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Role of Nuclear Receptor Coactivator 3 (Ncoa3) in Pluripotency Maintenance*[S]

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Background: Transcriptional regulation plays an important role in pluripotency maintenance.
Results: Knockdown of the nuclear receptor coactivator 3 (Ncoa3) compromises the pluripotency of mouse embryonic stem cells.
Conclusion: Ncoa3 binds to and activates the *Nanog* promoter, thus promoting self-renewal of mouse embryonic stem cells.
Significance: Uncovering the novel role and mechanism of Ncoa3 in pluripotency maintenance.

Nuclear receptors, including Esrrb, Dax1, and Nr5a2, have been shown to be involved in pluripotency maintenance. Yet, the role of their coactivators in mouse embryonic stem cells remains unexplored. Here, we demonstrated that the nuclear receptor coactivator 3 (Ncoa3) is essential for pluripotency maintenance. Knockdown of Ncoa3 not only compromises the expression of pluripotency markers but also impairs *in vitro* and *in vivo* differentiation potential of mouse ESCs. Ncoa3 binds to the *Nanog* promoter and recruits the histone acetyltransferase CREB binding protein (CBP) and the histone arginine methyltransferase CARM1 to activate *Nanog* expression. Moreover, glycogen synthase kinase 3 GSK3 signaling down-regulates the Ncoa3 protein level to suppress *Nanog* expression. Thus, Ncoa3 not only contributes to self-renewal by activating *Nanog* but also facilitates ESC differentiation as a break point to disrupt the core transcriptional circuitry of pluripotency.

Embryonic stem cells (ESCs)[2], derived from the inner cell mass (ICM) of blastocysts, are able to self-renew indefinitely while maintaining the differentiation potential into all types of cells in the body (1, 2). Transcription factors play important roles in pluripotency maintenance. It is well established that transcription factors Oct4, Sox2, and Nanog, act as core pluripotency regulators that form a transcriptional circuitry to promote the expression of pluripotency-associated genes and to suppress differentiation-related genes (3–7). In addition, nuclear receptors (NRs), including Esrrb, Dax1 (also known as Nr0b1), and Nr5a2, have been shown to regulate the expression of pluripotency factors and to be critical for pluripotency maintenance. Esrrb interacts with Nanog and Oct4 and activates *Nanog* and *Oct4* in ESCs (8–10). Moreover, overexpression of Esrrb allows ESCs to self-renew in the absence of leukemia inhibitory factor (LIF) (9). Dax1 enhances *Oct4* gene expression but inhibits the transcriptional activity of Oct4 protein (11–13). Interestingly, either knockdown or overexpression of Dax1 leads to ESC differentiation, suggesting a critical dose-dependent activity of Dax1 in ESC maintenance (11, 14). Nr5a2 is required for maintaining proper levels of *Oct4*, *Nanog*, and *Tbx3* in ESCs as well as for Oct4 expression at the epiblast stage of embryonic development (12, 15–17). The importance of NRs in pluripotency has been demonstrated further by their reprogramming activities in induced pluripotent stem cell derivation. Esrrb, together with Oct4 and Sox2, allows efficient derivation of iPS cells from mouse embryonic fibroblasts (MEFs) (8). Nr5a2 can replace Oct4 in reprogramming mouse somatic cells to the pluripotent state (15).

NR-mediated gene transcription usually requires nuclear receptor coactivators that are directly recruited by NRs to the promoter/enhancer regions of target genes. The three members of the p160 steroid receptor coactivator (SRC) family (Src1, Src2, and Src3, also known as Ncoa1, Ncoa2, and Ncoa3) are the first identified nuclear receptor coactivators (18–21) and have been implicated in somatic growth, reproductive function, uterine growth, blastocyst implantation, and mammary gland development as well as many types of cancers (22–28). After binding to target genes through the interaction with NRs or other transcription factors, SRCs recruit histone acetyltransferases and methyltransferases for chromatin remodeling and, consequently, activate gene expression. Three structural domains of SRCs are critical for their transcriptional activity. The amino-terminal basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain is the most conserved region, interacting

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[S] This article contains supplemental Figs. S1–S3, Tables S1–S4, and Experimental Procedures.

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[2] The abbreviations used are: ESC, embryonic stem cell; ICM, inner cell mass; NR, nuclear receptor; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts; SRC, steroid receptor coactivator; NRID, nuclear receptor interacting domain; CBP, CREB binding protein; EB, embryoid body; AD, activation domain; CREB, cAMP-responsive binding protein.

with several transcription factors such as myogenin, MEF2c, and transcriptional enhancer proteins (29–31). The central nuclear receptor interacting domain (NRID), containing three L*XX*LL motifs, mediates the interaction with NRs (32–34). The carboxyl terminus is composed of two transcriptional activating domains (AD1 and AD2). AD1 binds CREB binding protein (CBP) and the histone acetyltransferase p300, and AD2 interacts with coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine *N*-methyltransferase 1 (PRMT1) (35–37). Moreover, in response to several upstream signaling pathways, the transcriptional activities of SRCs are regulated by various posttranslational modifications, including phosphorylation, ubiquitylation, SUMOylation, acetylation, and methylation (38–45).

Despite the importance of NRs in pluripotency maintenance, the function of nuclear receptor coactivator in ESCs has not been fully investigated. Here, we studied the functions of the p160 family coactivators in mouse ESCs and showed that Ncoa3 is required for the expression of pluripotency genes in mouse ESCs. We further demonstrated that Ncoa3 is essential but not sufficient for pluripotency maintenance. Ncoa3 binds to the *Nanog* promoter and recruits CBP and CARM1, which in turn increase the levels of histone acetylation and histone arginine methylation at the *Nanog* promoter and activate *Nanog* expression. Moreover, Ncoa3 is essential for GSK3 signaling to repress *Nanog*. Taken together, Ncoa3 serves as a bridge to link transcription factors and epigenetic regulators as well as GSK3 signaling to regulate mouse ESC self-renewal and differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—V6.5 mouse ESCs were cultured in growth medium consisting of 85% DMEM (high-glucose, Invitrogen), 15% FBS (Hyclone), 2 mM L-glutamine, 5000 units/ml penicillin and streptomycin, 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1000 units/ml LIF (ESGRO, Chemicon). MEFs and NIH3T3 were cultured in growth medium consisting of 90% high-glucose DMEM, 10% FBS, 2 mM L-glutamine, and 5000 units/ml penicillin and streptomycin.

Embryo Culture—Female ICR mice (4–6 weeks) were induced to superovulate by intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (Calbiochem) and, 48 h later, 5 IU human chorionic gonadotropin (Sigma). Then females were paired with ICR males overnight and checked for vaginal plugs the following morning. Two-cell embryos were flushed from oviducts at 42–48 h post-human chorionic gonadotropin and cultured in groups of 20–30 in a 50-μl droplet of potassium simplex optimization medium with amino acids (Millipore) covered by mineral oil (Sigma, for embryo culture) in a 37 °C incubator with 6.5% CO_2.

Immunofluorescence—Embryos at desired stages were fixed in 4% paraformaldehyde for 20 min and then permeabilized with 0.2% Triton X-100 for 30 min. After blocking with 5% goat serum for 2 h, embryos were incubated with anti-Ncoa3 (Cell Signaling Technology) and anti-Cdx2 (Biogenex) antibodies overnight at 4 °C. Then embryos were washed and incubated with Alexa Fluor 488 anti-mouse and Alexa Fluor 594 anti-rabbit secondary antibodies (Molecular Probes). Hoechst 33342 (Sigma) was used for nucleus staining. Confocal images were captured with a Leica TCS SP5 confocal microscope.

Quantitative RT-PCR—Total RNA was extracted from cells using the RNeasy mini kit (Qiagen). cDNA synthesis was performed using the TransScript II First-Strand cDNA Synthesis SuperMix kit (Transgen) according to the instructions of the manufacturer. PCR reactions were performed with SYBR Green Real-time PCR MasterMix (TOYOBO) in a Bio-Rad iQ5 system. PCR cycling conditions were 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30s, and then a melting curve of the amplified DNA was acquired. Quantification of target genes was normalized with β-actin. Primer sequences are shown in supplemental Table S1.

Western Blot Analysis—Cells were lysed in lysis buffer (Beyotime), and protein concentration was measured using a BCA protein assay kit (Beyotime) to ensure equal loading. The samples were resolved by SDS-PAGE, followed by transfer onto a PVDF membrane (Millipore). Membranes were probed with anti-SRC3 (Cell Signaling Technology), anti-β-tubulin (Huada, Beijing), anti-Nanog, anti-CARM1 (Bethyl Laboratories), anti-SRC1, anti-SRC2, anti-Oct3/4, anti-CBP (Santa Cruz Biotechnology), and anti-Esrrb (R&D Systems). Bound primary antibodies were recognized by HRP-linked secondary antibodies (GE Healthcare). Immunoreactivity was detected by ECL Plus (Beyotime) and Kodak light film. Digital images of films were taken with Bio-Rad Molecular Imager Gel Doc XR. The intensity of bands was quantified with the Quantity One analysis software (Bio-Rad).

Alkaline Phosphatase Staining—Ncoa3 knockdown or control ESCs cultured on feeder were fixed in 3.7% formaldehyde solution for 30 min at room temperature. Cells were washed once with PBS solution and incubated with alkaline phosphatase substrate kit III (Vector) for 20 min at room temperature.

Embryoid Body Formation—Control and Ncoa3 knockdown ESCs were cultured in suspension in ESC medium without LIF in Petri dishes for 3 days and then plated on gelatin-coated tissue culture dishes. Cells were collected at indicated points.

Teratoma Formation—MEF cells, control, and Ncoa3 knockdown ESCs were harvested by trypsin treatment and resuspended in their culture medium accordingly. 1.5×10^6 cells in 150 μl were subcutaneously injected to the dorsolateral site. Every cell line was injected into three mice.

Luciferase Reporter Assay—The luciferase reporter containing the 6-kb *Nanog* promoter was constructed previously (46). A 250-bp *Nanog* promoter DNA fragment, immediately upstream of the *Nanog* transcription initiation site, was amplified by PCR and inserted into the pGL3 vector (Promega) to make the 250-bp *Nanog* reporter. V6.5 ESCs were seeded at a density of 1×10^5 cells per well in 24-well plates and transfected using Lipofectamine 2000 (Invitrogen) with the *Nanog* reporter plasmid (200 ng) and pRV-SV40 (8 ng, Promega) together with 600-ng control, Ncoa3 knockdown, or overexpression plasmids. At 24 h after transfection, luciferase activities were measured with the dual-luciferase reporter assay system (Promega) according to the instructions of the manufacturer. Each experiment was performed in triplicate and repeated three times.

Chromatin Immunoprecipitation—6×10^7 cells were cross-linked with 1% formaldehyde and sonicated to an average size of 0.5–1 kb. Chromatin extract from 1×10^7 cells was used for each immunoprecipitation. Anti-Ncoa3 (Santa Cruz Biotechnology), anti-CARM1 (Bethyl Laboratories), anti-CBP (Santa Cruz Biotechnology), anti-acetyl-histone H3 (Upstate), or anti-H3R17 dimethylation antibodies (Abcam) were used. Purified ChIP DNA and input DNA were analyzed by real-time PCR. Fold enrichments were calculated by comparing ChIP DNA to input DNA and normalized with the actin level defined as 1. Primer sequences are shown in supplemental Table S2.

Coimmunoprecipitation—V6.5 ESCs were collected, and cell extracts were prepared in lysis buffer (150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris (pH 8.0), 1 mM PMSF) with protease inhibitor (Roche). The cell extract was precleared using protein A-Sepharose (GE Healthcare) at 4 °C for 2 h. The supernatant was incubated with anti-Ncoa3 antibody (Cell Signaling Technology) or rabbit IgG (Santa Cruz Biotechnology) at 4 °C overnight. Then protein A-Sepharose was added and mixed for 4 h. After washing the pellet three times with lysis buffer, the bound proteins were released from the protein A-Sepharose by boiling for 5 min in 2× SDS loading buffer. A Western blot analysis was performed to detect the proteins present in the immunoprecipitation samples.

RNAi and Generation of Stable Ncoa3 Knockdown or Overexpression ESC Lines—Gene knockdown was performed with the pSuper-puro system (Oligoengine) following the instructions of the manufacturer. Briefly, for the expression of small hairpin RNA targeting a specific gene, a 60-nt oligo containing the specific 19-nt targeting sequence was cloned into the BglII/HindIII site of the pSuper-puro vector. A control plasmid targeting GFP was constructed at the same time. The 19-nt targeting sequences used in this study are listed in supplemental Table S3. The mouse *Ncoa3* expression vector pSport-Ncoa3 was purchased from Thermo Fisher. The *Ncoa3* coding region from pSport-Ncoa3 was cloned into the pCAGIPuro expression plasmid (a gift from Dr. Hitoshi Niwa) to make pCAGIPuro-Ncoa3. Empty pCAGIPuro, Ncoa3 overexpression, shGFP, shN3-1, or shN3-2 plasmids were transfected into V6.5 ESCs with Lipofectamine 2000. After 7- to 10-day puromycin selection, single clones were picked and tested for knockdown or overexpression efficiency by quantitative RT-PCR.

Microarray Analysis—V6.5 ESCs were transfected by shGFP control, shN3-1, or shN3-2 plasmids. At 48 h after transfection, cells were harvested for RNA purification. Hybridization and scanning of the chips (Affymetrix mouse genome 430 2.0 array) was performed as outlined in the Affymetrix technical manual by CapitalBio Corp., Beijing, China. The gene ontology analysis was performed with a molecule annotation system 3.0 (MAS 3.0)

Statistical Analysis—Data were analyzed by Student's *t* test. Statistically significant *p* values were indicated in figures as follows: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

RESULTS

Ncoa3 Is Required for Pluripotency Maintenance—To explore the potential role of the p160 family coactivators in pluripotency maintenance, we performed shRNA knockdown experiments in mouse ESCs and quantified the expression levels of the pluripotency genes *Nanog*, *Oct4*, and *Sox2*. Despite knockdown efficiencies of the *Ncoa1*, *Ncoa2*, and *Ncoa3* mRNAs varied to some extent, high efficient knockdown of Ncoa1, Ncoa2, and Ncoa3 proteins was achieved (Fig. 1, *A–D*). Knockdown of *Ncoa1* or *Ncoa2* only slightly reduced the expression of *Oct4* and *Sox2* but not *Nanog*. Moreover, not all three shRNA plasmids targeting Ncoa1 or Ncoa2 consistently affected *Oct4* and *Sox2* expression (Fig. 1, *A* and *B*). Therefore, Ncoa1 and Ncoa2 are not required for pluripotency maintenance in mouse ESCs. In contrast, down-regulation of *Ncoa3* impaired *Nanog*, *Oct4*, and *Sox2* expression, suggesting that Ncoa3 is required for pluripotency maintenance (Fig. 1*C*).

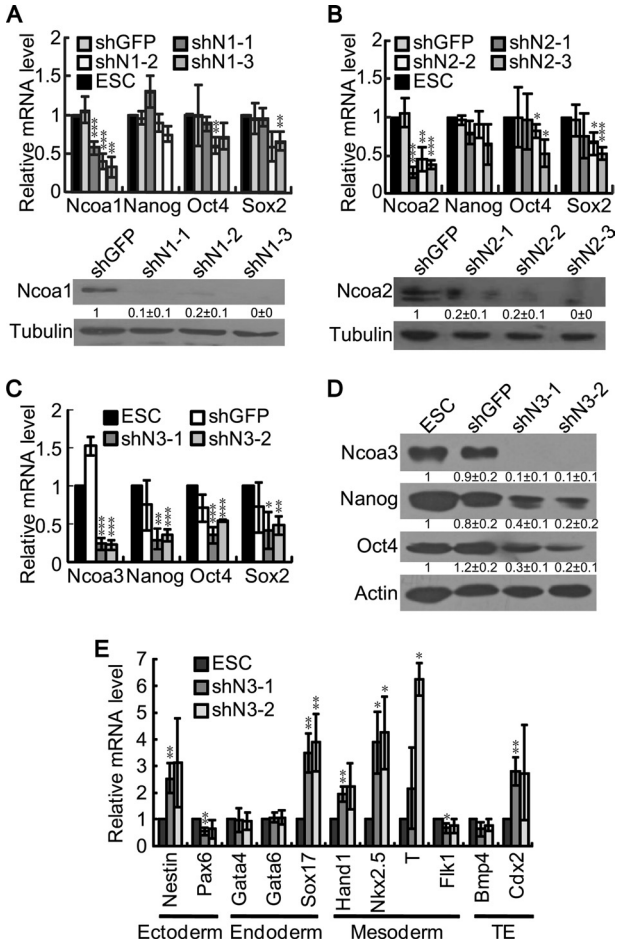

FIGURE 1. **Down-regulation of *Ncoa3* compromises the expression of pluripotency markers.** *A–C*, V6.5 ESCs were transfected by pSuper-puro empty vector, shGFP control, or shRNA plasmids targeting Ncoa1 (*A*), Ncoa2 (*B*), or Ncoa3 (*C*). Cells were harvested 48 h after transfection and subjected to RNA purification and cDNA synthesis as well as protein extraction. The knockdown efficiency on *Ncoa1*, *Ncoa2*, and *Ncoa3* and the expression levels of the pluripotency genes *Nanog*, *Oct4*, and *Sox2* were examined by real-time PCR with normalization to β-actin. A Western blot analysis was performed to detect the expression of Ncoa1 and Ncoa2. Tubulin served as a loading control. Quantification results of the Western blot analysis are shown below corresponding bands as mean ± S.D. ($n = 3$). *D*, V6.5 ESCs were transfected as described in *C*. The expression of Ncoa3, Nanog, and Oct4 proteins were examined by Western blot analysis. β-Actin served as a loading control. Quantification results of the Western blot analysis were shown below corresponding bands as mean ± S.D. ($n = 3$). *E*, V6.5 ESCs were transfected as described in *C*. Quantitative RT-PCR was carried out to determine the expression levels of differential markers upon *Ncoa3* knockdown; *TE*, trophectoderm. Averages and S.D. from three independent experiments are plotted. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

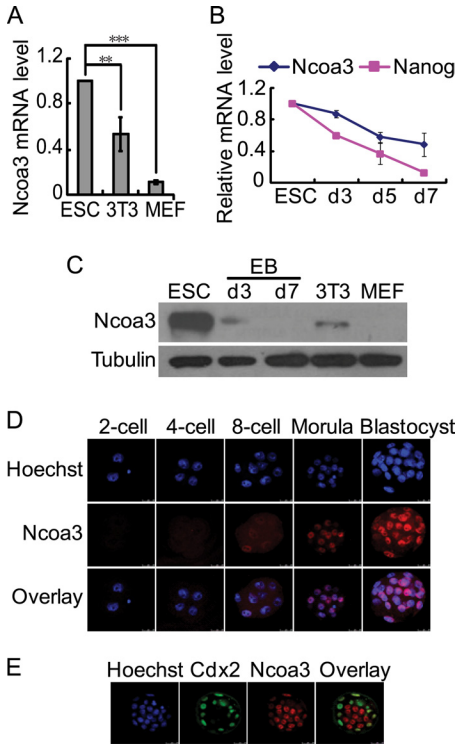

FIGURE 2. **Ncoa3 expression is associated with pluripotent cells.** *A*, mRNA levels of endogenous *Ncoa3* in V6.5 ESCs, NIH3T3, and MEFs. **, $p < 0.01$; ***, $p < 0.001$. *B*, the expression pattern of *Nanog* and *Ncoa3* mRNA in ESCs and day 3, day 5, and day 7 EBs. Means ± S.D. from three independent experiments are plotted. *C*, Western blot analysis of Ncoa3 in V6.5 ESCs, NIH3T3, and MEFs as well as day 3 and day 7 EBs. β-tubulin served as a loading control. *D*, expression dynamics of Ncoa3 during early embryogenesis. Mouse embryos from the two-cell to the blastocyst stages were immunostained with anti-Ncoa3 antibody. *E*, reciprocal expression of Ncoa3 and Cdx2 in blastocysts. Blastocysts were immunostained for Cdx2 (*green*) and Ncoa3 (*red*). Hoechst 33342 staining (*blue*) marked the nucleus. The overlay picture of Cdx2 and Ncoa3 is displayed. Confocal images are shown.

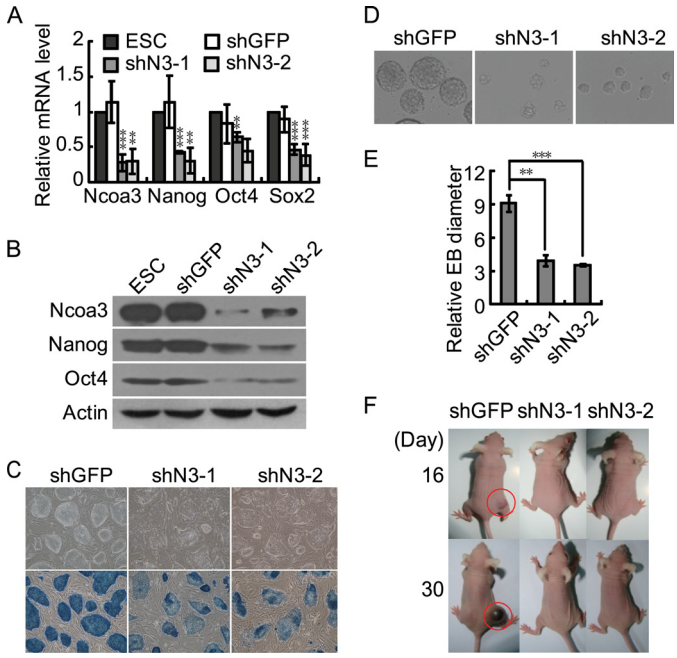

FIGURE 3. **Ncoa3 depletion impairs ESC differentiation *in vitro* and *in vivo*.** *A*, reduced expression of pluripotency genes in two stable Ncoa3 knockdown ESC lines. Stable shGFP control and Ncoa3 knockdown ESC lines were established by transfection of shGFP or shRNA plasmids targeting Ncoa3 (shN3-1 and shN3-2) into V6.5 ESCs, followed by puromycin selection. The expression levels of *Ncoa3*, *Nanog*, *Oct4*, and *Sox2* in these cell lines were quantified with quantitative RT-PCR. Means ± S.D. from three independent experiments were plotted. *B*, Western blot analysis showed that the protein levels of Ncoa3, Nanog, and Oct4 in stable Ncoa3 knockdown ESC lines were decreased. β-Actin served as a loading control. *C*, alkaline phosphatase staining in stable shGFP and Ncoa3 knockdown ESCs. *D*, phase contrast images of day 2 EBs formed by stable shGFP and Ncoa3 knockdown ESCs. The size of day 2 Ncoa3 knockdown EBs was smaller than that of shGFP control EBs. *E*, 20 each of randomly selected shGFP and Ncoa3 knockdown EBs were scored, and their relative diameters from images taken under identical magnification were calculated. The means ± S.D. of relative diameters are presented. *F*, representative pictures of mice at day 16 and day 30 after injection of ESCs. Stable shGFP and Ncoa3 knockdown ESCs were injected subcutaneously into nude mice. Teratoma (*red circles*) formed by shGFP ESCs were observed as early as 16 days after injection. However, no teratoma was formed from shN3-1 or shN3-2 ESCs up to 30 days. **, $p < 0.01$; ***, $p < 0.001$.

Western blot analysis further confirmed that Ncoa3 depletion reduced the expression of Nanog and Oct4 proteins (Fig. 1*D*). In addition to decreased expression of pluripotency genes, differentiation markers, including *Nestin* for ectoderm; *Sox17* for endoderm; *Hand1*, *Nkx2.5*, and *T* for mesoderm; and *Cdx2* for trophectoderm, were activated in *Ncoa3* knockdown cells, indicating a random differentiation of these cells (Fig. 1*E*). Taken together, knockdown of Ncoa3 in ESCs led to reduced expression of pluripotency markers and elevated expression of differentiation markers, suggesting a pivotal role of Ncoa3 in pluripotency maintenance.

We then examined the expression of *Ncoa3* in ESCs and differentiated cells as well as developing embryos. The *Ncoa3* mRNA level was expressed at a higher level in ESCs than in MEFs and NIH3T3 cells (Fig. 2*A*). As ESCs differentiated into embryoid bodies (EBs), *Ncoa3* mRNA expression dropped gradually (Fig. 2*B*). Ncoa3 protein was detected by Western blot analysis in undifferentiated ESCs at a high level and in NIH3T3 cells and day 3 EB cells at much lower levels but not in MEFs or day 7 EB cells (Fig. 2*C*). A more dramatic change of Ncoa3 at protein level than at mRNA level suggested that Ncoa3 is not only regulated at transcriptional level but also at posttranscriptional steps. During early embryogenesis, Ncoa3 protein was first detected in some blastomeres at the eight-cell embryo stage and was present in all cells in morula and blastocysts (Fig. 2*D*). However, in blastocysts, Ncoa3 was apparently expressed at a higher level in ICM cells than in trophectodermal cells (Fig. 2*E*). These data were consistent with the role of Ncoa3 in pluripotency maintenance.

Depletion of Ncoa3 Compromises the in Vitro and in Vivo Differentiation Potential of Mouse ESCs—To further investigate whether Ncoa3 is essential for pluripotency maintenance in ESCs, multiple stable Ncoa3 knockdown ESC lines were established with two shRNA plasmids targeting *Ncoa3* (shN3-1 and shN3-2). Consistent with transient transfection result, the expression levels of pluripotency markers *Nanog*, *Oct4*, and *Sox2* decreased in stable Ncoa3 knockdown ESC lines (Fig. 3, *A* and *B*). Meanwhile, these Ncoa3 knockdown ESC lines displayed flattened colony morphology as well as reduced alkaline phosphatase staining, suggesting a compromised pluripotent state (Fig. 3*C*). We then focused on two stable Ncoa3 knockdown cell lines derived with different plasmids, shN3-1 and shN3-2, to evaluate their *in vitro* and *in vivo* differentiation potential. EBs formed by Ncoa3 knockdown cells were smaller

than EBs from control ESCs (Fig. 3, *D* and *E*). To understand how knockdown of Ncoa3 affects EB size, we first analyzed the expression of differentiation markers in ESCs and day 2 EB cells. In contrast to transient knockdown of Ncoa3 in ESCs (Fig. 1*E*), most differentiation markers, except for *T*, were not up-regulated in stable Ncoa3 knockdown ESC lines (supplemental Fig. S1). However, many differentiation markers, including *Nestin*, *Sox17*, *Nkx2.5*, *Flk1*, *Bmp4*, and *Cdx2*, were expressed higher in day 2 Ncoa3 knockdown EBs compared with control shGFP EBs (supplemental Fig. S1). These data implied that during the selection and maintenance of stable knockdown ESCs, ESCs could compensate for the loss of Ncoa3 and suppress the expression of differentiation markers. Yet, because of the reduced expression of Ncoa3 as well as pluripotency genes, stable Ncoa3 knockdown ESCs have a stronger tendency to differentiate. The accelerated differentiation may be one reason for the reduced EB size. In addition, EB size could be affected by both cell death and proliferation. We then examined the rate of apoptosis and necrosis in both ESCs and day 2 EBs by annexin V and propidium iodide staining. The results showed that knockdown of Ncoa3 did not increase cell death in both ESCs and EBs (supplemental Fig. S2). Microarray and gene ontology term analysis revealed that knockdown of Ncoa3 affects the expression of many genes involved in cell proliferation regulation (Fig. 5*A*). Indeed, some of these genes, such as *Nanog* and *Tbx3*, are also involved in pluripotency maintenance. Thus, compromised pluripotency, accelerated differentiation, and reduced cell proliferation are tightly connected. All these factors contribute to the smaller size of Ncoa3 knockdown EBs.

More importantly, when subcutaneously injected into immune-deficient mice, Ncoa3 knockdown ESCs did not support teratoma formation up to 30 days, whereas teratoma formed by control ESCs were observed as early as 16 days (Fig. 3*F*), indicating a compromised pluripotency of Ncoa3-deficient ESCs.

Ncoa3 Is Insufficient to Maintain ESC Self-renewal in the Absence of LIF—So far, we have demonstrated that Ncoa3 is critical for pluripotency maintenance in ESCs. We then asked whether forced expression of Ncoa3 is sufficient to maintain ESC self-renewal in the absence of LIF. Several stable Ncoa3 overexpression ESC lines were established. Elevated Ncoa3 expression was confirmed at both mRNA and protein levels in Ncoa3 overexpression ESCs (Fig. 4, *A* and *B*). Nevertheless, colony morphology and pluripotency markers expression were not affected by Ncoa3 overexpression (Fig. 4, *C* and *D*). When cultured on gelatin-coated dishes after LIF withdrawal, Ncoa3 overexpression ESCs differentiated similar to control ESCs containing empty vector, with flattened colony morphology and decreased expression of pluripotency markers (Fig. 4, *C* and 4*D*). Therefore, Ncoa3 is essential but not sufficient for pluripotency maintenance.

Ncoa3 Binds to and Activates the Nanog Promoter—To further understand how Ncoa3 regulates pluripotency, microarray experiments were carried out to characterize the change of the global expression profile upon Ncoa3 knockdown in mouse ESCs. 311 genes were up-regulated, and 398 genes were down-regulated more than 1.5-fold by both shN3-1 and shN3-2 (supplemental Table S4). Gene ontology term analysis revealed that

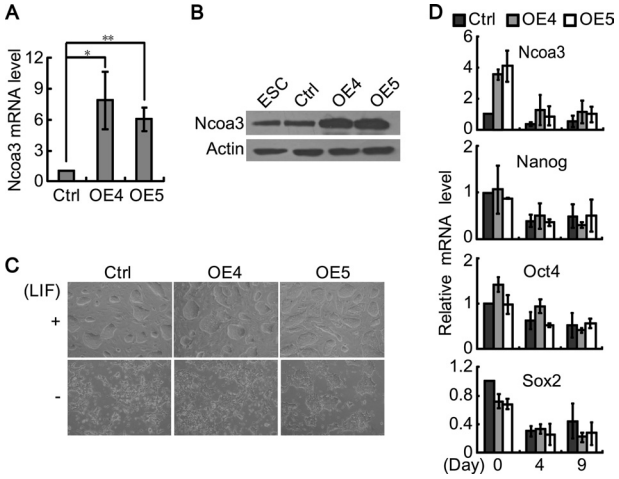

FIGURE 4. **Ncoa3 is insufficient to maintain the self-renewal of ESCs.** *A*, stable control and Ncoa3 overexpression (*OE*) ESC lines were established by transfection of pCAGIPuro or pCAGIPuro-Ncoa3 plasmids into V6.5 ESCs, followed by puromycin selection. The expression levels of *Ncoa3* in these cells were quantified with quantitative RT-PCR. Means ± S.D. from three independent experiments were plotted. *, $p < 0.05$; **, $p < 0.01$. *B*, Western blot analysis of Ncoa3 in stable control and Ncoa3 overexpression ESC lines. β-Actin served as a loading control. *C*, phase contrast images of control and Ncoa3 overexpression ESCs grown with or without LIF at day 2. *D*, quantitative RT-PCR analyzed the expression levels of *Ncoa3*, *Nanog*, *Oct4*, and *Sox2* in control and Ncoa3 overexpression ESCs cultured without LIF at days 0, 4, and 9. Means ± S.D. from three independent experiments were plotted.

genes affected by Ncoa3 knockdown are enriched for genes encoding transcription factors and genes involved in stem cell maintenance (Fig. 5*A*). Among the 399 down-regulated genes, five genes, *Esrrb*, *Klf4*, *Nanog*, *Dax1*, and *Tbx3*, have been shown to be involved in pluripotency maintenance (4, 5, 8–10, 12, 13, 47, 48). To identify direct target gene(s) of Ncoa3 among these pluripotency genes, ChIP was carried out to examine the occupancy of Ncoa3 at these genes as well as core pluripotency genes, including *Oct4* and *Sox2*. Indeed, the binding of Ncoa3 was only detected at the promoter of *Nanog* but not the promoters of other tested pluripotency genes (Fig. 5*B*). Transient overexpression of Ncoa3 enhanced the level of endogenous *Nanog* mRNA (Fig. 5*C*). Luciferase assays using a 6-kb *Nanog* promoter reporter also demonstrated that overexpression of Ncoa3 enhanced the activity of the *Nanog* reporter, whereas knockdown of Ncoa3 suppressed the *Nanog* promoter activity (Fig. 5, *D* and *E*).

Three known structural domains of Ncoa3, bHLH/PAS, NRID, and C-terminal activation domains, are critical for the activity of Ncoa3. The bHLH/PAS and NRID domains mediate the interaction with transcription factors and nuclear receptors, respectively (29–34). The C terminus activation domain is composed of two transcriptional activating domains, AD1 and AD2, which are responsible for the recruitment of the histone acetyltransferases CBP and p300 and the histone arginine methyltransferase CARM1 and PRMT1, respectively (35–37). To identify the crucial motifs of Ncoa3 for *Nanog* activation, we constructed four truncation mutants of Ncoa3, deleting the bHLH/PAS domain (ΔN), the NRID domain (ΔNRID), the AD1/AD2 domains (ΔAD), or the C-terminal end (ΔC) separately. Overexpression of the ΔNRID and the ΔAD mutants failed to activate the *Nanog* promoter, whereas the ΔN and the ΔC mutants activated the *Nanog* reporter activity to the same

A

GO analysis of genes regulated by Ncoa3

GO Term	Count	p-Value	Representative genes
nucleus	206	2.06E-33	Dax1,Prmt8, Notch,Gli2,Prmt8
protein binding	261	8.10E-29	Notch4, Bmp4, Dnmt3a, Trim23, Ube2n,Rnf125
transcription factor activity	59	2.42E-15	Klf4, Dax1, Tbx3, Esrrb, Nr5a2, Nanog, Pou4f2
zinc ion binding	95	4.86E-14	Ebf1, Wnt8b, Fgf5, Lefty1/2, Bmp8b, Ebf1, Foxp1,
regulation of transcription, DNA-dependent	80	1.49E-08	Jmjd1a, Pitx2, Nkx2.2
stem cell maintenance	6	9.30E-06	Klf4, Esrrb, Nanog, Tcl1
positive regulation of cell proliferation	21	2.41E-06	Tbx3, Nanog, Tgfb2, Tnfsf12
embryonic development	14	6.12E-04	Ttn, Tbx3, Gna13, Ube2a

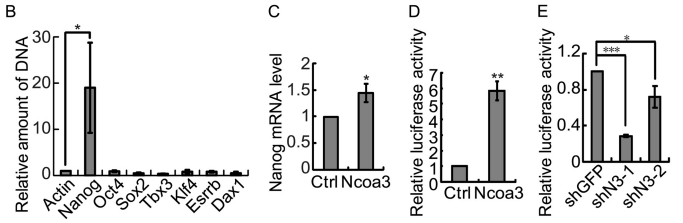

FIGURE 5. **Ncoa3 binds to and activates the *Nanog* promoter.** *A*, GO term analysis of genes regulated by Ncoa3 knockdown in ESCs. *B*, chromatin immunoprecipitation in V6.5 ESCs with anti-Ncoa3 antibody demonstrated that Ncoa3 binds to the *Nanog* promoter but not the promoters of *Sox2* or *Oct4*. *C*, overexpression of Ncoa3 enhanced endogenous *Nanog* expression. V6.5 ESCs were transfected by empty pCAGIPuro vector or pCAGIPuro-Ncoa3 overexpression plasmid. Twenty-four hours after transfection, cells were harvested. Nanog expression was measured by quantitative RT-PCR. *D*, luciferase assay showed that overexpression of Ncoa3 increased the *Nanog* promoter activity. Empty pCAGIPuro vector or pCAGIPuro-Ncoa3 overexpression plasmid, together with pRV-SV40 and the *Nanog* promoter reporter plasmids, were cotransfected into V6.5 ESCs. Luciferase activities were measured 24 h after transfection. Means ± S.D. from three independent experiments were plotted. *E*, knockdown of Ncoa3 suppresses the *Nanog* promoter activity. A luciferase assay was performed as described in *B*, except that shGFP control or two shNcoa3 plasmids (shN3-1 and shN3-2) were used. Means ± S.D. from three independent experiments were plotted. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

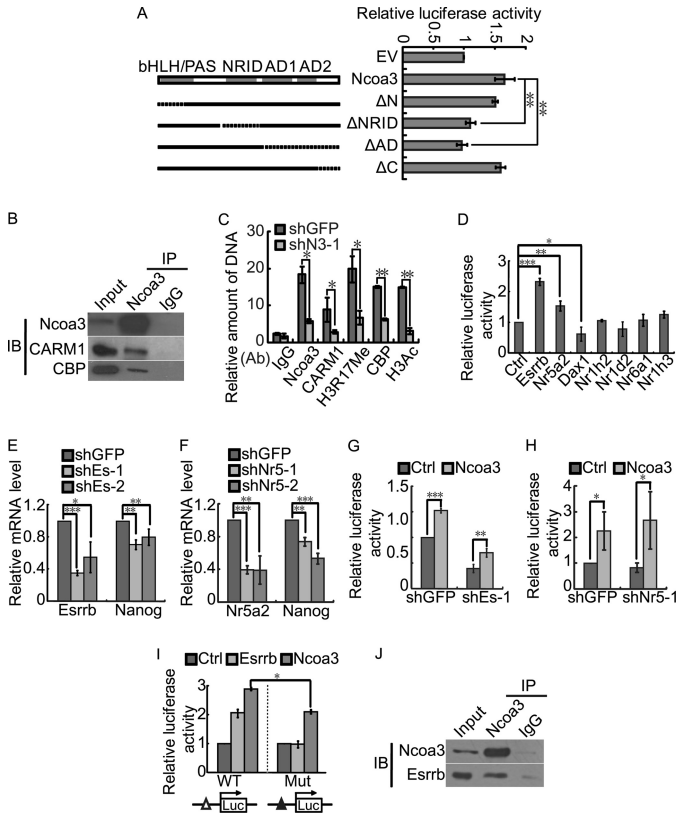

FIGURE 6. **Ncoa3 recruits histone acetyltransferase CBP and histone arginine methyltransferase CARM1 to activate *Nanog*.** *A*, schematic illustration of Ncoa3 deletion mutants (*left panel*). V6.5 ESCs were cotransfected with the *Nanog* promoter reporter, pRV-SV40, and pSPORT plasmids expressing WT or Ncoa3 deletion mutants. Luciferase activities were measured 24 h after transfection (*right panel*). *EV*, empty vector; Δ*N*, deletion of the N-terminal region; Δ*NRID*, deletion of the nuclear receptor interacting domain; Δ*AD*, deletion of the activation domains; Δ*C*, deletion of the C-terminal of Ncoa3. Means ± S.D. from three independent experiments were plotted. *B*, immunoprecipitation was performed with V6.5 ESC protein extract and Ncoa3 antibody or rabbit IgG. The immunoprecipitation (*IP*) samples were then immunoblotted (*IB*) with Ncoa3, CARM1, and CBP antibodies. *C*, ChIP was performed in stable shGFP and Ncoa3 knockdown (shN3-1) ESCs with anti-Ncoa3, anti-CARM1, anti-CBP, anti-acetyl-Histone H3, or anti-H3R17 dimethylation antibodies (*Ab*). *D*, Esrrb and Nr5a2 activated the *Nanog* promoter. Empty vector, Esrrb, Nr5a2, Dax1, Nr1h2, Nr1d2, Nr6a1, or Nr1h3 overexpression plasmid, together with pRV-SV40 and the *Nanog* promoter reporter plasmids, were cotransfected into V6.5 ESCs. Luciferase activities were measured 48 h after transfection. *E* and *F*, V6.5 ESCs were transfected by shGFP control or shRNA plasmids targeting Esrrb (*E*, shEs-1, 2) or Nr5a2 (*F*, shNr5–1, 2). Cells were harvested 48 h after transfection. Knockdown efficiency and *Nanog* expression were examined by real-time PCR. *G* and *H*, V6.5 ESCs were transfected by pRV-SV40 and the *Nanog* promoter reporter plasmids, shGFP control, shEs-1 (*G*), or shNr5–1(*H*) plasmids, together with empty pCAGIPuro vector or pCAGIPuro-Ncoa3. Luciferase activities were measured at 48 h after transfection. *I*, A 250-bp Nanog promoter containing a known Esrrb binding site (△) was used. V6.5 ESCs were transfected by pRV-SV40 together with empty pCAGIPuro control, Esrrb, or Ncoa3 overexpression plasmids and the wild-type or mutant 250-bp *Nanog* promoter reporter plasmids. Luciferase activities were measured 48 h after transfection. Mutation of the Esrrb binding site (▲) not only abolished the activation effect of Esrrb on the *Nanog* reporter but also compromised the reporter activation by Ncoa3. *J*, immunoprecipitation was performed with V6.5 ESC protein extract and Ncoa3 antibody or rabbit IgG. The immunoprecipitation samples were then immunoblotted with Ncoa3 and Esrrb antibodies. Means ± S.D. from three independent experiments were plotted. *, $p < 0.05$; **, $p < 0.01$.

extent as WT Ncoa3 (Fig. 6*A*). Quantitative RT-PCR and Western blot analysis demonstrated similar or even higher expression levels of these WT and truncated Ncoa3 at both mRNA and protein levels (supplemental Fig. S3), thus ruling out the possibility that the inefficient activation of *Nanog* by the ΔNRID and the ΔAD mutants is due to reduced expression of these mutants. These data suggest that the binding of Ncoa3 at the *Nanog* promoter requires the interaction between the NRID domain and a certain nuclear receptor. In addition, the recruitment of histone acetyltransferase and/or histone arginine methyltransferase is likely essential for the activation of the *Nanog* promoter.

To elucidate the role of histone acetylation and histone arginine methylation in *Nanog* activation by Ncoa3, a coimmunoprecipitation assay was performed in V6.5 ESCs. Both CBP and CARM1 were detected in a Ncoa3 immunoprecipitated sample but not in an IgG control sample, confirming that Ncoa3 interacts with CBP and CARM1 in ESCs (Fig. 6*B*). Next, ChIP was carried out to determine the binding of CBP and CARM1 at the *Nanog* promoter in stable shGFP and Ncoa3 knockdown ESCs. Ncoa3 knockdown reduced the occupancy of Ncoa3, as well as CBP and CARM1, at the *Nanog* promoter, suggesting that Ncoa3 recruits both CBP and CARM1 to the *Nanog* promoter (Fig. 6*C*). Consequently, the levels of histone H3 acetylation and histone H3 arginine 17 dimethylation (H3R17 di-Me) at the *Nanog* promoter decreased in Ncoa3 knockdown ESCs. Taken together, Ncoa3 recruits the histone acetyltransferase CBP and

the histone arginine methyltransferase CARM1 to acetylate histones and methylate histone arginine at the *Nanog* promoter, leading to the activation of *Nanog*.

Ncoa3, as a nuclear receptor coactivator, activates gene transcription in cooperation with nuclear receptors. We have demonstrated that the ΔNRID mutant failed to activate the *Nanog* promoter (Fig. 6*A*). We set out to identify the nuclear receptor, which recruits Ncoa3 to the *Nanog* promoter, and activate *Nanog* expression. Seven nuclear receptors, including Esrrb, Nr5a2, Dax1, Nr1h2, Nr1d2, Nr6a1, and Nr1h3, which are highly expressed in ESCs and down-regulated upon differentiation (49), were tested for their ability to activate the *Nanog* promoter. Only Esrrb and Nr5a2 were able to activate the *Nanog* luciferase reporter (Fig. 6*D*). Consistently, knockdown of *Esrrb* and *Nr5a2* reduced the expression of *Nanog* in ESCs (Fig. 6, *E* and *F*). However, Ncoa3 still activated the *Nanog* reporter when either Esrrb or Nr5a2 was knocked down (Fig. 6, *G* and *H*). With these data, we could not rule out the possibility that Esrrb or Nr5a2 recruit Ncoa3 to the *Nanog* promoter. The knockdown efficiency was only around 60% for both Esrrb and Nr5a2 (Fig. 6, *E* and *F*). The remaining Esrrb or Nr5a2 could still facilitate Ncoa3 to activate *Nanog*. To avoid this problem, a 250-bp *Nanog* promoter luciferase reporter harboring an Esrrb binding site was used (10). Mutation in the Esrrb binding site abolished the activation effect of Esrrb on the *Nanog* reporter. Ncoa3 activated both the WT and mutant 250-bp *Nanog* promoter reporter, but the mutation in the Esrrb binding site compromised the activation efficiency of Ncoa3 (Fig. 6*I*). A coimmunoprecipitation experiment also detected the interaction between Esrrb and Ncoa3 in ESCs (Fig. 6*J*). These data suggest that Esrrb is involved in recruiting Ncoa3 to the *Nanog* promoter but that other factor(s) independent of Esrrb might also recruit Ncoa3 to activate *Nanog*.

Ncoa3 Is Required for GSK3 Signaling to Repress Nanog—It has been demonstrated that the transcriptional activity of Ncoa3 is regulated by phosphorylation in response to various upstream signaling pathways (38, 41, 42, 45). We then asked whether the phosphorylation status of Ncoa3 affects *Nanog* activation. Point mutations were introduced to five known phosphorylated residues: serine 497, 534, 846, and 849 and tyrosine 1336 of Ncoa3. Mutation to alanine or phenylalanine prevents phosphorylation, whereas mutation to aspartate mimics the phosphorylated status. A luciferase assays showed that mutations to alanine and phenylalanine at these sites did not affect the activation of *Nanog* by Ncoa3. However, the Ncoa3 mutant harboring S497D and S534D was less efficient in activating the *Nanog* promoter compared with WT Ncoa3. Interestingly, when these five residues were all mutated into aspartate, the transcriptional activity of this Ncoa3 mutant (5D) on the *Nanog* promoter was similar to WT Ncoa3 (Fig. 7*A*). These data suggested that the transcriptional activity of Ncoa3 on the *Nanog* promoter is indeed regulated by its phosphorylation status. Western blot analysis showed that the Ncoa3 mutant harboring S497D and S534D was expressed at a lower level than other Ncoa3 mutants and WT Ncoa3, whereas no significant difference was detected at the mRNA level (Fig. 7, *B* and *C*), suggesting that the phosphorylation status of Ncoa3 regulates the protein stability, hence the transcriptional activity.

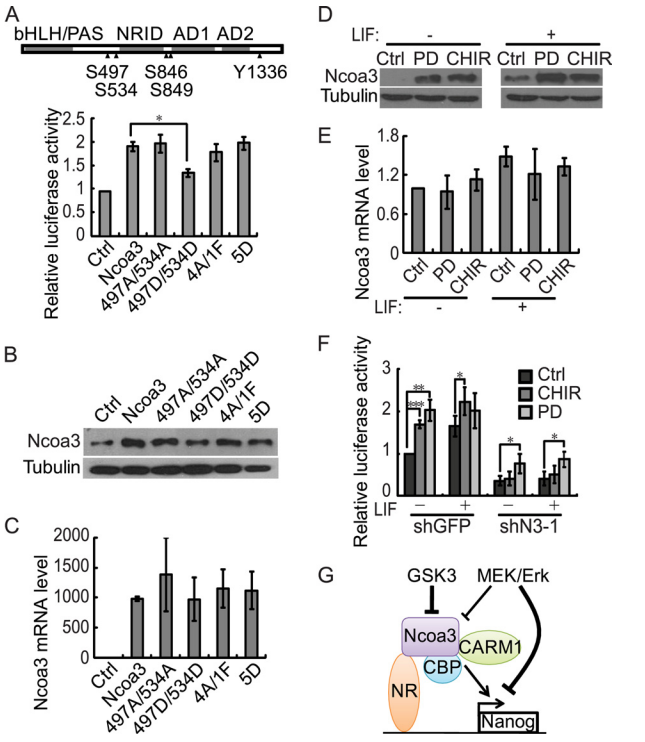

FIGURE 7. **The GSK3 pathway regulates the *Nanog* promoter through Ncoa3.** *A*, the transcriptional activity of Ncoa3 is regulated by its phosphorylation status. A schematic illustration of Ncoa3 is shown. Five phosphorylation sites, Ser-497, 534, 846, and 849 and Tyr-1336, are marked (▲). *497A/534A* represents the Ncoa3 mutant with S497A and S534A mutations. *497D/534D* means that Ser-497 and Ser-534 were mutated to aspartate. In the 4A/1F mutant, Ser-497, Ser-534, Ser-846, and Ser-849 were replaced with alanine, and Tyr-1336 was mutated into phenylalanine. Ser-497, Ser-534, Ser-846, Ser-849, and Tyr-1336 were all replaced by aspartate in the 5D mutant. V6.5 ESCs were cotransfected with the *Nanog* promoter reporter, pRV-SV40, and pSPORT plasmids expressing WT or Ncoa3 mutants with mutations in phosphorylation sites. Luciferase activities were measured 24 h after transfection. Means ± S.D. from three independent experiments were plotted. *B*, Ncoa3 protein levels in V6.5 ESCs described in *A* were detected by Western blot analysis. *C*, Ncoa3 mRNA expression in V6.5 ESCs described in *A* was measured by quantitative RT-PCR. *D*, GSK3 and Mek/Erk signaling repress the expression of Ncoa3 protein. V6.5 ESCs were cultured in medium supplemented with CHIR99021 (3 μM) (*CHIR*) or PD0325901 (1 μM) (*PD*), with or without LIF, for 48 h. Cell lysates were analyzed by Western blot analysis. β-tubulin served as a loading control (*Ctrl*). *E*, *Ncoa3* mRNA expression in V6.5 ESCs described in *D* was measured by quantitative RT-PCR. *F*, inhibition of GSK3 signaling does not enhance the activity of the *Nanog* promoter in stable Ncoa3 knockdown ESCs. Stable shGFP and Ncoa3 knockdown (shN3-1) ESCs were cotransfected with the *Nanog* promoter reporter and pRV-SV40 plasmids and cultured in medium containing CHIR99021 (3 μM) or PD0325901 (1 μM) with or without LIF. Luciferase activities were measured 24 h after transfection. Means ± S.D. from three independent experiments were plotted. *G*, a working model for Ncoa3 in pluripotency maintenance. Through interaction with the nuclear receptor, Ncoa3 binds to the promoter of *Nanog* and subsequently recruits the histone acetyltransferase CBP and the histone arginine methyltransferase CARM1 to modify the histones and activate the *Nanog* promoter. Upon differentiation, GSK3 and Mek/Erk signaling down-regulate Ncoa3 protein, leading to *Nanog* repression. In addition to Ncoa3, Mek/Erk might suppress *Nanog* through other mechanism(s). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

It has been shown that ERK2 phosphorylates NCOA3 *in vitro* and that GSK3 signaling regulates the stability of NCOA3 protein through phosphorylation of serine 505 (equivalent to Ser-497 in mouse Ncoa3) in human breast cancer cells (41, 50). Moreover, inhibition of Mek/Erk and GSK3 signaling allows ESCs to self-renew in the absence of LIF (51). Thus, we asked whether Mek/Erk and GSK3 signaling regulate ESC self-renewal through Ncoa3. ESCs cultured with the Mek inhibitor

PD0325901 or the GSK3 inhibitor CHIR99021 expressed more Ncoa3 than control ESCs, no matter whether LIF was present or not (Fig. 7*D*). Despite the elevated Ncoa3 protein expression, the Ncoa3 mRNA level was not increased by PD0325901 or CHIR99021 treatment, indicating that Mek/Erk and GSK3 signaling regulate Ncoa3 expression at a posttranscriptional step (Fig. 7*E*). Residue(s) of Ncoa3 phosphorylated by Mek/Erk and GSK3 signaling in ESCs remains to be characterized.

Inhibition of Mek/Erk and GSK3 signaling facilitate pluripotency maintenance in ESCs (51). Our data showed that Mek/Erk and GSK3 signaling suppress the expression of Ncoa3 protein, which can activate *Nanog* to maintain pluripotency in ESCs. We then asked whether Mek/Erk and GSK3 signaling act through Ncoa3 to repress *Nanog* expression. Luciferase reporter assays showed that inhibition of Mek/Erk or GSK3 signaling indeed enhanced the *Nanog* promoter activity in shGFP control ESCs, especially in the absence of LIF. However, when Ncoa3 was depleted, the *Nanog* promoter activity was not increased by CHIR99021 treatment, whereas PD0325901 still activated the *Nanog* promoter (Fig. 7*F*). These data suggest that GSK3 signaling represses *Nanog* expression mainly through down-regulation of Ncoa3 protein. Mek/Erk signaling may also inactivate the *Nanog* promoter. However, Ncoa3 is not the key downstream mediator of Mek/Erk to suppress *Nanog* expression.

DISCUSSION

Previous studies have demonstrated the importance of certain nuclear receptors, including Essrb, Nr5a2, and Dax1, in pluripotency maintenance (9, 11, 12, 14–16). However, it is not clear whether nuclear receptor coactivators are also required for pluripotency and ESC self-renewal. Here, we demonstrated that the nuclear receptor coactivator Ncoa3 is essential for pluripotency maintenance in mouse ESCs. Depletion of *Ncoa3* in ESCs compromises the expression of the pluripotency genes *Nanog*, *Oct4*, and *Sox2* as well as the *in vitro* and *in vivo* differentiation potential of ESCs. However, Ncoa3 appears to be dispensable for the pluripotency of ICM cells. *Ncoa3* null mice are viable but associated with a pleiotropic phenotype showing growth retardation, delayed puberty, reduced female reproductive function, and blunted mammary gland development (22, 23). This discrepancy might be caused by redundant factors during embryogenesis. Alternatively, ESCs, as the *in vitro* counterpart of the ICM, are not completely identical to the ICM. For example, LIF is required for *in vitro* culture of ESCs, but knockout of LIF or the LIF receptor gp130 has no effect on the pluripotency of ICM cells (52, 53).

Ncoa3 might maintain pluripotency through activating *Nanog* expression. The NRID and AD domains of Ncoa3 are necessary for the transcriptional activation of *Nanog* by Ncoa3, suggesting that the interactions with nuclear receptor and histone modifying enzymes are required for the function of Ncoa3. Essrb and Nr5a2 have been shown to bind to and activate the *Nanog* promoter (8, 10, 15, 16). Here, we have demonstrated that Esrrb interacts with Ncoa3 in mouse ESCs, and contributes to the activation of *Nanog* by Ncoa3. Our data also suggest that other transcription factors might facilitate Ncoa3 to activate the *Nanog* promoter independently of Esrrb. More experiments are required to identify other transcription factor responsible for Ncoa3 recruitment to the *Nanog* promoter. Upon binding to the *Nanog* promoter, Ncoa3 recruits the histone acetyltransferase CBP and the histone arginine methyltransferase CARM1 to acetylate histones and methylate histone H3 arginine 17. Consequently, *Nanog* expression is activated (Fig. 7*G*).

ESCs have two critical properties, namely self-renewal and differentiation potential into all types of cells in the body. Self-renewal requires the maintenance of a pluripotency-associated transcriptional profile. In contrast, during differentiation, the pluripotency-associated transcriptional profile has to be altered in response to various environmental cues. It has been shown that β-catenin, together with Tcf3, activates the expression of *Nr5a2*, which, in turn, promotes the expression of *Nanog*, *Oct4*, and *Tbx3*. GSK3 signaling stimulates the degradation of β-catenin, thus repressing downstream *Nr5a2*, *Nanog*, *Oct4*, and *Tbx3* (16). Our data suggest that Ncoa3 might be another downstream mediator for GSK3 signaling to down-regulate *Nanog* expression. Activated GSK3 signaling might phosphorylate Ncoa3 and accelerate Ncoa3 degradation, leading to suppression of *Nanog*. Thus, down-regulation of Ncoa3 by GSK3 signaling might facilitate disrupting the core transcriptional circuitry of pluripotency and allow ESCs to differentiate. Moreover, during ESC differentiation by LIF withdrawal, multiple signaling pathways might work on many downstream targets simultaneously to regulate transcriptional profile change. For example, Mek/Erk signaling could suppress *Nanog* independent of Ncoa3 (Fig. 7*F*). This explains why Ncoa3 overexpression is insufficient to maintain ESC self-renewal in the absence of LIF.

In summary, Ncoa3 plays a dual role in pluripotency maintenance. It activates the expression of *Nanog* to promote the self-renewal of ESCs. Meanwhile, Ncoa3 protein is subjected to various posttranslational modifications, which are regulated by signaling pathways such as GSK3 and Mek/Erk. These signaling pathways may stimulate the degradation of Ncoa3 or suppress the transcriptional activity of Ncoa3, subsequently reducing *Nanog* expression and allowing ESCs to differentiate. Given the reprogramming activities of potential Ncoa3-interacting nuclear receptor Esrrb and Nr5a2 (8, 15), Ncoa3 might cooperate with Esrrb or Nr5a3 to enhance reprogramming efficiency. Further investigation is required to demonstrate the function of Ncoa3 in iPS derivation.

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