found that the expression of Septin4 increased while the expression of HIF-1 α decreased (Fig. 4a, b). What's more, the knockdown of Septin4 with the three Septin4 siRNA sequences resulted in an obviously decreased expression of Septin4 but a significantly increased expression of HIF-1 α (Fig. 4c, d).

The above two findings suggested that Septin4 may mediate the degradation of HIF-1 α in some way. Therefore, we investigated whether septin4 affected the stability of HIF-1 α by the use of protein synthesis inhibitor CHX to inhibit transcription activity or proteasome inhibitor MG132 to inhibit the proteasome degradation activity.

In the CHX assay, transfection of Flag-Septin4 in H9c2 cells led to an obvious reduction in the expression of endogenous HIF-1 α compared to the empty vector group (Fig. 4e, f), while in the MG132 assay with

Flag-Septin4 transfected in H9c2 cells, the accumulation of HIF-1 α was more faster and marked than the empty vector group (Fig. 4g, h), suggesting that Septin4 reduced the expression of HIF-1 α by the proteasome degradation pathway.

Septin4 mediates the polyubiquitination of HIF-1 α

Given that HIF-1 α commonly degraded by proteasome[25], we then transfected Flag-Septin4 in H9c2 cells whether followed by the treatment of MG132 or not, and found that the interaction between Septin4 and HIF-1 α was enhanced by MG132 (Fig. 5a). The results of endogenous co-immunoprecipitation assay also showed a MG132 enhanced interaction between Septin4 and HIF-1 α (Fig. 5b).

Furthermore, after co-transfecting Flag-Septin4 and HA-UB in H9c2 cells followed by the treatment of MG132, we found that Septin4 significantly increased HIF-1 α ubiquitination levels by co-immunoprecipitation assay (Fig. 5c). What's more, Septin4 silencing was found to reduce HIF-1 α ubiquitination levels in Septin4 knockdown H9c2 cells after overexpression of HA-UB (Fig. 5d). These results suggested that Septin4-mediated degradation of HIF-1 α by the proteasome pathway was dependent on polyubiquitination.

Septin4 promotes the ubiquitin-proteasome degradation of HIF-1 α by mobilizing von Hippel-Lindau protein (VHL)

The existence of VHL in an E3 ubiquitin ligase complex, which can mediate the rapid proteasome degradation of HIF-1 α , makes HIF-1 α levels low on normoxia [26, 27]. We next bought HIF-PHDs inhibitor BAY85-3934, which negatively affect the VHL- HIF-1 α interaction and then clarified whether Septin4 mediates the ubiquitin-proteasome degradation of HIF-1 α via VHL.

First, we evaluated the effect of Septin4 and BAY85-3934 on HIF-1 α in hypoxic H9c2 cells and found that the expression levels of HIF-1 α significantly decreased under hypoxia after the overexpression of Septin4, while BAY85-3934 can rescue this result (Fig. 6a, b). Second, to explain the phenomenon, we then transfected Flag-Septin4 in H9c2 cells and found that the interaction between HIF-1 α and VHL was enhanced (Fig. 6c) while Septin4 silencing was found to reduce this binding effect. (Fig. 6d). Lastly, after co-transfecting Flag-Septin4 and HA-UB in H9c2 cells, we found that BAY85-3934 significantly decreased the ubiquitination levels of HIF-1 α (Fig. 6e).

These results confirmed that Septin4 mediated HIF-1 α polyubiquitination via VHL and promoted HIF-1 α degradation by the proteasome pathway (Fig. 6f).

Discussion

The main findings of the current study are that, Septin4 is first found to interact with HIF-1 α mainly via its GTPase domain and negatively regulates HIF-1 α through the ubiquitin-proteasome pathway by enhancing the binding effect between von Hippel-Lindau protein (VHL) and HIF-1 α . In the hypoxic

cardiomyocytes, Septin4 had obviously increased expression levels and enhanced binding effect with HIF-1 α , which result in significant reduction of HIF-1 α and then aggravated cardiomyocytes apoptosis.

Sept4_i2 (ARTS) is one of the splice variants encoded by Septin 4 gene, and is the only one with the proapoptotic function [28]. In recent years, several studies have shown that Septin4 may be a target of preventing cardiovascular diseases. Zhang et al. [29] first reported that Septin4 aggravated oxidative stress induced endothelial cell apoptosis by interacting with PARP1. Another study[30] performed by Zhang et al. showed that Septin4 protected human vascular smooth muscle cells from phenotypic transformation, proliferation and migration induced by PDGF-BB by promoting SIRT1-mediated STAT3 acetylation at K685 site and phosphorylation at Y705 site. These two findings provided a theoretical basis of the treatment of atherosclerosis. In addition, Wang et al. [31] found that Septin4 inhibited Ang-II induced vascular smooth muscle cells proliferation and migration. Our previous study[32] revealed that WWP2 bound with Septin4 in human umbilical vein endothelial cells and degraded it through the ubiquitin-proteasome pathway at K174 site of Septin4 to prevent hypertensive angiopathy. The present study first confirmed that the expression levels of Septin4 were increased from hypoxia. Knockdown of Septin4 decreased cardiomyocytes apoptosis while overexpression of Septin4 on the basis of Septin4 silencing increased it. To explore the phenomenon, we found HIF-1 α , which is beneficial to adaptations to hypoxia, binding with the GTPase domain of Septin4 and was down-regulated by Septin4.

HIF-1 α and HIF-1 β are the two subunits of HIF-1, in which HIF-1 α subunit plays the central role in protecting cells from local hypoxia by increasing expression of its downstream target genes in many cardiovascular diseases [5]. The target genes of HIF-1 α are those involved in angiogenesis, vascular reactivity (including secreted factors and cell surface receptors) and glucose metabolism (including metabolic enzymes, transporters and mitochondrial proteins)[6, 33]. For example, neovascularization stimulated by expression of vascular endothelial growth factor (VEGF) is induced by HIF-1 α mediated transcription of VEGF gene on hypoxia [34]. In addition, injection of AdCA5, an adenovirus providing an active form of HIF-1 α showed the improved vascular remodeling and angiogenic responses [35-37]. In the present study, we performed rescue experiment, CHX, MG132 and poly-ubiquitination assays and found the stability of the protective factor HIF-1 α was negatively affected by Septin4 through the ubiquitin-proteasome pathway, which suggested that Septin4 indeed aggravated the hypoxia-induced cardiomyocytes injury by down-regulating HIF-1 α .

Under normoxia, HIF-1 α is continually synthesized and rapidly degraded by the hydroxylation at Pro402 or Pro564 within the oxygen-dependent degradation domain (ODDD) particularly by PHD2 [38] before VHL, the recognition subunit of an E3 ubiquitin ligase complex, targets the hydroxylated HIF-1 α for proteasome degradation [39]. And under hypoxia, the accumulation of HIF-1 α results from the inhibited proline residues hydroxylation and the following ubiquitination and proteasome degradation [39]. In the current study, we found BAY85-3934 (a PHDs inhibitor) significantly inhibited the down-regulation and ubiquitination levels of HIF-1 α mediated by Septin4. Additionally, overexpression of Septin4 showed enhanced interaction between HIF-1 α and VHL while Septin4 silencing reduced the binding effect. This confirmed the molecular mechanism of down-regulation of HIF-1 α by Septin4 was targeted to VHL-

mediated proteasome degradation, which explained how Septin4 was involved in hypoxia-induced cardiomyocytes injury in detail.

There are some limitations of the present study. First, although HIF-1 α does have a protective effect through its target genes under hypoxic condition, we have not yet determined which gene played the protective role in our experiment. Second, the role of Septin4 in myocardial ischemic patients remains to be further studied.

Conclusions

Septin4 is found to be involved in hypoxia-induced cardiomyocytes injury and HIF-1 α is confirmed as a novel protein mainly binding with the GTPase domain of Septin4. Additionally, HIF-1 α is down-regulated by Septin4 and the underlying mechanism is the VHL-mediated ubiquitin-proteasome degradation. These findings may be beneficial to provide effective strategies for clinical treatment of myocardial ischemia and ischemic heart disease.

Materials And Methods

Cell culture and hypoxia treatment

The rat cardiomyocyte H9c2 cell line and the human embryonic kidney 293T cell line were procured from the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS (HyClone). All cells were situated in an incubator containing 5% CO₂ and 95% air at 37 °C. To establish the hypoxia model, H9c2 cells were cultured with serum-free DMEM in an incubator containing 94% N₂, 5% CO₂, and 1% O₂ for 24 hours unless otherwise noted.

Plasmids construction and transfection

Flag-Septin4 (full length) and HA-UB were purchased from GeneChem (China). Four truncated Septin4 plasmids containing different domains with a Flag-tag were obtained: N-terminal and GTPase domains; N-terminal domain; C-terminal domain; C-terminal and GTPase domains. Lipofectamine 3000 (Invitrogen, California, USA) was used for plasmid transfections following the manufacturer's instructions.

Septin4 knockdown in H9c2 cells

Normal Control and Septin4 shRNAs were obtained from GeneChem (China). Septin4 silencing was performed with lentivirus. To prevent off-target effects, three sequences were employed:

Septin4 shRNA-1: GCGGAAGAGAGGATCATGCAA

Septin4 shRNA-2: CTGCATCAGCGGGTCAACATT

Septin4 shRNA-3: TGGCCTGAATCGCAAGAACAT

Control and Septin4 siRNAs were obtained from RIBOBIO (China). Septin4 silencing was performed with jetPRIME transfection reagent from PolyPlus (France). To prevent off-target effects, three sequences were employed:

Septin4 siRNA-1: CGGTGGATATAGAAGAGAA

Septin4 siRNA-2: CTATCAGTTCCCAGATTGT

Septin4 siRNA-3: ATGCAAACCGTGGAGATTA

The efficiency of Septin4 knockdown by shRNA and siRNA was confirmed by Western blot analysis.

Antibodies and reagents

Polyclonal goat anti-Septin4 (abcam, USA), polyclonal rabbit anti-HIF-1 α (abcam), polyclonal rabbit anti-VHL (proteintech, USA), polyclonal rabbit anti-caspase-3 (Cell Signaling Technology, USA), monoclonal mouse anti-HA (Cell Signaling Technology), monoclonal mouse anti-Flag (abcam), and monoclonal mouse anti-GAPDH (abcam). CHX (A8244) and MG132 (A2585), used in DMSO, were purchased from ApexBio (USA). HIF-PHDs inhibitor BAY85-3934 was also purchased from ApexBio.

Cell viability assay

Cell viability was assessed with a Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan). Briefly, H9c2 cells were seeded in 96-well plates (NEST Biotechnology) at a density of 5000 cells/well in DMEM with 10% FBS. After being performed the indicated treatments, cells were incubated with 110 μ l of CCK-8 solution (serum-free DMEM/CCK-8 reagent = 10/1) per well for 2 hours. Finally, the absorbance value of each well was measured at a wavelength of 450 nm with a Microplate Reader Bio-Rad microplate reader (Model 680; Bio Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry analysis

To detect cell apoptosis, Annexin V-FITC/PI Kit (Beyotime Institute of Biotechnology) were used to stain treated cells according to the manufacturer's instruction, followed by flow cytometry analysis. Briefly, treated H9c2 cells were collected carefully and resuspended in 500 μ l binding buffer containing 3.5 μ l Annexin V-FITC and 3.5 μ l PI to react with H9c2 cells in the dark for 15 min at RT. Finally, apoptotic cells were identified and quantified with a FACSCalibur flow cytometer.

Protein preparation

For protein analysis, the H9c2 cells were washed with cold PBS, harvested and lysed as quickly as possible on ice in cell lysis buffer (50 mM Tris, 137 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, 1% NP-40, 1 mM DTT, 10% glycerol, pH 7.8) with both protease (Roche, Switzerland) and phosphatase inhibitors (Bimake, USA), then centrifuged at 13300 rpm/min for 15 minutes at 4 °C.

Co-immunoprecipitation and immunoblotting

The lysates were incubated with specific antibodies (antibody/cell lysate = 1 µg/mg) for 3 hours followed by adding 30-35 µl Protein A/G beads (Bimake) and incubating for another 12 hours at 4°C. Next, the lysates including antibodies and beads were washed with cell lysis buffer for three times and resolved by 8% or 12% SDS-PAGE gels and then transferred to the PVDF membranes (Millipore USA). Afterwards, the PVDF membranes were blocked by 5% BSA in TBST for 1 hour at RT before incubations with primary antibodies overnight at 4 °C and with secondary antibodies for 1 hour at RT the next day. Image J 1.52v (National Institutes of Health, USA) was used to quantify the immunoreactive bands. The expression levels of target protein were showed as fold changes relative to expression levels of corresponding GAPDH.

Statistical analysis

Data are presented as mean ± standard deviation (SD). F-test was performed to evaluate the homogeneity of variance and the normality of data was evaluated by Shapiro–Wilk test. To determine the significance of differences between several groups of one related study factor or two, one-way ANOVA and two-way ANOVA were performed respectively, followed by Tukey's multiple comparisons test. All data were processed by SPSS 25.0 (SPSSInc, Chicago, USA) and GraphPad Prism 8 for Windows, p <0.05 was considered significant.

List Of Abbreviations

IHD: Ischemic heart disease

PARP1: Poly(ADP-ribose) polymerase-1

HIF-1: Hypoxia-inducible factor 1

VHL: von Hippel-Lindau protein

IAPs: Inhibitors of apoptosis XIAP (X-linked IAP)

CHX: Cycloheximide

VEGF: vascular endothelial growth factor

PHD: Prolyl hydroxylase domain proteins

ODDD: Oxygen-dependent degradation domain

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.

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Authors’ contribution: Yingxian Sun guided this study. Shaojun Wu conducted the cells and mechanism part of the experiments. Shilong You and Sien Lu contributed in the cells and mechanism part of the experiments. Ying Zhang and Naijin Zhang designed the experiments. Shaojun Wu and Naijin Zhang wrote the manuscript. All authors read and approved the final manuscript.

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Figures

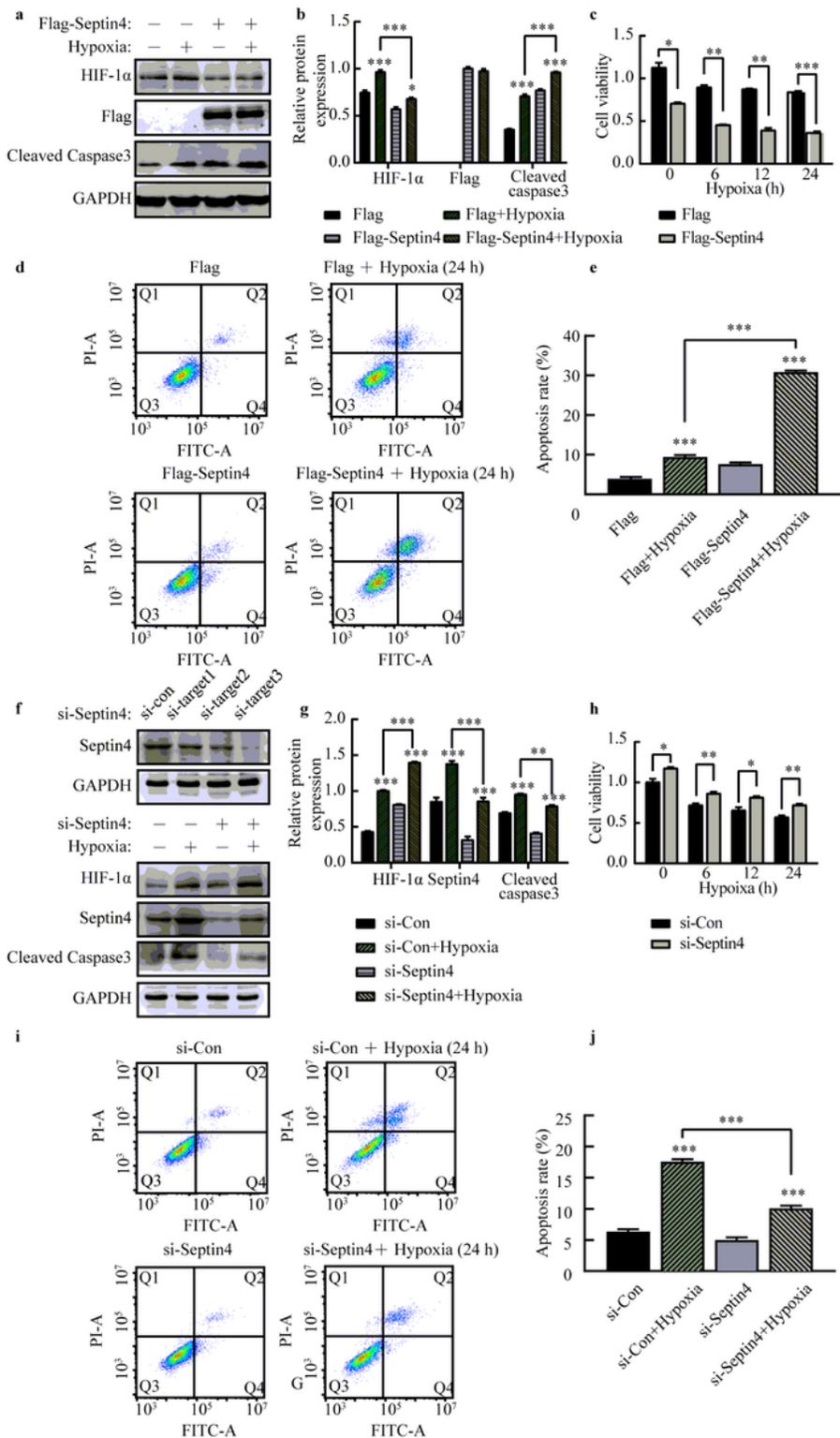


Figure 1

Septin4 participates in hypoxia-induced cardiomyocytes injury a b Western blot analysis of the expression levels of HIF-1α, Septin4 and cleaved caspases3 in H9c2 cells transfected with vector empty or Flag-Septin4 under hypoxia for 24 h. c Cell viability assay of H9c2 cells' viability after transfected with vector empty or Flag-Septin4 under hypoxia for 24 h. d e Flow cytometry analysis of H9c2 cells' apoptosis rate after transfected with vector empty or Flag-Septin4 under hypoxia for 24 h. f g Western blot analysis

of the expression levels of HIF-1 α , Septin4 and cleaved caspases3 in H9c2 cells transfected with si-Con or si-Septin4 under hypoxia for 24 h. h Cell viability assay of H9c2 cells' viability after transfected with si-Con or si-Septin4 under hypoxia for 24 h. i j Flow cytometry analysis of H9c2 cells' apoptosis rate after transfected with si-Con or si-Septin4 under hypoxia for 24 h. All data are presented as mean \pm SD of three repeated experiments; *** < 0.001, ** < 0.01, * < 0.05. One-way ANOVA with Tukey's multiple comparisons test (b, e, g, and j); two-way ANOVA with Tukey's multiple comparisons test (c, h).

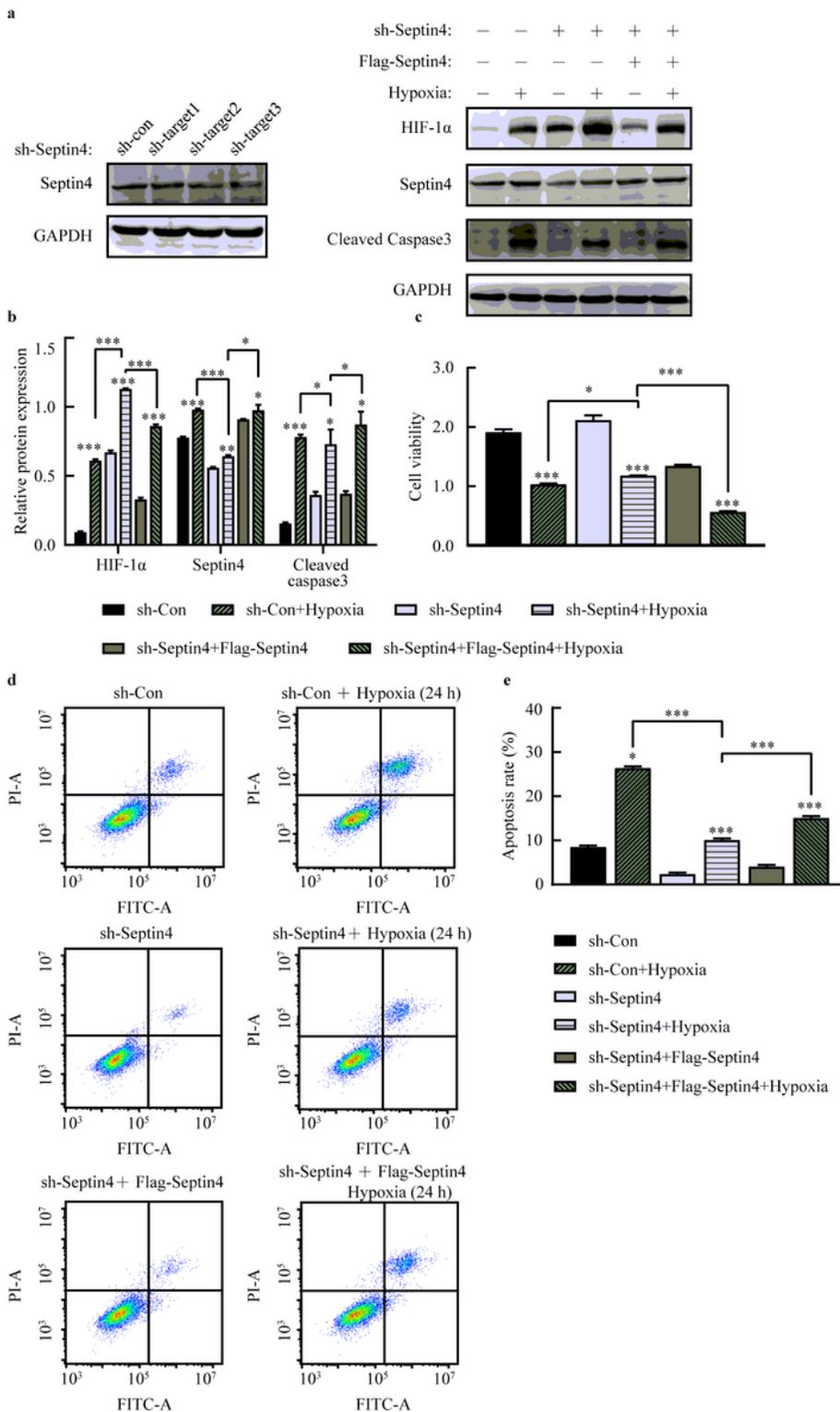


Figure 2

Septin4 participates in hypoxia-induced cardiomyocytes injury by down-regulating expression levels of HIF-1α a b Western blot analysis of the role of Septin4 in regulating the expression levels of HIF-1α in sh-Septin4 H9c2 cells transfected with vector empty or Flag-Septin4 under hypoxia for 24 h. c Cell viability assay of sh-Septin4 H9c2 cells' viability after transfected with vector empty or Flag-Septin4 under hypoxia for 24 h. d e Flow cytometry analysis of sh-Septin4 H9c2 cells' apoptosis rate after transfected with vector empty or Flag-Septin4 under hypoxia for 24 h. All data are presented as mean ± SD of three repeated experiments; *** < 0.001, ** < 0.01, * < 0.05. One-way ANOVA with Tukey's multiple comparisons test (b, c, and e).

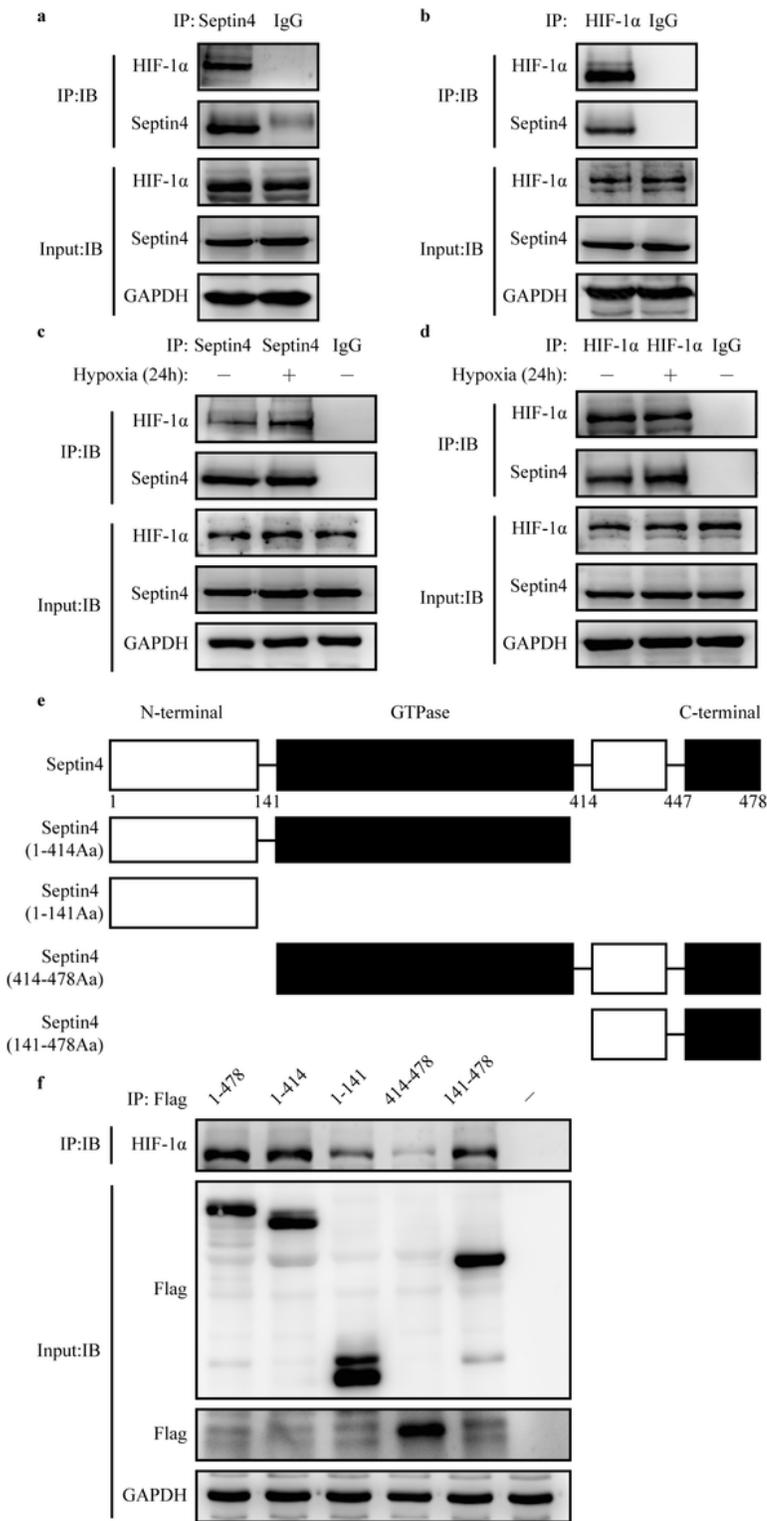


Figure 3

Septin4 interacts with HIF-1α mainly through its GTPase domain a b Co-immunoprecipitation analysis of the endogenous interaction between Septin4 and HIF-1α. c d Co-immunoprecipitation analysis of the endogenous interaction between Septin4 and HIF-1α under hypoxia for 24 h. e f Co-immunoprecipitation analysis of the interaction between different truncations of Septin4 (exogenously overexpressed) and HIF-1α.

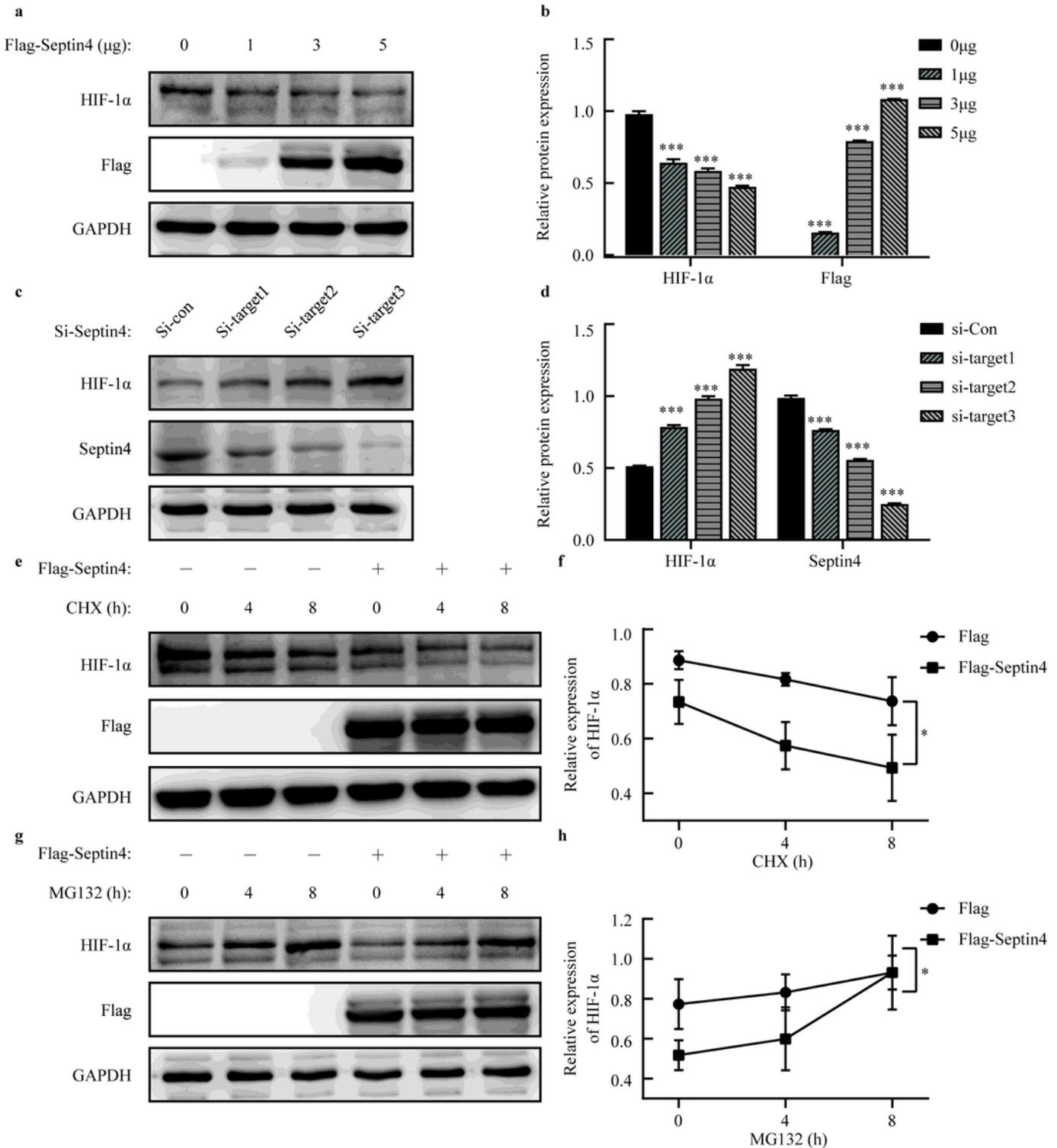


Figure 4

Septin4 mediates the proteasome degradation of HIF-1α a b Western blot analysis of the gradient overexpression of Septin4 and the changes of HIF-1α protein expression levels. c d Western blot analysis of the knockdown of Septin4 with three different sequences and the changes of HIF-1α protein expression levels e f Western blot analysis of the expression levels of HIF-1α under the treatment of CHX for different hours in H9c2 cells transfected with vector empty or Flag-Septin4. g h Western blot analysis of the

expression levels of HIF-1 α under the treatment of MG132 for different hours in H9c2 cells transfected with vector empty or Flag-Septin4. All data are presented as mean \pm SD of three repeated experiments; *** < 0.001, ** < 0.01, * < 0.05. One-way ANOVA with Tukey's multiple comparisons test (b, d). Two-way ANOVA with Tukey's multiple comparisons test (f, h).

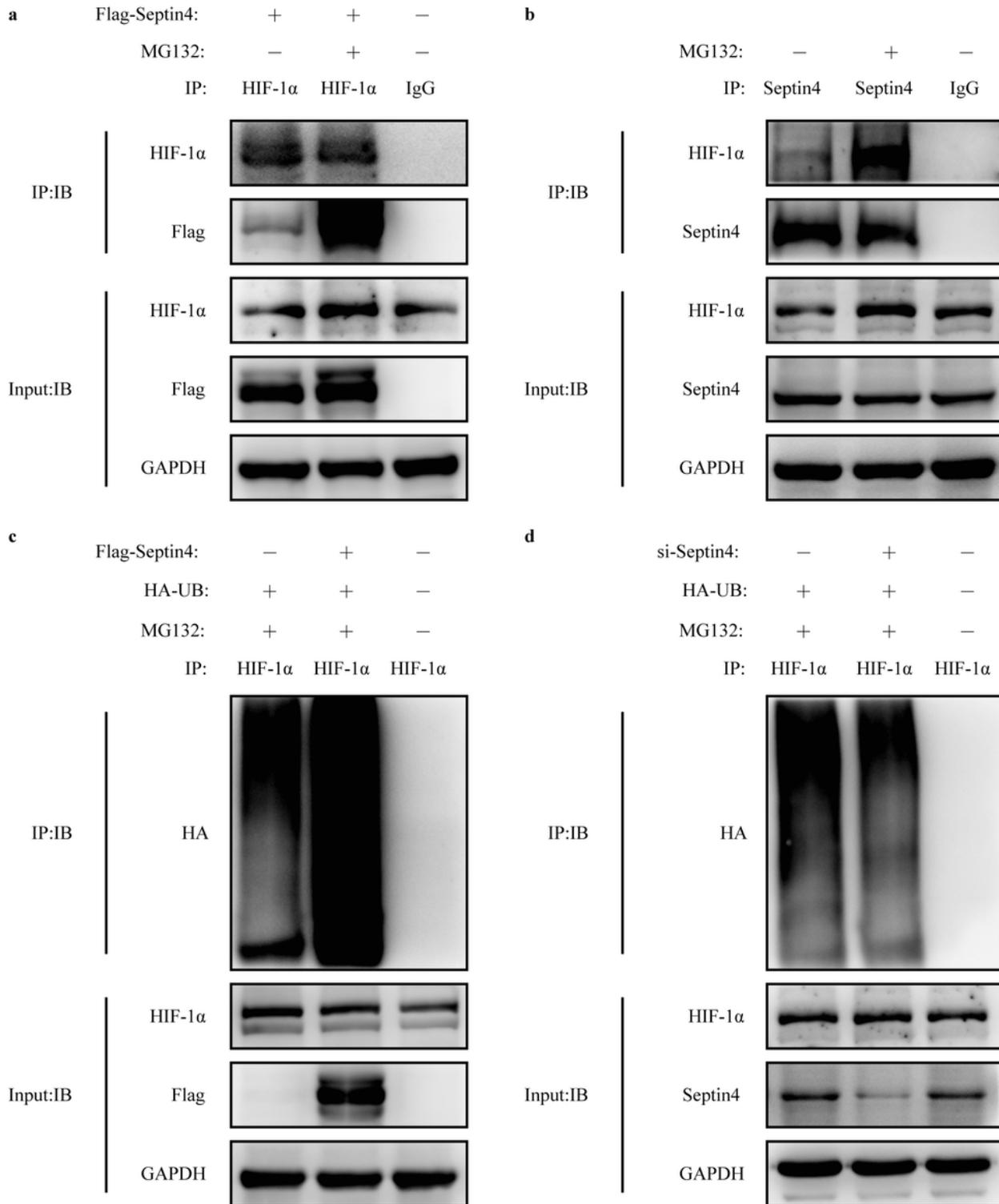


Figure 5

Septin4 mediates the polyubiquitination of HIF-1 α a Co-immunoprecipitation analysis of the interaction between exogenous Septin4 and endogenous HIF-1 α under the treatment of MG132 for 8 hours. b Co-immunoprecipitation analysis of the interaction between endogenous Septin4 and endogenous HIF-1 α under the treatment of MG132 for 8 hours. c Co-immunoprecipitation analysis of the role of Septin4 in regulating the ubiquitination levels of HIF-1 α in H9c2 cells transfected with vector empty or Flag-Septin4. d Co-immunoprecipitation analysis of the role of Septin4 in regulating the ubiquitination levels of HIF-1 α in H9c2 cells transfected with si-Con or si-Septin4.

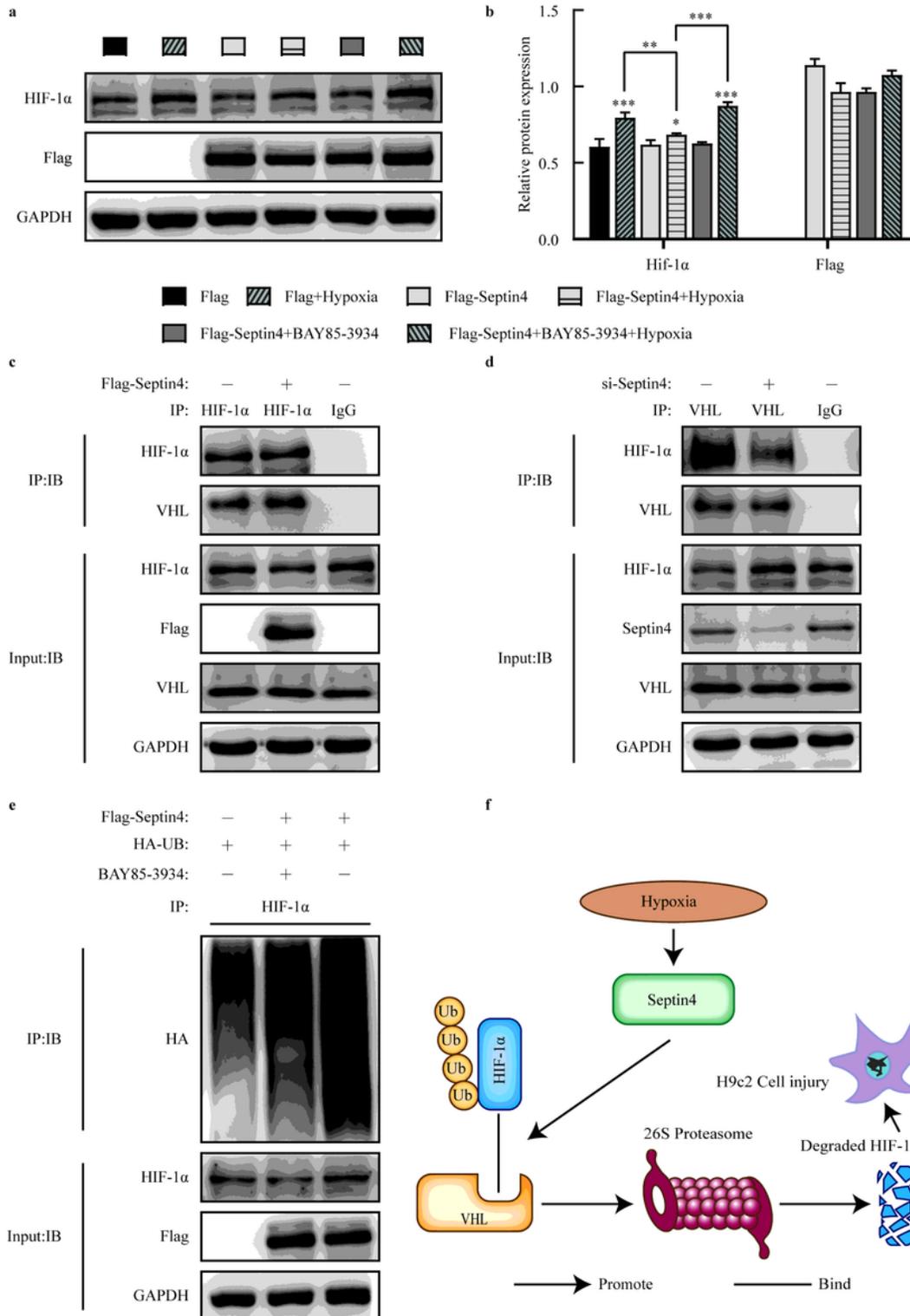


Figure 6

Septin4 promotes the ubiquitin-proteasome degradation of HIF-1 α by mobilizing von Hippel-Lindau protein (VHL) a b Western blot analysis of the expression levels of HIF-1 α rescued by BAY85-3934 in H9c2 cells transfected with vector empty or Flag-Septin4. c Co-immunoprecipitation analysis of the role of Septin4 in regulating the endogenous interaction between VHL and HIF-1 α in H9c2 cells transfected with vector empty or Flag-Septin4. d Co-immunoprecipitation analysis of the role of Septin4 in regulating the endogenous interaction between VHL and HIF-1 α in H9c2 cells transfected with si-Con or si-Septin4. e Co-immunoprecipitation analysis of BAY85-3934's effect on the necessity of Septin4 regulating the ubiquitination levels of HIF-1 α in H9c2 cells transfected with vector empty or Flag-Septin4. f Proposed working mode of Septin4 as a negative regulator of HIF-1 α by targeting VHL-mediated HIF-1 α ubiquitin-proteasome degradation. All data are presented as mean \pm SD of three repeated experiments; *** < 0.001, ** < 0.01, * < 0.05. One-way ANOVA with Tukey's multiple comparisons test (b).

Supplementary Files

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