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Regulation of the stability and transcriptional activity of NFATc4 by ubiquitination

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Abstract Nuclear factor of activated T cells (NFATc4) has been implicated as a critical regulator of the cardiac development and hypertrophy. However, the mechanisms for regulating NFATc4 stability and transactivation remain unclear. We showed that NFATc4 protein was predominantly ubiquitinated through the formation of Lysine 48-linked polyubiquitin chains, and this modification decreased NFATc4 protein levels and its transcriptional activity. Furthermore, activation of GSK3β markedly enhanced NFATc4 ubiquitination and decreased its transactivation, whereas inhibition of GSK3β had opposite effects. Importantly, ubiquitination and phosphorylation induced by GSK3β repressed NFATc4-dependent cardiac-specific gene expression. These results demonstrate that the ubiquitin–proteasome system plays an important role in regulating NFATc4 stability and transactivation.

Structured summary:
MINT-6798349:
NFATc4 (uniprotkb:Q14934) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6798334:
NFATc4 (uniprotkb:Q14934) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6798321:
Ubiquitin (uniprotkb:P62988) *physically interacts* (MI:0218) with *NFATc4* (uniprotkb:Q14934) by *pull down* (MI:0096)

Keywords: Ubiquitination; Degradation; NFATc4; Transcriptional activity; Glycogen synthase kinase-3β; Cardiac gene expression

1. Introduction

Nuclear factors of activated T cells (NFATs) are members of REL-homology domain (RHD)-containing transcription factors [1,2]. Today, five members of the NFAT family of transcription factors have been identified. Four of them are regulated by the calcium/calmodulin-dependent phosphatase calcineurin, including NFAT1 (NFAT c2/p), NFAT2 (NFATc1/c), NFAT3 (NFATc4), and NFAT4 (NFATc3/x). In resting cells, NFAT proteins exist in the cytoplasm as phosphoproteins and their activation requires dephosphorylation. Upon stimulation, elevation of cytoplasmic free Ca^{2+} concentration enhances calcineurin activity, which dephosphorylates NFATs and leads to their activation [1,2]. Conversely, NFATs are phosphorylated by several protein kinases, including glycogen synthase kinase-3β (GSK3β) [3,4] and deactivated in the cytoplasm in resting cells. Thus, nuclear translocation of NFATs is essential for their transcriptional activity.

Earlier studies have reported the presence of NFATc1, NFATc2, and NFATc3 mainly in T cells regulating the expression of cytokine genes such as interleukin-2 (IL-2) [1,2]. However, recent studies demonstrate that all five NFAT isoforms are expressed in the ventricular cardiomyocytes [1,5–7]. The cardiovascular system is dependent on NFAT activity for embryonic development and for adult adaptation to cardiac stress [8]. Mice with disruption of NFATc3/c4 genes die around E11 with defects in vessel wall assembly [9]. Recent studies demonstrate that NFATc4 is an important regulator for cardiac hypertrophy [10–12]. However, the mechanisms for regulating NFATc4 protein stability and transactivation are still poorly understood. In the present study, we showed that NFATc4 protein is predominantly ubiquitinated through the formation of K48-linked polyubiquitin chain. The modifications of ubiquitination and phosphorylation induced by GSK3β play an important role in regulating NFATc4 stability and transactivation.

2. Materials and methods

2.1. Plasmids and reagents

The Luciferase reporter plasmids NFAT/AP-1-Luc and PPAR-Luc were provided by Dr. Chiwing Chow. His- or HA-ubiquitin (wild-type, K48R) and antisense HA-Ub were from Dr. Kuan-Teh Jeang. Active form and dominant-negative form of GSK3β (Ac GSK3β and Dn-GSK3β) were obtained from Dr. Renliang Wu. The IL-2-Luc reporter and Flag-NFATc4 plasmids have been described previously [13]. The RNA Oligo GSK3ß siRNA (sc-35525) and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology. The antibodies used: anti-Flag (M2, Sigma), anti-HA (12CA5, Roche), anti-ubiquitin and anti-β-actin (Biomol), anti-NFATc4 (Santa Cruz), anti-His, anti-GSK3β, anti-mouse- and anti-rabbit-conjugated

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antibodies (Cell signaling Technology). Cycloheximine and MG-132 were obtained from Biomol. LiCl, phenylephrine (PE) and cyclosporine A (CsA) were purchased from Sigma.

2.2. Cell culture, transfection and Luciferase assay

293T cells and myocardial cells H9c2 were obtained from ATCC and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) or siRNA Transfection Reagent (SantaCruz) according to manufacturer's instruction. Luciferase assay was performed using the Luciferase Assay System (Promega) as described [13]. Data represent means ± S.E.M. of three independent assays.

2.3. Immunoprecipitation and immunoblotting

Lysates were extracted in cell lysis buffer containing 50 mm Tris (pH 7.4), 150 mm NaCl, 1% Nonidet P-40, and protease and phosphatase inhibitor cocktail (Sigma). For immunoprecipitations, 1000 μg proteins were incubated overnight at 4 °C with anti-Flag antibody and then precipitated the next day for 2 h with protein A/G (Upstate Biotechnology). Immunoprecipitates were resolved by 6–8% SDS–PAGE and analyzed by immunoblotting using appropriate antibodies [13]. The intensities of protein bands were measured using Gel-pro 4.5 Analyzer (Media Cybernetics, USA).

2.4. Nickel–agarose chromatography and ubiquitination assay

Cells were transfected with indicated plasmids and lysed in Ni–agarose lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 0.05% Tween 20, 10 mM of *N*-ethylmaleimide, and complete protease inhibitor). Ubiquitin-conjugated proteins were purified by nickel chromatography (Ni–NTA–agarose, Qiagen) and detected by immunoblotting [14]. Ubiquitination assay in vivo was performed as described [13].

2.5. Quantitative real-time RT-PCR analysis

Total RNA was purified from H9c2 cells with Trizol (Invitrogen). Reverse-transcription was performed using ImProm-II reverse-transcriptase (Promega) with oligo-dT priming according to manufacturer's instruction. PCR was performed using Bio-RAD iQ5 Multicolor Real-Time PCR Detection System with SYBR Green (Takara) as fluorescent and ROX (Takara) as reference dyes. PCR primers for atria natriuretic factor (ANF), ß-myosin heavy chain (ß-MHC) and GAPDH were used as described [13].

2.6. Statistical analysis

Data are presented as means ± S.E.M. Differences between groups were analyzed for statistical significance using *ANOVA* tests and Student's *t*-test. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Modification of NFATc4 by ubiquitination

The expression level and consequent transactivity of many transcription factors are regulated by ubiquitin-mediated proteolysis. To investigate whether NFATc4 is modified by ubiquitination in vivo, nickel chromatography assay was performed. As shown in Fig. 1A, ubiquitination of Flag-NFATc4 was readily detected in the presence of His-ubiquitin (His-Ub). To further verify whether the endogenous NFATc4 is ubiquitinated, H9c2 cells were transfected with HA-Ub and immunoprecipitated with anti-NFATc4 antibodies followed by immunoblotting with anti-HA or anti-NFATc4 antibodies. As shown in Fig. 1B, marked ubiquitination of NFATc4 was also detected in a dose-dependent manner (Fig. 1B). These results suggest that NFATc4 is ubiquitinated in vivo.

3.2. Proteasome-dependent degradation of NFATc4 reduces its transcriptional activity

Ubiquitination modification has been associated with proteasome-dependent degradation of target proteins. To assess whether ubiquitination promotes NFATc4 degradation by proteasome, the stability of NFATc4 protein in 293T cells was examined. The half-life of NFATc4 protein was markedly reduced in the presence of HA-Ub compared with vector control (Fig. 2A). Moreover, overexpression of HA-Ub significantly decreased the level of endogenous NFATc4 proteins, whereas treatment with MG-132 resulted in marked stabilization of NFATc4 proteins compared with the cells without MG-132 (Fig. 2B). Furthermore, treatment with MG-132 dramatically enhanced the level of ubiquitinated proteins of NFATc4

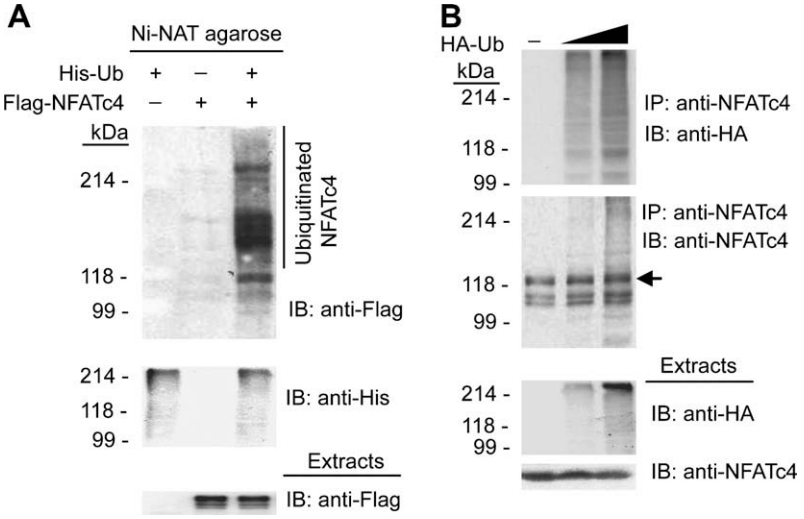

Fig. 1. Modification of NFATc4 was mediated by ubiquitination. (A) 293T cells were transfected with Flag-NFATc4 and His-Ub plasmids and treated with MG-132 (10 μM) for 6 h before harvesting. His-Ub bound proteins were purified by nickel chromatography and analyzed by immunoblotting with anti-Flag (upper) or anti-His (middle) antibodies. The expression of NFATc4 in the cell extracts was examined with anti-Flag antibodies (lower). (B) H9c2 cells were transfected with HA-Ub plasmids. Immunoprecipitation (IP) was performed with anti-NFATc4 antibodies and analyzed by immunoblotting with anti-HA (upper) or anti-NFATc4 (middle) antibodies. The expression of Ub and NFATc4 in the cell extracts was examined with anti-HA or anti-NFATc4 (lower) antibodies.

(Fig. 2C). These findings indicate that ubiquitinated NFATc4 is regulated through proteasome-mediated degradation.

To further examine the effect of ubiquitination on NFATc4 transcriptional activity, Luciferase assay was performed with IL-2-luc reporter [1,8]. As shown in Fig. 2D, NFATc4 markedly enhanced the IL-2-luc reporter activity, whereas overexpression of HA-Ub repressed NFATc4-mediated activation of the IL-2-luc reporter. As expected, treatment with MG-132 increased the transcriptional activity of NFATc4. In contrast, overexpression of antisense HA-Ub moderately increased IL-2-luc activation (Fig. 2E). Together these results suggest that ubiquitination inhibits the transcriptional activity of NFATc4.

3.3. Degradation of NFATc4 is mediated by the K48-linked polyubiquitin chains

To determine the mechanism of ubiquitination on NFATc4 stability, ubiquitination assay was performed in 293T cells transfected with wild-type ubiquitin or ubiquitin K48R mutant, in which the lysine at position 48 of ubiquitin was replaced by arginine [15,16]. Transfection with wild-type ubiquitin increased the NFATc4 ubiquitination and decreased its protein level. In contrast, transfection with ubiquitin K48R mutant markedly decreased the NFATc4 ubiquitination and increased its protein level (Fig. 3A and B). Furthermore, overexpression of wild-type ubiquitin markedly decreased NFATc4 transcriptional activity, while K48R-ubiquitin mutant had no effect (Fig. 3C). The results indicate that the degradation of NFATc4 is mediated by the K48-linked polyubiquitin chains, thereby leading to inhibition of its transcriptional activity.

3.4. GSK3β activation enhances NFATc4 ubiquitination and inhibits its transcriptional activity

Previous findings revealed that NFATc4 is phosphorylated by protein kinase GSK3β in cardiac myocytes and other cell types [3,4]. This promoted us to investigate whether the

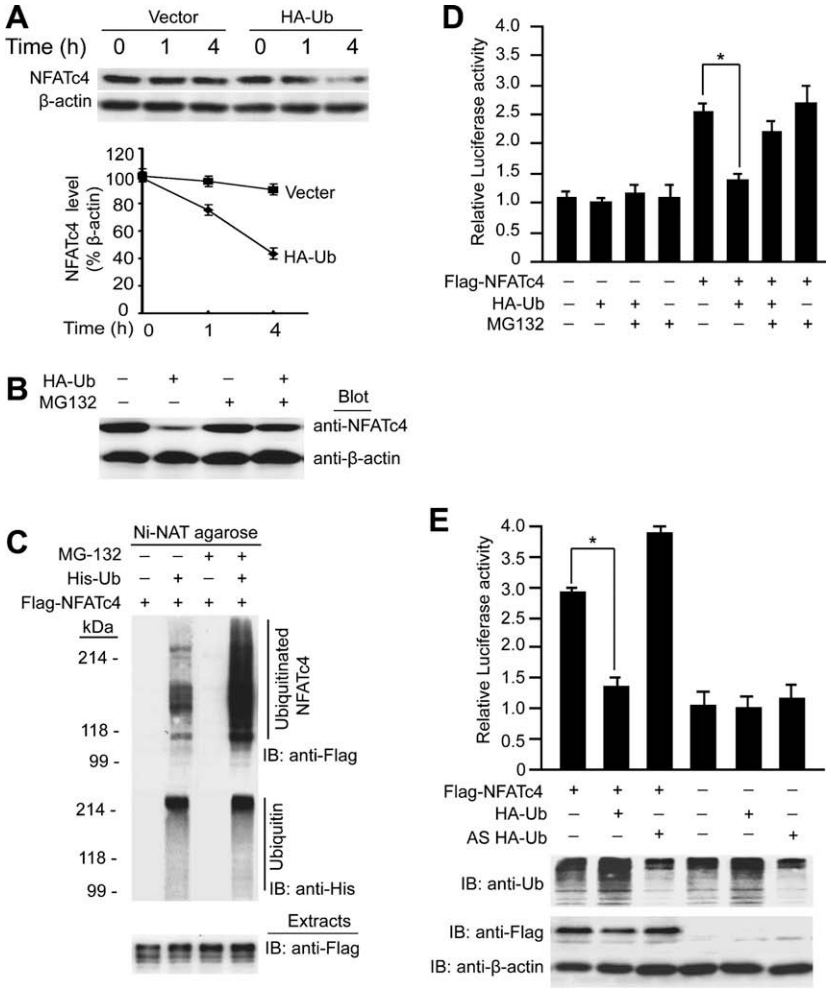

Fig. 2. Ubiquitinated-NFATc4 was degradated by proteasome. (A) H9c2 cells were transfected with HA-Ub plasmids and treated with cycloheximide (50 μg/ml) over time as indicated. The expression of NFATc4 in the cell extracts was examined with anti-NFATc4 antibodies (upper) or β-actin (middle) antibodies. The NFATc4 band intensities were measured (lower panel). (B) H9c2 cells were transfected with HA-Ub plasmids and treated with MG-132 (10 μM) for 6 h before harvesting. The expression of proteins in the cell extracts was examined with anti-NFATc4 (upper) or anti-β-actin (lower) antibodies. (C) 293T cells were transfected with Flag-NFATc4 and His-Ub plasmids and treated with MG-132 (10 μM) for 6 h before harvesting. His-Ub bound proteins were analyzed as in Fig. 1A. (D) 293T cells were cotransfected with the IL-2 Luciferase reporter, Flag-NFATc4 and HA-Ub with or without MG-132 treatment. Luciferase activity was measured 24 h after transfection. $^{*}P < 0.01$. (E) 293T cells were transfected with the reporter IL-2 alone or with NFATc4, HA-Ub or antisense HA-ubiquitin (AS HA-Ub). Luciferase activity was measured. Protein levels were analyzed by immunoblotting as indicated antibodies. $^{*}P < 0.01$ vs. NFATc4 alone.

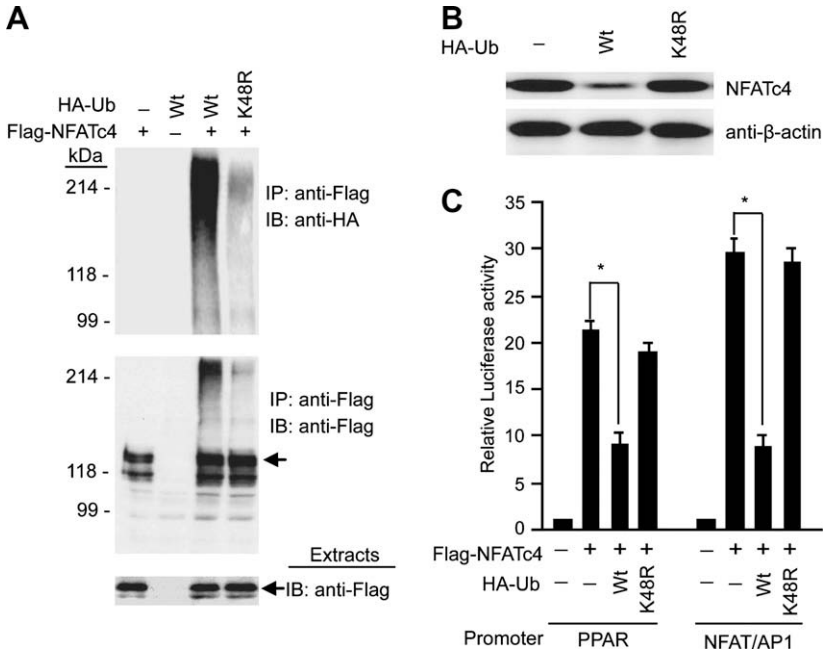

Fig. 3. Degradation of NFATc4 is dependent on the K48-linked polyubiquitin chains. (A) 293T cells were transfected with the Flag-NFATc4 and HA-Ub wt. or HA-Ub K48R mutant and treated with MG-132 (10 μM) for 6 h before harvesting. Immunoprecipitation was performed with anti-Flag antibodies and analyzed by immunoblotting with anti-HA (upper) or anti-Flag (middle) antibodies. The expression of NFATc4 in the cell extracts was examined with anti-Flag antibodies (lower). (B) H9c2 cells were transfected with the HA-Ub wt. or HA-Ub K48R mutant. The expression of NFATc4 in the cell extracts was examined with anti-NFATc4. (C) 293T cells were transfected with Luciferase reporters (PPAR or NFAT/AP-1), NFATc4, HA-Ub wt. or HA-Ub K48R mutant. Luciferase activity was assayed 24 h after transfection. $^{*}P < 0.01$ vs. NFATc4 alone.

GSK3β is involved in the regulation of NFATc4 ubiquitination and transactivation. As shown in Fig. 4A and B, overexpression of active GSK3β markedly increased NFATc4 ubiquitination and repressed its transcriptional activity. Conversely, dominant-negative GSK3β had an opposite effect. Furthermore, inhibition of endogenous GSK3β activation by siRNA or LiCl treatment inhibited NFATc4 ubiquitination and enhanced its transcriptional activity compared with control (Fig. 4C and D). These results suggest that GSK3β dependent phosphorylation plays a significant role for NFATc4 ubiquitination and transcriptional activity.

3.5. Calcineurin activation by PE affects NFATc4 ubiquitination and transcriptional activity

It is known that NFATs are dephosphorylated by calcineurin in the cytoplasm and imported to the nucleus for their activation [1,2], we therefore tested the effect of calcineurin on NFATc4 ubiquitination and transcriptional activity. Cells were treated with hypertrophic agent, PE or calcineurin inhibitor, cyclosporine A (CsA). As shown in Fig. 5A and B, treatment with PE significantly decreased NFATc4 ubiquitination and increased its transcriptional activity, whereas CsA substantially had an opposite effect. These results indicate that activation of calcineurin inhibits the ubiquitination of NFATc4 and enhances its transcriptional activity.

3.6. Both ubiquitin and GSK3β inhibit NFATc4-induced cardiac gene expression

Because NFATc4 plays an important role in cardiac gene expression and hypertrophy [6,10–12], we further asked whether ubiquitin or GSK3β is able to affect NFATc4-dependent cardiac gene expression in H9c2 cells. Active NFATc4 or wild-type NFATc4 alone markedly induced the expression of cardiac ANF and β-MHC (Fig. 6A and B). However, cotransfection with ubiquitin or Ac-GSK3β significantly inhibited these gene expressions (Fig. 6A–D). Conversely, transfection with Dn-GSK3β or LiCl treatment had an opposite effect (Fig. 6C and D). These findings indicate that the modifications of ubiquitination and phosphorylation inhibit NFATc4-dependent cardiac-specified gene expression.

4. Discussion

In the present study, we demonstrated that NFATc4 is predominantly ubiquitinated and degradated through the proteasome system. Ubiquitin-mediated modification reduces NFATc4-dependent transcriptional activity. Furthermore, GSK3β dependent phosphorylation is required for efficient ubiquitination of NFATc4 and inhibition of its transactivation. Finally, both ubiquitination and phosphorylation induced by GSK3β can regulate NFATc4-dependent cardiac gene expression.

Previous studies have shown that NFATc4 plays important role in the development of cardiovascular system [1,8]. However, the molecular mechanisms that regulate the stability and activity of NFATc4 remain unclear. There is a growing belief that posttranslational modification by ubiquitin acts as an important regulator of transcription factor activity [17]. Recent reports demonstrated that proteasome pathway is involved in degradation of NFAT members such as NFATc2 and NFAT5 [18,19]. Consistent with this view, we found that overexpression of ubiquitin promoted the ubiquitination and degradation of NFATc4, and treatment with proteasome

inhibitor MG-132 led to the accumulation of NFATc4 proteins (Figs. 1 and 2), indicating that ubiquitination of NFATc4 is mediated through the proteasome-dependent degradation. It is also known that that the ubiquitin lysine 48-linked polyubiquitin chain functions as a signal for targeting substrates to proteasome degradation, whereas ubiquitin lysine 63-linked is involved in several non-degradative processes such as receptor endocytosis and sorting, translation, as well as DNA damage repair [14,20]. In this study, we demonstrated that ubiquitination of NFATc4 was ubiquitin lysine-48-dependent (Fig. 3A). Furthermore, we clearly observed that wild-type ubiquitin resulted in decreased NFATc4 protein level and its transactivation. However, the ubiquitin K48R mutant had no effect (Fig. 3B and C). Taken together, these data suggest that NFATc4 is a target of the ubiquitin–proteasome system in vivo.

Phosphorylation has been considered to play a critical role in influencing protein ubiquitination, and in several cases the target proteins were first marked by phosphorylation for ubiquitin-mediated degradation [21,22]. In the case of NFATc4, GSK3β as a ubiquitous serine/threonine protein kinase phosphorylates a series of sites in the N-terminal regulatory regions of NFAT proteins and inactivates NFATs activity by preventing their accumulation in the nucleus [3,4]. We found that cotransfection of active GSK3β significantly enhanced NFATc4 ubiquitination and repressed its transcriptional activity. In contrast, dominant-negative GSK3β, siRNA-GSK3β or treatment with LiCl decreased NFATc4 ubiquitination and enhanced its transactivation (Fig. 4A–D), indicating that GSK3β dependent phosphorylation is required for the efficient ubiquitination of NFATc4 and inhibition of its transcriptional activity. Recent evidences suggest that the calcineurin/NFATc4 pathway can be activated by classical hypertrophy inducers, including PE, angiotensin II (ANG II), endothelin-1 (ET-1) [13,23,24]. Activated calcineurin dephosphorylates NFATc4 for nuclear import [24]. In our case, we demonstrated that treatment with PE, which is known to induce calcineurin activity, decreased NFATc4 ubiquitination and enhanced its tran-

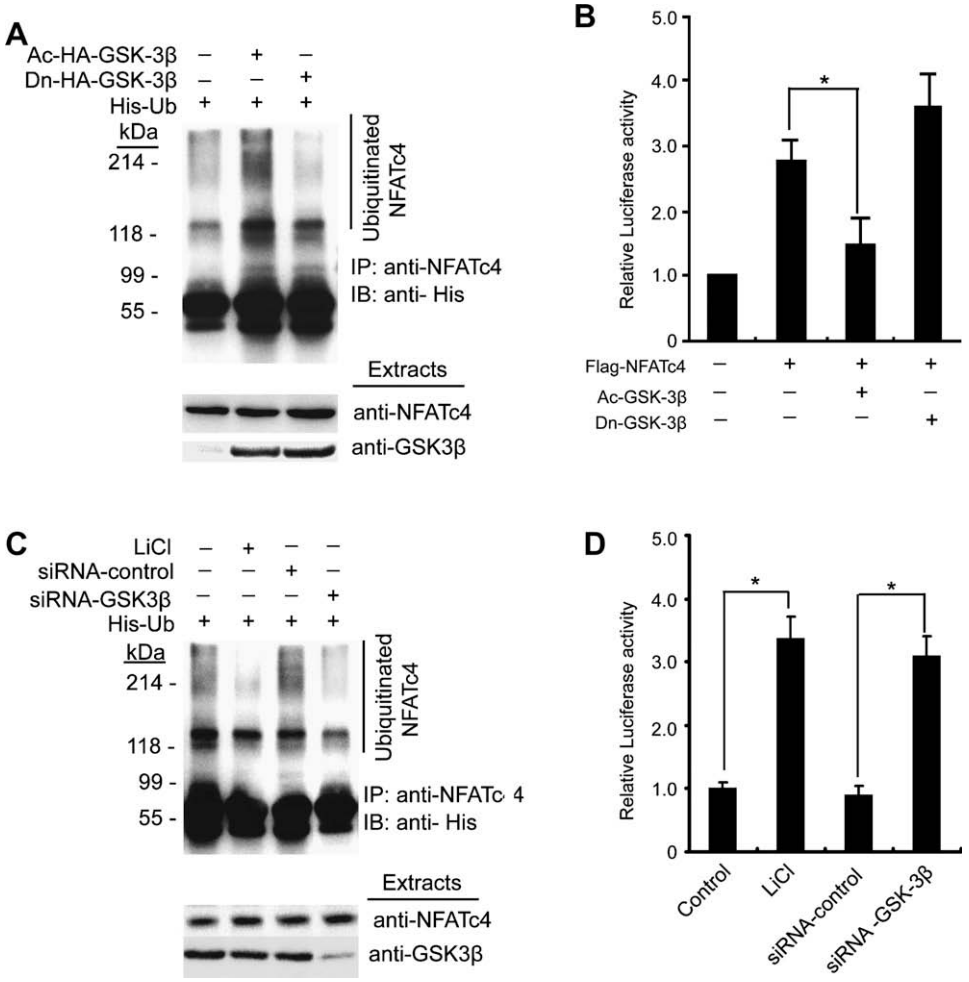

Fig. 4. GSK3β regulates the NFATc4 ubiquitination and transcriptional activity. (A) H9c2 cells were cotransfected with His-Ub and Ac-GSK3β or Dn-GSK3β and treated with MG-132 (10 μM) for 6 h before harvesting. Immunoprecipitation was performed with anti-NFATc4 antibodies and analyzed by immunoblotting with anti-His (upper) antibodies. The expression of NFATc4 and GSK3β in the cell extracts was examined by immunoblotting as indicated (lower). (B) 293T cells were transfected with IL-2 Luciferase reporter and Ac-GSK3β or Dn-GSK3β plasmids and Luciferase activity was assayed. $^{*}P < 0.01$ vs. NFATc4 alone. (C) H9c2 cells were cotransfected with His-Ub, siRNA-control or siRNA-GSK3β or treatment with LiCl (10 mM) for 6 h. Cells were treated with MG-132 (10 μM) for 6 h before harvesting. Immunoprecipitation was performed as in (A). (D) 293T cells were cotransfected with IL-2 Luciferase reporter and siRNA-GSK3β or siRNA-control for 36 h or treatment with LiCl (10 mM) for 6 h, and Luciferase activity was assayed. $^{*}P < 0.01$ vs. control.

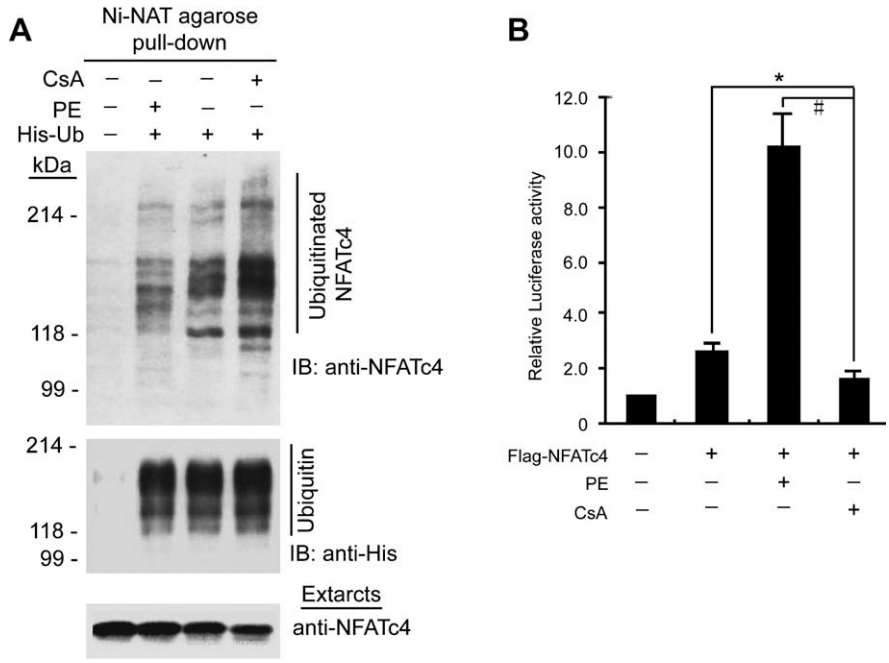

Fig. 5. The effect of calcineurin activation by PE on NFATc4 ubiquitination and transcriptional activity. (A) H9c2 cells were cotransfected with His-Ub and treated with PE (100 μM) or CsA for 6 h. Immunoprecipitation was performed with anti-NFATc4 antibodies and analyzed by immunoblotting with anti-His (upper) or anti-NFATc4 (middle) antibodies. The expression of NFATc4 in the cell extracts was examined by immunoblotting (lower). (B) 293T cells were transfected with IL-2 Luciferase reporter and treated with PE (100 μM) or CsA for 6 h, and Luciferase activity was assayed. $^{*}P < 0.01$ vs. NFATc4 alone.

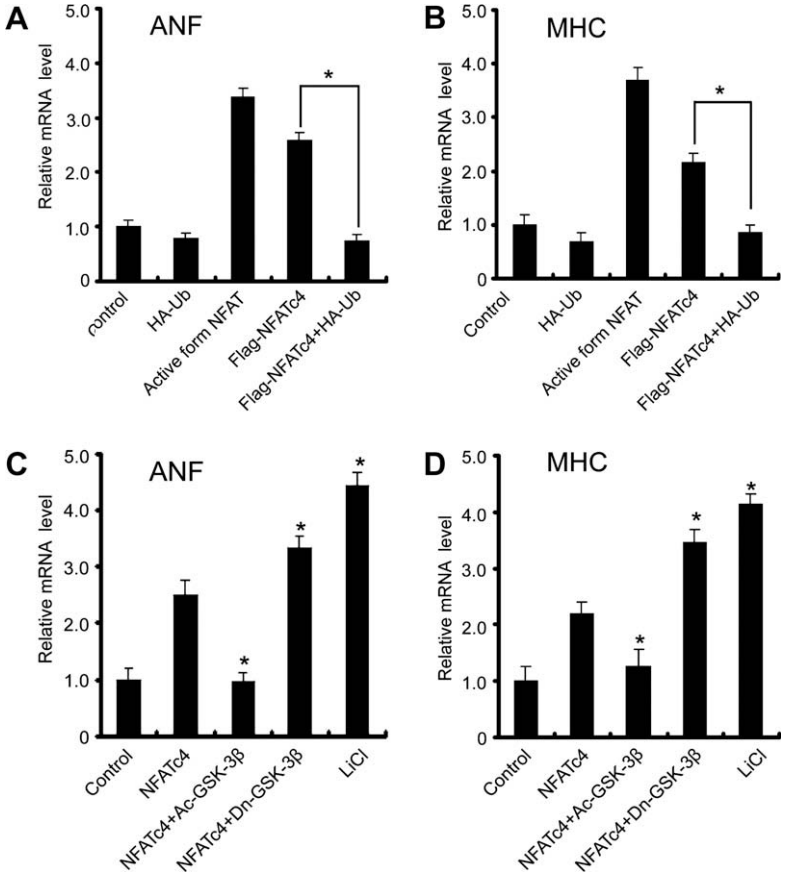

Fig. 6. Both ubiquitin and GSK3β repress NFATc4-induced cardiac-specific gene expression. (A, B) H9c2 cells were transfected with plasmids Ub, active form of NFATc4 or wild-type NFATc4 as indicated. The mRNA level of ANF and β-MHC was determined by RT-PCR analysis. (C, D) H9c2 cells were transfected with plasmids Ub, NFATc4, Ac-GSK3β or Dn-GSK3β or treatment with LiCl as indicated. The mRNA level of ANF and β-MHC was determined as in A and B. $^{*}P < 0.001$ vs. NFATc4 alone.

scriptional activity, whereas CsA had opposite effects (Fig. 4B and C), indicating that activation of cacineurin by PE inhibits NFATc4 ubiquitination and increases its transcriptional activity.

Cardiac hypertrophy is accompanied by the activation of a set of fetal cardiac genes, including those encoding ANF and β-MHC [24]. Activation of several transcription factors, including NFATc1-c4, contributes in part to the changes in cardiac gene expression [7,10,25,26]. Among these factors, NFATc4 is the major form of NFAT family member, and its nuclear localization is sufficient to induce cardiac hypertrophy [10–12]. Importantly, cardiac-specific expression of activated GSK3β diminished stimuli-induced hypertrophy, at least in part, by preventing nuclear localization of NFAT [25]. In the present study, we found that both ubiquitination and GSK3β dependent phosphorylation of NFATc4 repressed the expression of cardiac ANF and β-MHC. However, dominant-negative GSK3β or LiCl markedly increased the expression of these genes (Fig. 6A–D). These data demonstrate that modifications of ubiquitination and phosphorylation induced by GSK3β play an important role in regulating NFATc4-dependent cardiac gene activity.

In conclusion, our results demonstrate that the ubiquitin–proteasome system plays a crucial role in the stability of NFATc4 protein and its transcriptional activity. We proposed a model depicting the antagonistic effects of GSK3β and calcineurin on NFATc4 ubiquitination and transcriptional activity. GSK3β activation antagonizes calcineurin signaling by phosphorylation of NFATc4 proteins and cytoplasmic translocation, thereby leading to ubiquitination and proteasomal degradation. Further studies to identify the E3 ligases for NFATc4 will open a new view into the mechanism for modulating cardiac development and hypertrophy.

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