

Original Article

Lysine residues 639 and 673 of mouse Ncoa3 are ubiquitination sites for the regulation of its stability

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Ncoa3 is a transcriptional coactivator involved in a wide range of biological processes. Regulation of Ncoa3 protein stability is important to control its activity precisely. Here, we found that deleting amino acid residues 614–740 of Ncoa3 enhances the protein expression level. Replacing two lysine residues, K639 and K673, within this region by arginine, increases the stability of the luciferase fusion protein as well as Ncoa3 protein. When these two lysine residues are mutated to arginine, the overall ubiquitination level of Ncoa3 decreases, indicating that lysine 639 and 673 are its ubiquitination sites. Taken together, we identified two ubiquitination sites at lysine 639 and 673 of Ncoa3. Ubiquitination of these two lysine residues leads to proteasomal degradation of Ncoa3.

Keywords Ncoa3; ubiquitination; protein stability; proteasomal degradation

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Introduction

Nuclear receptor coactivator 3 (Ncoa3, also known as Aib1, Src-3, Rac3, pCip and Actr), belonging to the p160 steroid receptor coactivator (SRC) family, is involved in transcriptional activation by nuclear receptors and other transcription factors [1]. It plays important roles in many developmental processes, including somatic growth [2], placental morphogenesis [3], female reproductive function, and mammary gland development [4]. Moreover, *NCOA3* is frequently over-expressed or amplified in various human tumors, such as breast, ovarian, prostate, pancreatic, and gastric cancers [1,5–8]. The oncogenic function of *Ncoa3* has been directly demonstrated in a transgenic mouse model over-expressing *Ncoa3* [9]. Recently, it has been reported that Ncoa3 is also required for pluripotency maintenance in mouse embryonic stem cells [10,11].

Given the importance of Ncoa3 in normal development as well as diseases, the transcriptional activity of Ncoa3 has to be tightly regulated. Indeed, various post-translational modifications, such as phosphorylation, sumoylation, methylation, ubiquitination, and acetylation, modulate the transcriptional activity of Ncoa3 protein [12–17]. In addition to transcriptional activity, the protein stability of Ncoa3 is also regulated by post-translational modifications, and the degradation of Ncoa3 is mediated by the ubiquitin–proteasome pathway as well [18–20]. For example, in MCF7 cells, phosphorylation of NCOA3 S505 by GSK3 promotes NCOA3 ubiquitination at lysine 723 and 786 by the E3 ubiquitin ligase Fbw7 α , and consequently the degradation of NCOA3 protein [12]. Similarly, CKI ϵ phosphorylates S101/S102 on NCOA3, thus enhancing NCOA3 ubiquitination by speckle-type POZ protein (SPOP) and degradation by proteasome [21]. CARM1-dependent arginine methylation of NCOA3 leads to fast turnover of NCOA3 protein [15].

To further understand the mechanism for regulating Ncoa3 protein stability, we constructed a series of Ncoa3 truncation mutants, and found that the deletion of amino acid residues 614–740 significantly enhances the expression level of truncated protein. We then fused this fragment with the luciferase gene, and found that the expression of luciferase fusion protein was decreased. Two lysine residues, K639 and K673, in this region are identified to be critical for protein stability regulation. Mutational analysis showed that these two lysine residues are ubiquitination sites, leading to proteasomal degradation upon ubiquitination.

Materials and Methods

Cell culture

HEK293T cells were maintained in growth medium consisting of 90% Dulbecco's modified Eagle's medium of high glucose (Invitrogen, Carlsbad, USA), 10% fetal bovine serum (Hyclone, Logan, USA), 2 mM L-glutamine, 5000 U/ml penicillin and streptomycin, and incubated in a 37°C incubator

(Thermo Fisher, Waltham, USA) with 5% CO₂ and 95% humidity.

Transfection

HEK293T cells were transfected with plasmid DNA using LipoFiter™ Liposomal Transfection Reagent (Hanbio, Shanghai, China) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were harvested for RNA isolation or protein lysate preparation.

Plasmids and site-directed mutagenesis

The full-length Ncoa3 over-expression vector was purchased from Thermo Fisher. The truncation mutants were amplified by polymerase chain reaction (PCR) and inserted into pCMV-Sport6 (Thermo Fisher). PCR cycling conditions were 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 120 s. To make the luciferase fusion reporter plasmids, fragments of *Ncoa3* were amplified by PCR and inserted into the *NcoI* site of pGL3 promoter vector (Promega, Madison, USA). PCR conditions were 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Site-directed mutagenesis was performed with PfuUltra II Fusion HS DNA Polymerase (Stratagene, Santa Clara, USA) following the manufacturer's instruction. Primers are listed in **Supplementary Table S1**.

Western blot analysis

Whole cell lysates were prepared in lysis buffer (Beyotime, Suzhou, China), and protein concentration was measured using BCA Protein Assay Kit (Beyotime). Extracts were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto a PVDF membrane (Millipore, Boston, USA). Membranes were probed with anti-Ncoa3 (Cell Signaling Technology, Danvers, USA), anti-β-tubulin (Huada, Beijing, China), and anti-GFP (Clontech, Mountain View, USA). Bound primary antibodies were recognized by HRP-linked secondary antibodies (GE Healthcare, Piscataway, USA). Immunoreactivity was detected by ECL Plus (Beyotime) and a Tanon 5500 chemiluminescence detection system (Tanon, Shanghai, China). β-Tubulin was used as a loading control.

Quantitative RT–PCR

Total RNA from cells was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using a TransScript II First-Strand cDNA Synthesis SuperMix Kit (Transgen, Beijing, China) according to manufacturer's instruction. Quantitative real-time PCR was performed with a SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) in an iQ5 system (Bio-Rad, Hercules, USA) under the following conditions: 95°C for 2 min, 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s, and then a melting curve of the amplified

DNA was acquired. Primers are listed in **Supplementary Table S1**. Quantification of target genes was normalized with *Actin*.

Luciferase reporter assay

HEK293T cells (1×10^5) were seeded in regular growth medium described above in 24-well plates, and transfected with 600 ng Ncoa3 fragment-luciferase fusion reporter plasmid and 8 ng pRL-SV40 (Promega) using LipoFiter™ Liposomal Transfection Reagent. Twenty-four hours after transfection, luciferase activities were measured with the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Immunoprecipitation

HEK293T cells were transfected with plasmids over-expressing Myc-Ubiquitin and Flag-Ncoa3 (wild type or mutants). Two days after transfection, cells were treated with 10 μM MG132 for 5 h and harvested. The cells were lysed in lysis buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.2% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM NaF, 10 mM NEM, 1 mM Na₃VO₄, and protease inhibitor mixture (Sigma, San Francisco, USA). The Flag-Ncoa3 protein was purified by anti-Flag M2 magnetic beads (Sigma) at 4°C overnight. The pellet was washed three times with lysis buffer, and the bound proteins were eluted by boiling for 10 min in 2 × SDS loading buffer. Western blot was performed to detect the proteins in IP samples with the anti-Myc (Sigma) and anti-Flag (Sigma) antibodies.

Statistical analysis

Each experiment was performed in triplicates. Data were analyzed by Student's *t*-test. $P < 0.05$ was considered of significant difference.

Results

Deleting the aa 614–740 region of Ncoa3 increases protein stability

The transcriptional activity of Ncoa3 is regulated at multiple levels, including protein stability. Previous studies have identified phosphodegron at S505–S509 and S101/S102, and ubiquitination sites at K723 and K786 on human NCOA3 [12,22]. To address whether other regions of Ncoa3 also contribute to protein stability regulation, we constructed two truncation mutants of mouse Ncoa3, the ΔA and ΔC mutants. The C-terminal activation domain (AD) was deleted in the ΔA mutant, whereas both the nuclear receptor interacting domain and the AD were truncated in the ΔC mutant (**Fig. 1A**). The ΔC protein is expressed at a higher level than the wild type (WT) and the ΔA protein in HEK293T cells (**Fig. 1B**). In contrast, the ΔC mRNA level is only slightly higher than the WT and the ΔA *Ncoa3*

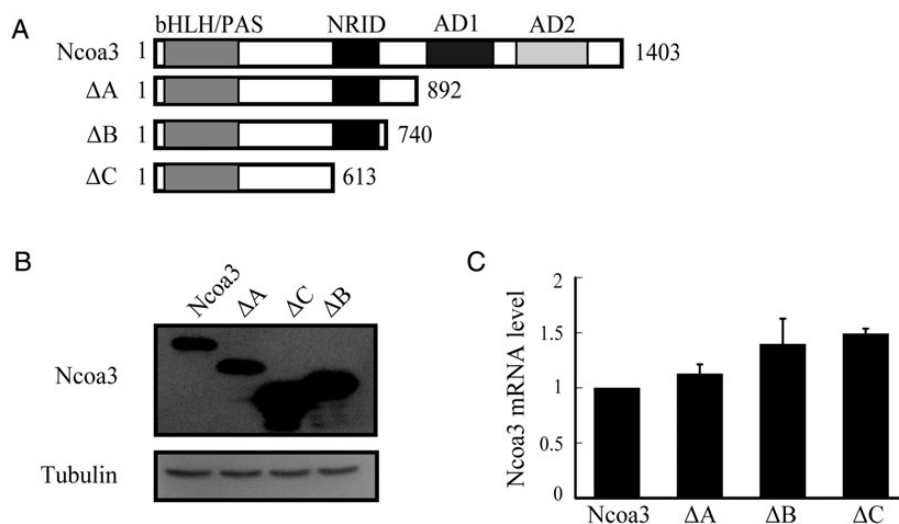


Figure 1. The expression of Ncoa3 truncation mutants (A) Schematic illustration of full-length Ncoa3 and truncation mutants of Ncoa3. (B) HEK293T cells were transfected by WT and truncation mutants of Ncoa3. Cells were harvested at 24 h after transfection, and subjected to protein extraction and western blotting. β -Tubulin serves as a loading control. (C) Cells as described in B were subject to quantitative RT-PCR to determine the mRNA levels of WT and truncation mutants of Ncoa3. Data were represented as mean \pm SD of three independent experiments.

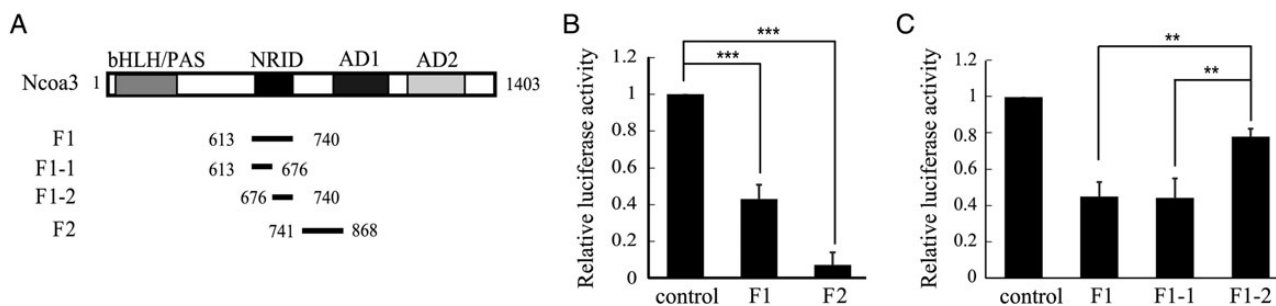


Figure 2. Peptide sequence aa 613–740 of Ncoa3 negatively regulates protein stability (A) To study the regulatory effect of different fragments of Ncoa3 protein, fusion genes containing fragments from Ncoa3, F1 (613–740), F1-1 (613–676), F1-2 (676–740) or F2 (741–848), and luciferase were constructed. (B,C) Empty pGL3 promoter vector or plasmids expressing luciferase fusion protein, together with pRV-SV40, were transfected into HEK293T cells. Luciferase activities were measured at 24 h after transfection. Data were represented as mean \pm SD of three independent experiments. $**P < 0.01$, $***P < 0.001$.

mRNA level (Fig. 1C). These data suggested that the region from aa 614 to 892 harbors sequences promoting protein degradation. The known ubiquitination site K723 on human NCOA3 is not conserved on mouse Ncoa3, while the other ubiquitination site K786 on human NCOA3 corresponds to K777 on mouse Ncoa3. To address whether the aa 614–892 region has additional protein degradation sequences other than K777, we further deleted the aa 741–892 region on the ΔA mutant, resulting in the ΔB mutant (Fig. 1A). In comparison to the WT and the ΔA mutant, deletion of the aa 741–892 region significantly enhances the ΔB protein expression, while the mRNA level remains stable (Fig. 1B,C). It is consistent with the known function of K786 on human NCOA3 (K777 on mouse Ncoa3). Yet, the ΔC protein level is still higher than that of the ΔB protein, suggesting that the

aa 614–740 region also contributes to negative regulation of protein stability (Fig. 1B).

Then we established a luciferase fusion protein reporter system to demonstrate the regulatory function of certain peptides on protein stability. Two peptide sequences, F1 (613–740) and F2 (741–868), are fused to the N-terminus of firefly luciferase (Fig. 2A). The fusion luciferase genes, as well as the luciferase WT gene, are transcribed from the same SV40 promoter. Presumably, the transcriptional activities of luciferase fusion genes are at the same level. Yet, the activity is reduced when F1 or F2 is fused to the luciferase gene (Fig. 2B). The reduced activity of F1- and F2-luciferase fusion protein is most likely due to the decreased protein stability. It remains possible that the fused peptides affect the activity of luciferase. However, when two lysine residues in

the F1-1 luciferase fusion gene. K639R or K673R alone modestly increases the luciferase activity, with no statistical significance. When both K639 and K673 are replaced with arginine, the luciferase activity is significantly elevated (**Fig. 3B**), implying that both lysine residues are involved in regulating protein stability. To demonstrate that K639 and K673 indeed affect the protein stability of Ncoa3, we introduced K639R and K673R mutations into the *Ncoa3* gene. Mutations of these two lysine residues do not affect the expression level of *Ncoa3* mRNA (**Fig. 3D**). Consistent with the luciferase fusion protein reporter result, individual mutation of K639 and K673 slightly enhances Ncoa3 protein expression, and simultaneous mutation of both K639 and K673 further elevates the expression level of Ncoa3 protein (**Fig. 3C**).

K639 and K673 are ubiquitination sites

Now we have proved that K639 and K673 play a role in negatively regulating Ncoa3 protein stability. Next, we asked whether these two lysine residues are involved in proteasomal degradation of Ncoa3. F1-1 luciferase fusion protein and the same fusion protein with K639R&K673R mutation were over-expressed in HEK293T cells. Before measuring the activities of luciferase, cells were treated with MG132 to inhibit proteasome. As shown in **Fig. 4A**, inhibition of proteasome enhances the WT F1-1 luciferase activity by ~2 folds, while MG132 treatment only modestly increases the K639R&K673R F1-1 luciferase activity by ~1.2 folds. These results indicated that K639 and K673 contribute to proteasomal degradation of Ncoa3 protein. We then addressed whether K639 and K673 are ubiquitination sites. To this end, as well as Myc-tagged ubiquitin, Flag-tagged WT and K639R&K673R Ncoa3 were over-expressed in HEK293T cells. Ncoa3 protein was immunoprecipitated with anti-Flag M2 beads, and the ubiquitination level was examined by anti-Myc immunoblot. Substitution of K639 and K673 with arginine reduces the ubiquitination level of Ncoa3 (**Fig. 4B**), suggesting that these two lysine residues are ubiquitination sites. Taken together, we have demonstrated that K639 and K673 of Ncoa3 protein are ubiquitination sites, and that ubiquitination of these two lysine residues promotes proteasomal degradation of Ncoa3 protein.

Discussion

We have demonstrated that the sequence aa 614–740 of Ncoa3 negatively regulates protein stability. Two lysine residues K639 and K673 within this region are ubiquitination sites, and facilitate proteasomal degradation of Ncoa3. Enhanced expression of NCOA3 has been observed in various tumors. Transcriptional activation and gene amplification are the major causes for NCOA3 overexpression in

tumors [1]. In addition, it is possible that mis-regulated protein stability could contribute to the up-regulation of NCOA3. It will be interesting to examine whether any NCOA3 mutations in human tumors affect NCOA3 protein stability. Two E3 ubiquitin ligases SPOP and Fbw7 α have been identified to ubiquitinate NCOA3 [12,21]. Whether these two E3 ligases or other E3 ligases catalyze the ubiquitination at K639 and K673 remains to be further explored.

Supplementary Data

Supplementary Data are available at *ABBS* online.

Acknowledgements

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References

- Xu J, Wu RC and O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. *Nat Rev Cancer* 2009, 9: 615–630.
- Wang Z, Rose DW, Hermanson O, Liu F, Herman T, Wu W and Szeto D, *et al.* Regulation of somatic growth by the p160 coactivator p/CIP. *Proc Natl Acad Sci USA* 2000, 97: 13549–13554.
- Chen X, Liu Z and Xu J. The cooperative function of nuclear receptor coactivator 1 (NCOA1) and NCOA3 in placental development and embryo survival. *Mol Endocrinol* 2010, 24: 1917–1934.
- Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C and O'Malley BW. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc Natl Acad Sci U S A* 2000, 97: 6379–6384.
- Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY and Sauter G, *et al.* AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 1997, 277: 965–968.
- Ghadimi BM, Schrock E, Walker RL, Wangsa D, Jauho A, Meltzer PS and Ried T. Specific chromosomal aberrations and amplification of the AIB1 nuclear receptor coactivator gene in pancreatic carcinomas. *Am J Pathol* 1999, 154: 525–536.
- Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, Masuda K and Kimura A, *et al.* Amplification and over-expression of the AIB1 nuclear receptor co-activator gene in primary gastric cancers. *Int J Cancer* 2000, 89: 217–223.
- Gnanaprasagam VJ, Leung HY, Pulimood AS, Neal DE and Robson CN. Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer. *Br J Cancer* 2001, 85: 1928–1936.
- Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M and Sellers WR, *et al.* High tumor incidence and activation of the PI3K/

- AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell* 2004, 6: 263–274.
10. Percharde M, Laval F, Ng JH, Kumar V, Tomaz RA, Martin N and Yeo JC, *et al.* Ncoa3 functions as an essential Esrrb coactivator to sustain embryonic stem cell self-renewal and reprogramming. *Genes Dev* 2012, 26: 2286–2298.
 11. Wu Z, Yang M, Liu H, Guo H, Wang Y, Cheng H and Chen L. Role of nuclear receptor coactivator 3 (ncoa3) in pluripotency maintenance. *J Biol Chem* 2012, 287: 38295–38304.
 12. Wu RC, Feng Q, Lonard DM and O'Malley BW. SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. *Cell* 2007, 129: 1125–1140.
 13. Wu RC, Qin J, Yi P, Wong J, Tsai SY, Tsai MJ and O'Malley BW. Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic responses to multiple cellular signaling pathways. *Mol Cell* 2004, 15: 937–949.
 14. Wu H, Sun L, Zhang Y, Chen Y, Shi B, Li R and Wang Y, *et al.* Coordinated regulation of AIB1 transcriptional activity by sumoylation and phosphorylation. *J Biol Chem* 2006, 281: 21848–21856.
 15. Naeem H, Cheng D, Zhao Q, Underhill C, Tini M, Bedford MT and Torchia J. The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation. *Mol Cell Biol* 2007, 27: 120–134.
 16. Chen H, Lin RJ, Xie W, Wilpitz D and Evans RM. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* 1999, 98: 675–686.
 17. Feng Q, Yi P, Wong J and O'Malley BW. Signaling within a coactivator complex: methylation of SRC-3/AIB1 is a molecular switch for complex disassembly. *Mol Cell Biol* 2006, 26: 7846–7857.
 18. Song X, Zhou T, Jia H, Guo X, Zhang X, Han P and Sha J. SProtP: a web server to recognize those short-lived proteins based on sequence-derived features in human cells. *PLoS One* 2011, 6: e27836.
 19. Yao T and Ndoja A. Regulation of gene expression by the ubiquitin–proteasome system. *Semin Cell Dev Biol* 2012, 23: 523–529.
 20. Schreiber A and Peter M. Substrate recognition in selective autophagy and the ubiquitin–proteasome system. *Biochim Biophys Acta* 2014, 1843: 163–181.
 21. Li C, Ao J, Fu J, Lee DF, Xu J, Lonard D and O'Malley BW. Tumor-suppressor role for the SPOP ubiquitin ligase in signal-dependent proteolysis of the oncogenic co-activator SRC-3/AIB1. *Oncogene* 2011, 30: 4350–4364.
 22. Li C, Liang YY, Feng XH, Tsai SY, Tsai MJ and O'Malley BW. Essential phosphatases and a phospho-degron are critical for regulation of SRC-3/AIB1 coactivator function and turnover. *Mol Cell* 2008, 31: 835–849.