Short Communication

Akt1 Decreases Gcn5 Protein Stability through Regulating The Ubiquitin-Proteasome Pathway in Mouse Embryonic Fibroblasts

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Abstract

General control non-derepressible 5 (Gcn5) is a member of histone acetyltransferase (HAT) that plays key roles during embryogenesis as well as in the development of various human cancers. Gcn5, an epigenetic regulator of *Hoxc11*, has been reported to be negatively regulated by Akt1 in the mouse embryonic fibroblasts (MEFs). However, the exact mechanism by which Akt1 regulates Gcn5 is not well understood. Using protein stability chase assay, we observed that Gcn5 is negatively regulated by Akt1 at the post-translational level in MEFs. The stability of Gcn5 protein is determined by the competitive binding with the protein partner that interacts with Gcn5. The interaction of Gcn5 and Cul4a-Ddb1 complex predominates and promotes ubiquitination of Gcn5 in the wild-type MEFs. On the other hand, in the Akt1-null MEFs, the interaction of Gcn5 and And-1 inhibits binding of Gcn5 and Cul4a-Dbd1 E3 ubiquitin ligase complex, thereby increasing the stability of the Gcn5 protein. Taken together, our study indicates that Akt1 negatively controls Gcn5 via the proteasomal degradation pathway, suggesting a potential mechanism that regulates the expression of *Hox* genes.

Keywords: Akt1, Gcn5, Proteasome Endopeptidase Complex, Ubiquitin

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The *Hox* genes are transcription factors that have a pivotal role in the anteroposterior axis determining during embryogenesis (1, 2). In addition, *Hox* genes are expressed in adult tissues and abnormal expression of those genes is associated with the development, progression, and metastasis of various cancers (3-5). This means that the expression of *Hox* genes should be precisely controlled in a specific spatiotemporal manner. Dynamic gene expression regulation is achieved by epigenetic changes by various histone modifying and chromatin remodeling enzymes, such as histone deacetylase (HDAC) and acetyltransferase (HAT) (6-8). General control non-derepressible 5 (Gcn5) is a member of the *GCN5*-related N-acetyltransferase *(GNAT)* superfamily of HAT (9). Histone acetylation by Gcn5, along with other types of histone modifications, has been reported as an important epigenetic factor regulating Hox gene expression during embryonic development (10).

Akt, a serine/threonine kinase, is required to regulate various biological responses (11). Interestingly, we have previously identified Akt1 as a *Hox* modulator (12, 13). Along with a long history of research on Akt, recent epigenetic studies have unveiled novel Akt substrates such as p300, EZH2, and BMI1, indicating a critical role of Akt in regulating epigenetic processes (14). According to our previous results, Gcn5 also binds directly to Akt1 and the protein level of Gcn5 is dependent on whether Akt1 is expressed (15, 16). This strongly suggests the Akt1 functions as a Gcn5 regulator, but the precise mechanism of this action has not yet been elucidated.

Therefore, we conducted a follow-up study to find out how Akt1 regulates the protein stability of Gcn5 in the mouse embryonic fibroblasts (MEFs). We observed that degradation of Gcn5 is mediated by Cul4a-Ddb1 E3 ligase complex, which is regulated by Akt1 expression. These results help us to understand how Akt1 and Gcn5 regulate *Hox* gene expression during embryonic development. Revealing that Akt1 acts as an epigenetic regulator of *Hox* gene expression further implicates a possible mechanism how PI3K/Akt pathway, which plays an important role in cancer, can affect the regulation of *HOX* gene expression in various human cancers.

The wild-type MEFs and Akt1-null MEFs were generated as described previously (17, 18). For preparation of embryonic fibroblast cells, embryos were dissected to remove the head and other viscera. Remaining tissues were finely minced and washed with phosphate buffered saline (PBS). Then, cells were trypsinized (LS 015-10, WelGENE Inc., Daegu, Korea) and plated in the Dulbecco's modified Eagle's medium (DMEM, LS 001-05, WelGENE Inc., Daegu, Korea) which is containing 10% fetal bovine serum (S 001-01, WelGENE Inc., Daegu, Korea) and 1% of penicillin-streptomycin (LS 203-01, WelGENE Inc., Daegu, Korea). The Akt1-null MEFs were generated to have neomycin insertion between exon 3 and exon 8 by homologous recombination. The procedure for preparation of Akt1-null MEFs are same as wild-type MEFs. Cells were grown in a humidified incubator of 5% CO_2 at 37˚C.

For Western blotting, cell lysates were extracted using NP40 and protein contents were determined using the Pierce BCA Protein Assay Kit (23227, Thermo Scientific, Rockfold, IL. USA). Protein samples were run on the 8-10% sodium dodecyl sulfate (SDS) poly-acrylamide gel, immobilized onto PVDF transfer membranes (IPVH00010, Bio-Rad, Hercules, CA, USA), and probed with appropriate antibodies. Anti-Gcn5l2 (#3305, Cell Signaling Technology, Danvers, MA, USA), anti-Akt1 (#2938, Cell Signaling Technology), anti-Cul4a (A300-739A, Bethyl, Montgomery, Texas, USA), anti-Ddb-1 (A300-462A, Sigma, St. Louis, MO, USA), anti-And1 (630301, BioLegend, San Diego, CA, USA), anti-Ubiquitin (#3936, Abcam, Cambridge, UK), and anti-β-actin (ab6276, Abcam, Cambridge, UK) were used to detect each protein.

Coimmunoprecipitation (Co-IP) assay, harvested cells were lysed with NP40 lysis buffer, containing protease inhibitor cocktail (11697498001, Roche, Darmstadt, Germany). The lysate was precleared with Protein A/G plus-agarose beads (sc-2003, Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at 4˚C. Anti-Gcn5 (A-11) (sc-365321, Santa Cruz Biotechnology) primary antibody or normal IgG (sc-2025, Santa Cruz Biotechnology, Dallas, TX, USA) was incubated at 4˚C for overnight with gentle rotation. The following day, target protein-antibody complexes were precipitated with Protein A/G plus-agarose beads for 3 hours. Target complexes were detached from the Protein A/G plus-agarose beads by heating at 95˚C for 5 minutes. Protein samples were resolved by SDS-PAGE and used for immunoblotting.

Using Trizol reagent (15596018, Invitrogen, Carlsbad, CA, USA), total RNA was isolated from wild-type MEFs and Akt1$^{-/-}$ MEFs. Reverse transcription was conducted to synthesize cDNA with RNA (2 µg) using ImProm-II™ Reverse Transcriptase. Quantitative PCR was carried out using StepOnePlus™ Real-Time PCR System (4376600, Applied Biosystems, Foster City, CA, USA) and Power SYBR Green PCR Master Mix (4367659, Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR results were analyzed by comparative cycle threshold (Ct) values and relative expression levels for target genes were normalized to that of β-actin. Primers for quantitative polymerase chain reaction (qPCR) were as follows: Mouse

Gcn5-F: 5′-ATTCCTGTCCATGCTTGAGG-3′
R: 5′-TCCAGGGTCAGGTTCTCAGG-3′ (195 bp)

β-*actin*-F: 5′-CATGTTTGAGACCTTCAACACCCC-3′
R: 5′-GCCATCTCCTGCTCGAAGTCTAG-3′ (318 bp)

For protein stability chase assay, wild-type MEFs and Akt1-null MEFs were treated with cycloheximide (CHX; 66-81-9, Sigma, St. Louis, MO, USA) at a concentration of 10 µg/ml for 1 to 8 hours or MG132 (1211877-36-9, Sigma, St. Louis, MO, USA) at a concentration of 20 µg/ml for 2 hours, followed by cell lysates isolation for western blot analysis. Quantification of protein bands was performed by using the ImageJ software v.1.8.0 (Wisconsin, U.S) according to the manufacturer's instructions and the measured values were displayed as a bar graph.

Data are represented as the mean values with the standard error of the mean (SEM). Statistically significant differences were determined by Student's t test. $P<0.05$ was considered statistically significant.

The *Gcn5* gene expression level was not significantly different between wild-type MEFs and Akt1-null MEFs, while the protein level was increased in the Akt1-null MEFs than in wild-type MEFs (Fig.1A, B). In addition, the results of the CHX (an inhibitor of de novo protein synthesis) chase assay showed that the Gcn5 protein half-life was about 4 hours in wild-type MEFs, whereas Gcn5 was stable for more than 8 hours in the Akt1-null MEFs (Fig.1C). Western blotting results of the CHX chase experiment were quantified (Fig.1D). These data suggest that Akt1 may deteriorate Gcn5 protein stability in the MEFs.

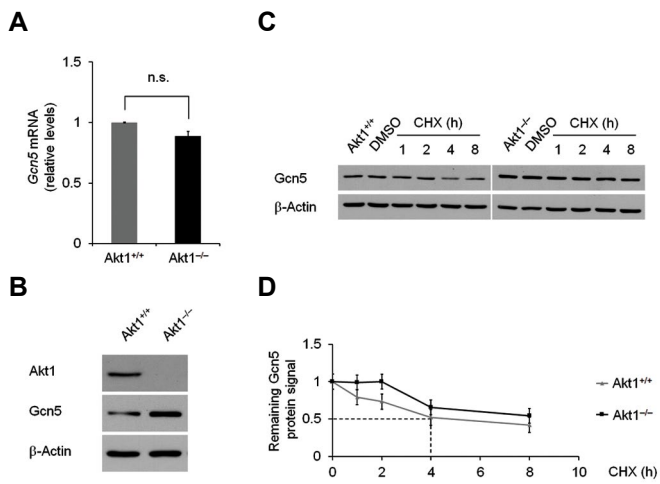

Fig.1: Gcn5 is post-translationally down-regulated by Akt1 in the MEFs. **A.** Real-time qPCR analysis for *Gcn5* transcription level detection in the wild-type and Akt1-null MEFs (P=0.0658). **B.** Western blotting analysis for detection of Gcn5 protein level in the wild-type and Akt1-null MEFs. Mouse β-actin was used as an internal control. **C.** Western blotting analysis of Gcn5 in the wild-type and Akt1-null MEFs after 10 µg/ml of cycloheximide treatment for 1, 2, 4, and 8 hours for Gcn5 protein stability. DMSO was used as a negative control. **D.** Quantification of immunoblotting results of cycloheximide chase which is calculated by using Image J software. MEFs; Mouse embryonic fibroblasts, qPCR; Quantitative polymerase chain reaction, DMSO; Dimethyl sulfoxide, and n.s.; Not significant.

To demonstrate that the degradation of Gcn5 occurs via the ubiquitin/proteasome pathway, we examined the effects of the proteasome inhibitor MG132 in the MEF cells. The stability of endogenous Gcn5 protein of wild-type MEFs was recovered by MG132 treatment (Fig.2). These results showed that the level of Gcn5 protein in the MEFs is controlled at the post-translational level through the ubiquitin/proteasome

pathway and Akt1 is involved in this process.

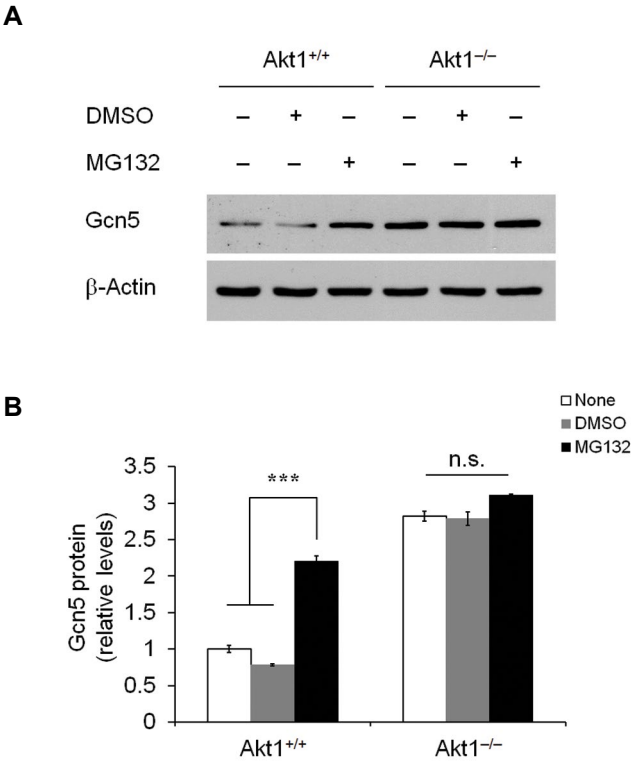

Fig.2: Gcn5 proteins are degraded in a proteasome-dependent manner in the MEFs. **A.** Western blotting analysis of Gcn5 in wild-type and Akt1-null MEFs after 20 μM of MG132 treatment for 2 hours for Gcn5 protein stability. DMSO was used as a negative control. **B.** Quantification of immunoblotting results of MG132 treatment which is measured by using Image J software. ***; P=7.28E-06, n.s.; Not significant, MEFs; Mouse embryonic fibroblasts, and DMSO; Dimethyl sulfoxide.

Cul4a mediates ubiquitination and degradation of specific substrates by constructing complexes with Ddb1 and ubiquitin ligase E3 (19). And-1, an HMG domain-containing protein, is known as a factor that associates with the Gcn5 protein stability in the cancer cells. So, we hypothesized that these protein complexes would be involved in the Gcn5 protein stability regulation in the MEFs. To figure out this hypothesis, we first examined the protein level of this complex in the MEF cells, wild-type and Akt1-null MEFs. The protein level of Cul4a-Ddb1 E3 ubiquitin ligase was decreased in the Akt1-null MEFs than the wild-type MEFs. Conversely, the And-1 was found to be elevated in the Akt1-null MEFs in compared with the wild-type MEFs (Fig.3A). Next, we performed co-immunoprecipitation experiments with anti-Gcn5 antibody to identify protein interactions between Gcn5 and these complexes in the wild-type and Akt1-null MEFs. The interaction between Gcn5 and Cul4a-Ddb1 complex was stronger than the wild-type MEFs in comparison with the Akt1-null MEFs. On the other hand, the interaction of Gcn5 with And-1 was inversely related to the Cul4a-Ddb1 complex. More interestingly, the ubiquitination of Gcn5 was only observed in the Akt1 wild-type MEFs, suggesting rapid Gcn5 protein degradation (Fig.3B). These observations demonstrated that And-1 contributes to Gcn5 protein stability via blocking the binding of Gcn5/Cul4a-Ddb1 complexes in the MEFs and is consistent with the previous report (20).

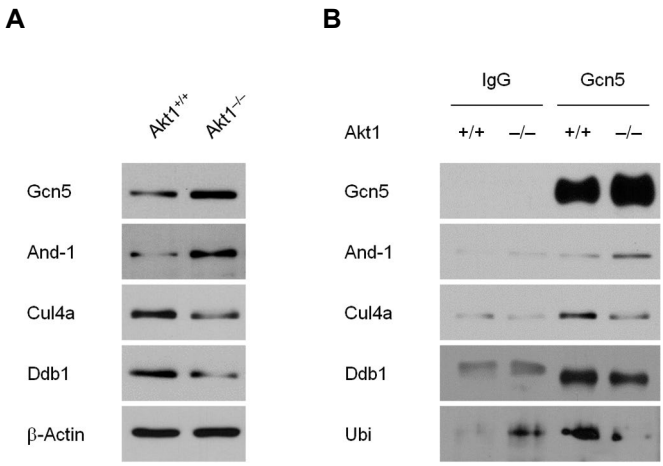

Fig.3: Degradation of Gcn5 protein is mediated by Cul4a-Ddb1 E3 ligase complex in the mouse embryonic fibroblasts (MEFs). **A.** The protein levels of Cul4a-Ddb1 E3 ubiquitin ligases and And-1 in the wild-type and Akt1-null MEFs. **B.** Co-immunoprecipitation of Gcn5 and Cul4a-Ddb1 E3 ubiquitin ligases in the wild-type and Akt1-null MEFs.

Recently, AKT has been shown to be directly involved in the ubiquitin-specific protease 4 (USP4) phosphorylation (21, 22). Zhang et al. (22) reported that USP4 contained the AKT consensus RXRXXpT phosphorylated site at Ser 445 through sequencing analysis. AKT-mediated phosphorylation of this motif resulted in increased USP4 stability and deubiquitylating enzymatic activity and also, relocated nuclear USP4 to the cytoplasm. On the other hand, previous reports revealed that phosphorylation on RXRXXpS/T Akt consensus motifs by Akt reduces HAT activity (23). More specifically, our previous study showed that mouse Gcn5 contains Akt consensus sequences [one RXRXXpS/T and several RXXpS/T sites] (15). Given these results, further investigation of phosphorylation may affect the Gcn5 protein regulation with Akt1 is suggested. In order to fully understand, Gcn5 regulation mechanism by Akt1 under specific conditions and its effect on downstream genes is important.

In conclusion, our data demonstrate the mechanism by which Akt1 regulates Gcn5 stability through the ubiquitin-proteasome pathway in MEFs.

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Authors' Contributions

D.S.J., J.H.O.; Experiments design, data analysis, and manuscript writing. D.S.J., Y.C.K.; *In vitro* studies performance. J.H.O., M.H.K.; Study management, supervision, and manuscript finalization. All authors discussed the results and commented on the manuscript and approved the final version.

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