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Coactivators p300 and CBP Maintain the Identity of Mouse Embryonic Stem Cells by Mediating Long-Range Chromatin Structure

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ABSTRACT

Master transcription factors Oct4, Sox2, and Nanog are required to maintain the pluripotency and self-renewal of embryonic stem cells (ESCs) by regulating a specific transcriptional network. A few other transcription factors have been shown to be important in ESCs by interacting with these master transcription factors; however, little is known about the transcriptional mechanisms regulated by coregulators (coactivators and corepressors). In this study, we examined the function of two highly homologous coactivators, p300 and CREB-binding protein (CBP), in ESCs. We find that these two coactivators play redundant roles in maintaining the undifferentiated state of ESCs. They are recruited by Nanog through physical interaction to Nanog binding loci, mediating the formation of long-range chromatin looping structures, which is essential to maintain ESC-specific gene expression. Further functional studies reveal that the p300/CBP binding looping fragments contain enhancer activities, suggesting that the formation of p300/CBP-mediated looping structures may recruit distal enhancers to create a concentration of factors for the transcription activation of genes that are involved in self-renewal and pluripotency. Overall, these results provide a total new insight into the transcriptional regulation mechanism of coactivators p300 and CBP in ESCs, which is important in maintaining self-renewal and pluripotency, by mediating the formation of higher order chromosome structures. STEM CELLS *2014;32:1805–1816*

INTRODUCTION

Expression of protein-coding genes in embryonic stem cells (ESCs) is regulated in a highly orchestrated and precise pattern by an ESC-specific transcriptional network to maintain self-renewal and pluripotency. A few sequence-specific DNA binding transcription factors, including master transcription factors Oct4, Sox2, and Nanog [1, 2], are found to be not only indispensable for the maintenance of ESC identity but also more strikingly capable of reprogramming differentiated cells to a pluripotent state [3]. Whole-genome mapping studies of binding sites further reveal that these master transcription factors bind to the regulatory elements of both pluripotency-associated and differentiation-associated genes [4]. This poses the possibility that these master transcription factors interact with basal transcription machinery complexes through coregulators to activate or repress transcription of cell-type-specific genes. One of the transcriptional coactivator, p300, is found to co-occur with Nanog, Oct4, and Sox2 in ESCs [4], suggesting that p300 may be recruited by these master transcription factors to facilitate ESC-specific gene expression.

p300 and CREB-binding protein (CBP) are homologous genes interacting with a large variety of transcription factors during proliferation, differentiation, and apoptosis [5, 6]. Homozygous mutations of either p300 or CBP cause lethality in mice, even their other paralogs are normally expressed. In addition, p300-/CBP-double heterozygotes are also lethal, leaving an open question in embryogenesis of whether p300 and CBP have nonoverlapping functions or they are functionally redundant and the total level of p300/CBP is critical for normal development [7–10]. p300 null ESCs are viable but are severely compromised in their abilities to differentiate [11], suggesting that p300 is dispensable for ESCs self-renewal, but critical for cellular differentiation. However, how p300 assists master transcription factors in the upregulation of gene expression in the context of ES-specific chromatin structure and whether other coactivators are involved in the process are poorly understood.

In this study, we characterize the function of p300 and CBP in mouse ESCs. By knocking down their expression levels, we find that they are functionally redundant in maintaining the undifferentiated state of ESCs. p300 and CBP

interact with Nanog via their KIX domain in ESCs and thus are thus recruited to ESC-specific enhancer regions to promote gene activation. In addition, their HAT domain with putative histone acetylation activity is also functionally required. Interestingly, we find that p300 and CBP are crucial for the formation of ESC-specific long-range looping structures, which are conserved in both mouse and human ESCs. Finally, we further demonstrate that looping structures mediated by p300 and CBP are important in maintaining ESC-specific gene expression. Our work sheds light on how transcription cofactors bridge master transcription factors to promote extensive crosstalk among multiple distal and proximal enhancers with promoters.

Materials and Methods

Cell Culture

E14 mouse ESCs, cultured in feeder-free conditions on surfaces coated with 0.1% gelatin, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, http://www.invitrogen.com), supplemented with 15% heat-inactivated fetal bovine serum (FBS; GIBCO), 0.055 mM β-mercaptoethanol (Gibco, Grand Island, NY, http://www.invitrogen.com), 2 mM L-glutamine (Gibco, Grand Island, NY, http://www.invitrogen.com), 0.1 mM minimal essential medium with nonessential amino acids (Gibco, Grand Island, NY, http://www.invitrogen.com), and 1,000 U/ml of leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA, http://www.chemicon.com). The human ESC-line H1 (WiCell, Madison, WI, http://www.wicell.org) was cultured feeder-free on Matrigel (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). Conditioned medium used for culturing human ESCs contained 20% KO serum replacement, 1 mM L-glutamine, 1% nonessential amino acids, and 0.1 mM β-mercaptoethanol and an additional 8 ng/ml of basic fibroblast growth factor (Invitrogen) supplemented to the human ESCs unconditioned medium. HEK293T(293) cells were cultured in DMEM supplemented with 10% FBS.

Construction of Short Hairpin RNA and RNAi-Resistant Constructs

Short hairpin RNA (shRNA) targeting specific genes was designed as previously described [12, 13]. For double knockdown constructs, *Cla*I-*Xho*I sites of pSUPER-puro vector were used to insert different shRNA cassette digested by *BstB*I-*Xho*I. Specific shRNA sequences for each gene are listed in Supporting Information Table S1. RNAi-resistant constructs were made by introducing four silent mutations in the shRNA-targeted region.

Transfection of shRNA and RNAi-Resistant Constructs

Transfection of plasmid DNA was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY, http://www.invitrogen.com) according to manufacturer's instructions. Puromycin (Sigma) selection was introduced 1 day after transfection at 1.0 mg/ml. For rescue experiment, RNAi-resistant constructs were cotransfected with corresponding shRNA.

Alkaline Phosphatase Staining

Medium for cells was gently aspirated and the cells were washed with Phosphate buffered saline (PBS). Cells were then fixed by 4% paraformaldehyde for 2 minutes. The fixed cells were rinsed twice by phosphate buffered saline with Tween-20 (PBST). Detection of alkaline phosphatase (AP) was carried out using a commercial ESC characterization kit (Chemicon, Temecula, CA, http://www.chemicon.com). Briefly, cells were covered by AP staining solution at room temperature for 15–30 minutes, protected from light. Then the AP staining solution is removed and the stained cells were washed twice with PBS.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Grand Island, NY, http://www.invitrogen.com) and purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany, http://www1.qiagen.com). Reverse transcription was performed using SuperScript II Kit (Invitrogen). Quantitative PCR analyses were performed in real time using an ABI PRISM 7900 sequence detection system and SYBR green master mix (Life Technologies, Grand Island, NY, https://www.lifetechnologies.com). The levels of the transcripts were normalized against control empty vector transfection. Data are presented as the mean $\pm$ SEM and derived from three independent experiments.

Embryoid Body Formation

ESCs transfected with shRNA and rescue constructs were trypsinized to small clumps containing three to five cells. The cell suspension was applied to ultra-low attachment culture dishes and kept in a 37°C incubator. Embryoid bodies (EBs) were formed by suspending ESCs in ESC culture medium without LIF and the photos were taken after 7 days.

Chromatin Immunoprecipitation and Real-Time PCR

Chromatin immunoprecipitation (ChIP) assays were carried out as described previously [14]. Briefly, cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature, followed by the addition of 0.2 M glycine to inactivate the formaldehyde. Cells were then lysed to obtain chromatin extracts, which were sonicated to obtain DNA fragments with an average size of 300–500 bp. The resulting chromatin extracts were immunoprecipitated by antibodies immobilized on Protein-G beads. The antibody information is shown in Supporting Information Table S5. Relative occupancy values (fold enrichment) were calculated by determining the apparent immunoprecipitation efficiency and normalized to the level observed at a control region, which was defined as 1.0. All ChIP experiments were repeated at least three times and data are presented as the mean $\pm$ SEM. Primer sequences are available in Supporting Information Table S2.

Coimmunoprecipitation

Transfected cells were lysed in cell lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, and 10% glycerol with protease inhibitor cocktail) for 1 hour. Whole cell extracts were collected and precleared. Beads coated with antibody were incubated with precleared whole cell extracts at 4°C overnight. The beads were washed with cell lysis buffer four times. Finally, the beads were boiled in 2$\times$ sample buffer for 10 minutes. The eluents were analyzed by Western blot.

Glutathione S-Transferase (GST) Pull Down Assay

Full-length Nanog and CBP (full-length and/or various deletion fragments) were cloned into pET42b (Novagen, Gibbstown, NJ, http://www.emdbiosciences.com). The sequence information can be found in Supporting Information Table S6. The plasmids were transformed into BL21 *Escherichia coli*. The proteins were expressed and purified with Glutathione (GSH)-sepharose beads (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com) followed by Ni-NTA beads (Qiagen, Hilden, Germany, http://www1.qiagen.com). The purified proteins bound to the GSH beads were incubated with recombinant CBP or Nanog proteins for 2 hours at 4°C. The beads were washed six times with cell lysis buffer. The eluents were analyzed by Western blot.

Chromosome Conformation Capture Assay and ChIP-Chromosome Conformation Capture Assay

The chromosome conformation capture (3C) assay was performed as described previously [2] with some modifications. Briefly, cells were crosslinked with 1% formaldehyde for 5 minutes at room temperature. The cells were then subjected to cell lysis. Nuclei were pelleted and resuspended in 1× NEB3 restriction buffer for overnight *Bgl* II digestion at 37°C with shaking. The enzyme was inactivated with SDS (1.3% final concentration) and shaking for 15 minutes at 65°C. Ligation buffer (1×) and Triton X-100 (1% final concentration) were then added to the nuclei and incubated for 1 hour at 37°C. An 800 μl ligation reaction was prepared with T4 DNA ligase (NEB, Ipswich, MA, https://www.neb.com), 8 μl 10 mg/ml Bovine Serum Albumin (BSA), and 8 μl 100 mM ATP. The sample was incubated for 4 hours at 16°C and 30 minutes at room temperature. DNA was then purified and subjected to PCR amplification of chimeric products using Hot-Star polymerase (Qiagen, Hilden, Germany, http://www1.qiagen.com). Presence or absence of amplicon is detected in a 1.5% agarose gel. The identities of all the PCR products were verified by sequencing. BAC clones of Children's Hospital Oakland Research Institute were used to prepare positive control template for the loci of interest. The following BAC clones were used to detect each gene loci: loci RP24-73P7 for mouse *Dppa3-Nanog-Slc2a3*; RP23-295I11 and RP24-313P9 for mouse *Tcf7l1* locus; RP11-277J24 for human *DPPA3-NANOG-SLC2A14* loci; RP11-312D1 for human *TCF7L1* locus. To assure that DNA templates prepared from 3C analyses are working and to standardize the cross-linking frequency in all cell samples, we chose a primer pair that targets two nearby restriction fragments for the *GAPDH* (RP11-72G18) and *Ndufa4* (RP23-230A2) loci to be used as a control for the 3C analyses as they are constitutively expressed genes in human and mouse cells, and they are also located on the same chromosome with the loci of interest.

ChIP-3C assays were performed as essentially described previously with slight modifications [15, 16]. Briefly, antibody-specific immunoprecipitated chromatin was obtained as described in ChIP assays. Chromatin was then digested with restriction enzyme, eluted, ligated with T4 DNA ligase, eluted, and decrosslinked. After purification, the ChIP-3C material was detected for long-range interaction by PCR. Primer sequences used for 3C and ChIP-3C assays are available in Supporting Information Table S3.

Luciferase Reporter Assays

Nanog and p300 cobinding fragments of about 500 bp were cloned downstream of a *luciferase* gene driven by the *Oct4* minimal promoter as described previously [17]. The primers for cloning the fragments are listed in Supporting Information Table S4. The constructs were cotransfected with either *p300/CBP* or empty vector construct into mouse ESCs and the selection was performed with 1.0 mg/ml puromycin treatment. Luciferase activity was determined 72 hours after transfection using the dual-luciferase reporter assay system (Promega, Madison, WI, http://www.promega.com). Data are presented as the mean ± SEM and derived from at least three independent experiments.

BAC Clones and BAC Recombineering Using *galK* Positive/Negative Selection

BAC (RP11-277J24)-containing bacterial stocks were propagated in Lysogeny broth (LB) medium supplemented with chloramphenicol. Modification of BACs was performed according to the procedure described previously [18]. To introduce galK at the desired position, the galK cassette with 50 bp arms homologous to RP11-277J24 was PCR-amplified using 2 ng pgalK and 25 pmol primers and the following PCR conditions: 94°C for 45 seconds, 58°C for 45 seconds, 72°C for 90 seconds, for 30 cycles. PCR primers for Del1, Del2, and Del3 are available in Supporting Information Table S4. Templates were removed from the PCR products by DpnI digestion and gel purification. For the positive selection, 50 ml Lysogeny broth (LB) medium supplemented with 12.5 μg chloramphenicol per milliliter was inoculated with SW102 bacteria containing pHB5 (SW102-pHB5); the bacteria were grown at 32°C until an A600 of 0.6 was reached. The culture was heat-shocked at 42°C for 15 minutes, then cooled briefly in an ice/water bath slurry and pelleted at 4,500*g* at 0°C for 5 minutes. Bacteria were washed twice with 20 ml double-distilled H_2O (ddH_2O) and were finally resuspended in ddH_2O. Subsequently, 25 μl of the electro-competent SW102-pHB5 bacteria were transformed with 150 ng PCR product in a 0.2 cm cuvette using a Bio-Rad Gene Pulser Pulse Controller (Bio-Rad, Hercules, CA, http://www.bio-rad.com) at 25 μF, 1.75 kV, and 200 Ω. Bacteria were recovered in 1 ml Lysogeny broth (LB) medium for 1 hour at 32°C and then washed three times in 1 ml 1× M9 salts. Bacteria were resuspended in 500 μl 1× M9 salts before plating serial dilutions onto M63 plates supplemented with 0.2% galactose, 1 mg D-biotin per liter, 45 mg L-leucine per liter, and 12.5 μg chloramphenicol per milliliter. Plates were incubated for 3 days at 32°C. Gal+ colonies were streaked sequentially twice onto Gal indicator plates (MacConkey agar) (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) supplemented with 0.2% galactose and 12.5 μg chloramphenicol per milliliter, and incubated overnight at 32°C. Using the same recombination procedure, galK was replaced by two complementary oligos. PCR fragments with arms complementary to 50 bp homologous arms used for deletion were amplified in order to replace galK. The conditions for PCR amplification are the same as outlined above. The sequences of complementary are available in Supporting Information Table S3. The plasmid template was removed by *Dpn*I digestion and gel purification. Phosphorylation of DOG by GalK into 2-deoxygalactose-1-phosphate is toxic, resulting in the suppression of bacteria that failed to replace galK. This led to the exclusive growth of clones that contained the desired recombinant BAC. BAC clones with deletion were analyzed by PCR, sequencing, and BAC DNA restriction analysis. For this, *Cla*I, *Pme*I, *Xho*I triple-digested BAC

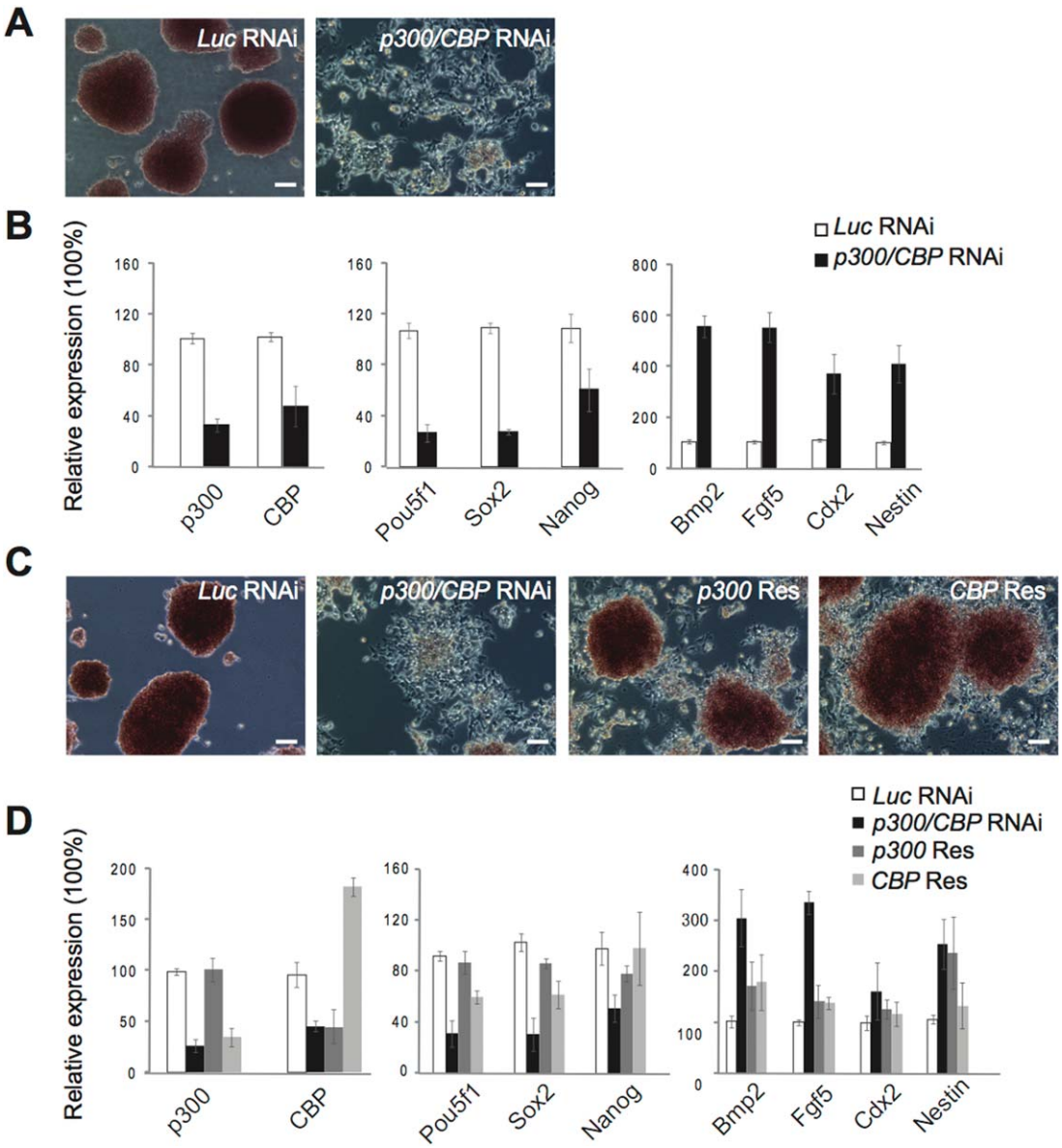


Figure 1. *p300* and *CBP* function redundantly roles for the maintenance of embryonic stem cells. **(A):** Alkaline phosphatase staining was performed on control cells (*Luc* RNAi) and *p300/CBP* knockdown cells (*p300/CBP* RNAi) 3 days after transfection. Scale bars = 100 μm. **(B, D):** Quantitative real-time PCR analysis of expression of *p300* and *CBP*, self-renewal, and differentiation-associated genes after knockdown. Data are the mean ± SEM of three biological replicates. **(C):** Alkaline phosphatase staining was performed on control cells (*Luc* RNAi), *p300/CBP* knockdown cells (*p300/CBP* RNAi), and rescue cells (*p300* Res, *CBP* Res). Scale bars = 100 μm. (D): Quantitative real-time PCR analysis of expression of *p300* and *CBP*, self-renewal, and differentiation-associated genes after knockdown and rescue. Data are the mean ± SEM of three biological replicates. Abbreviation: CBP, CREB-binding protein.

DNA was separated electrophoretically at 50–100 V for approximately 24 hours using a 0.6% agarose gel.

BAC Transfection and Generation of Stable Cell Lines

Stable cell lines were generated by transfection of mouse ESCs with engineered BAC plasmid using lipofectamine (Invitrogen, Grand Island, NY, http://www.invitrogen.com) and selection in 1 mg/ml neomycin (Gibco, Grand Island, NY, http://www.invitrogen.com) selection. Surviving ESC clones were submitted to a second round of selection with a higher (2 mg/ml) neomycin concentration. ESC clones survived for the second round were cultured for at least five passages and analyzed by RT-PCR to check the integration of complete engineered BAC clones. The correct clones were then extracted for RNA and analyzed for gene expression by RT-PCR.

Results

p300 and CBP Play Redundant Roles in Maintaining the Undifferentiated State of ESCs

To assess the functional roles of *p300* and its closely related gene *CBP* in ESCs, we knocked down endogenous *p300* or *CBP* transcript to less than 40%, respectively, by RNAi. AP activity and gene expression analysis showed that single knockdown of either p300 or CBP did not disrupt ESC identity,

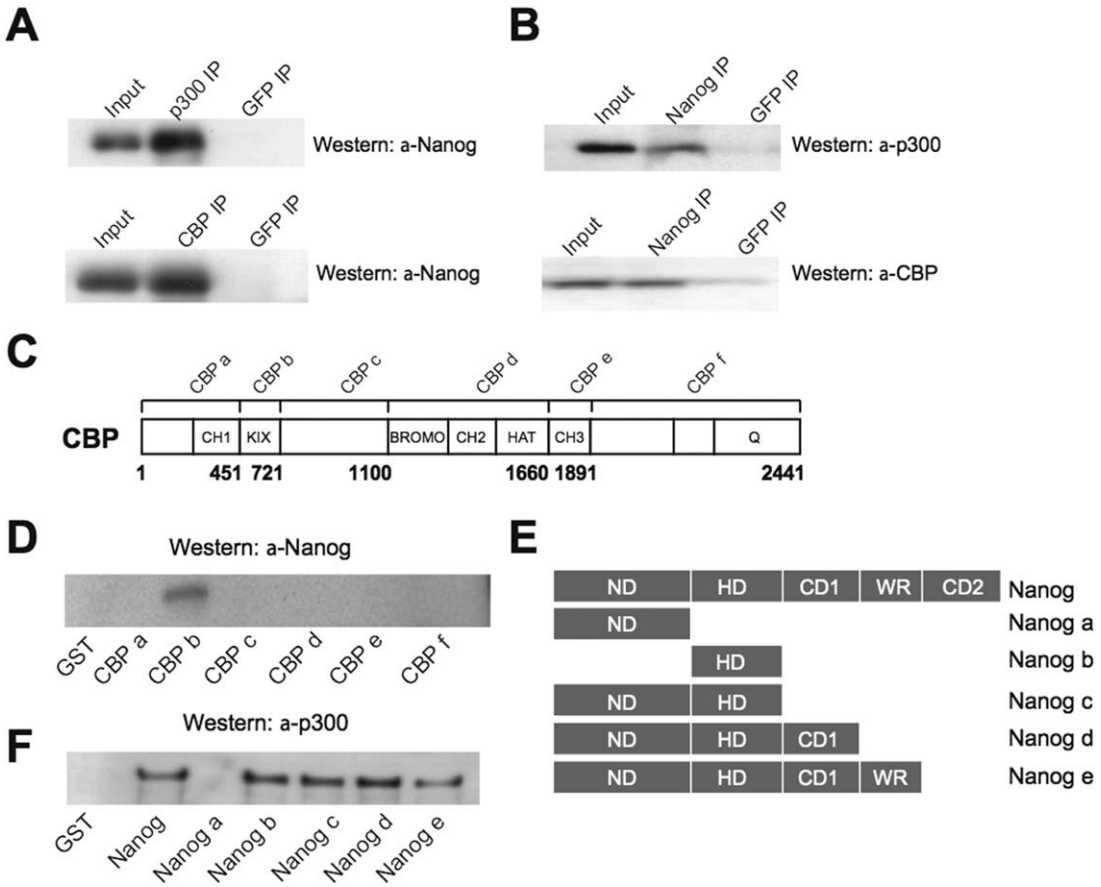


Figure 2. p300 and CBP physically interact with Nanog. **(A):** Co-IP using nuclear extracts of embryonic stem cells was performed using anti-p300 or anti-CBP antibody. Western blot was performed with anti-Nanog antibody. Control IP was performed using an anti-GFP antibody. **(B):** Reciprocal Co-IP using the ES nuclear extracts was performed using anti-Nanog antibody. Western blotting was carried out anti-p300 or anti-CBP antibody. Control IP was performed using an anti-GFP antibody. **(C):** Schematic diagram of CBP protein showing its functional domains. Each of the domains is expressed as GST fusion proteins for pull down study. **(D):** Pull-downs of CBP-GST fusion domains and purified Nanog protein. Western blot was performed with anti-Nanog antibody. GST served as negative control. **(E):** Schematic diagram of wild-type and deletion forms of Nanog protein. **(F):** GST pull down was carried out using GST-tagged Nanog proteins. Western blot was performed with anti-p300 antibody. GST served as negative control. Abbreviations: CBP, CREB-binding protein; CD1, C-terminal domain 1; CD2, C-terminal domain 2; GFP, green fluorescent protein; HD, homeobox domain; ND, N-terminal domain; WR, tryptophan repeat domain.

suggesting that p300 or CBP alone is enough for ESCs self-renewal (Supporting Information Fig. S1A, S1B). In order to test whether p300 and *CBP* play redundant roles for the maintenance of ESC identity, we knocked down *p300* and *CBP* simultaneously in ESCs using double shRNA constructs. We observed a reduction of RNA (Fig. 1B) and protein levels (Supporting Information Fig. S1E) of *p300* and *CBP* in double knockdown cells. Strikingly, knocking down *p300* and *CBP* simultaneously led to cell differentiation and consequently disrupted ESCs self-renewal. AP staining of pluripotent ESCs was reduced dramatically in the double knockdown cells, indicative of differentiation (Fig. 1A). Expression of self-renewal-associated genes *Pou5f1*, *Sox2*, *Nanog*, and *Esrrb* was reduced in response to *p300/CBP* double knockdown, while a strong induction of differentiation-associated genes was observed (Fig. 1B). In addition, double knockdown cells lose the capability to form EBs (Supporting Information Fig. S1F). To exclude the off-target effects, we performed rescue experiment to recover the results of double knockdown. We co-transfected p300 and CBP expressing contructs which is RNAi-resistant with p300/CBP shRNA into ESCs. RNA and protein levels of p300 and CBP have been recovered to a level similar to control *Luc* RNAi-treated cells (Supporting Information Fig. S1D, S1E). Consistent with the recovery of protein levels, cells with rescue constructs show similar AP staining (Supporting Information Fig. S1C) and gene expression profile (Supporting Information Fig. S1D) as control cells. Additionally, we further confirmed that double knockdown cells lose the capability to form EBs while the rescued cells regain the ability to form EBs (Supporting Information Fig. S1F).

Notably, we found that recovering the expression of either *p300* or *CBP* to its normal level was also able to rescue the phenotype caused by double knockdown (Fig. 1C). The expression of self-renewal and differentiation-associated genes was also rescued to a similar level as seen in the control cells (Fig. 1D). These results strongly suggest that *p300* and *CBP* are functionally redundant for the maintenance of ESCs identity.

p300 and CBP Are Recruited by Master Transcription Factors Through Physical Interaction

The genome wide binding sites of p300 in ESCs were found to be associated with the binding sites of master transcription factors. This suggests that master transcription factors may recruit p300 to specific loci in the genome [4]. To ascertain

whether it is also true for CBP, we tested CBP binding on Nanog cluster and Myc clusters. ChIP-qPCR results showed that CBP, similar to p300 and Nanog, bound preferentially to Nanog cluster and its binding was dependent on the presence of Nanog (Supporting Information Fig. S2A, S2B).

To investigate the possible physical interaction between p300/CBP and Nanog in ESCs, coimmunoprecipitation (Co-IP) experiments were performed using nuclear extracts of ESCs. Both p300 and CBP were able to coprecipitate with Nanog, as shown in the Co-IP experiments using anti-Nanog antibodies (Fig. 2A), and the reciprocal Co-IP using anti-p300 and anti-CBP antibodies (Fig. 2B). Control IP using anti-green fluorescent protein antibody and IgG further confirmed the specificity of Co-IP experiments (Fig. 2A, 2B, Supporting Information Fig. S2C, S2D).

p300 and CBP are large nuclear proteins with eight distinct functional domains (N terminal, CH1, KIX, Bromo, CH2, HAT, CH3, and glutamine-rich) [19, 20]. These domains mediate the interactions between p300/CBP and other nuclear proteins. To map Nanog-interacting domains on CBP, GST fusion proteins were generated with different segments of mouse CBP that collectively span the entire protein (Fig. 2C). These fusion proteins were used in pull-down experiments with purified Nanog protein. Only the fragment containing KIX domain immunoprecipitated Nanog proteins beyond background level (Fig. 2D). Conversely, to identify the p300-interacting domain on Nanog, we expressed and purified recombinant GST-fused full-length Nanog and various Nanog fragments (Fig. 2E). These proteins were immobilized onto GSH-Sepharose beads and incubated with purified p300 protein. Homeobox domain of Nanog and fragments containing homeobox domain are found to interact with p300 (Fig. 2F). Taken together, our data showed that the KIX domain of p300/CBP interacted with the homeobox domain of Nanog.

The KIX and HAT Domains of p300/CBP Are Critical for the Self-Renewal of ESCs

In order to dissect the functional domains of p300 and CBP in ESCs, we created a series of RNAi-resistant constructs, each expressing a specific mutant *p300* or *CBP* (Fig. 3A, 3D). The mutant *p300* or *CBP* constructs were then cotransfected with *p300/CBP* shRNA to test whether they were able to rescue double knockdown effect. Interestingly, except the mutants with deletion of the KIX or HAT domain, all the other mutant constructs were able to rescue the differentiation phenotype, resulting in positive AP staining (Fig. 3B, 3E). Expression analysis showed that these mutants could restore the expression level of key self-renewal-associated and differentiation-associated genes (Fig. 3C, 3F).These results suggest that the KIX and HAT domains are the functional domains of p300/CBP in mouse ESCs.

p300/CBP Mediate Intragenic and Intergenic Looping Interactions in ESCs

To further understand the molecular mechanism involved in p300/CBP regulation, we examined the binding sites of Nanog and p300 on the chromatin. Interestingly, we found that several loci cobound by Nanog and p300 were within proximity of a single gene or gene clusters. This raises an intriguing possibility that these binding loci may specifically interact with each other to form a unique higher order chromosomal structure in ESCs. To test our hypothesis, we studied an intragenic locus, *Tcf7l1*, and intergenic loci, *Dppa3-Nanog-Slc2a3*.

According to published ChIP-Seq data [4], there are three sites co-occupied by Nanog and p300 within *Tcf7l1* locus. They are located at 5′ end, middle, and 3′ end of the gene locus, which is about 200 kb long (red bar in Fig. 4A). The binding was further confirmed by ChIP-qPCR (Supporting Information Fig. S3C). To determine whether the binding sites confer conformational crosstalk, we performed 3C assay. We started the 3C analysis using an invariant primer (primer A, Fig. 4A), targeting the restriction fragment next to the binding site of Nanog and p300 at the 3′ end of *Tcf7l1*, together with one of a series of primers complementary to different restriction fragments along this gene. Chimeric DNA fragments were detected when primer A was used together with primers D, E, H, and I, indicative of the interactions (Fig. 4B). The detection of chimeric DNA fragments was ligation dependent in all 3C assays (Supporting Information Fig. S4C). The results indicated that DNA regions occupied by Nanog and p300 interact and form a looping structure. More importantly, the looping interactions appeared to be ESC-specific as chimeric DNA fragments were not detectable after subjecting ESCs to retinoic acid (RA)-induced differentiation (Supporting Information Fig. S4B).

It has been reported that Oct4 is required for maintaining the higher order chromatin structure at specific site in ESCs [21]. To test whether p300 and CBP are also involved in the formation of looping structure, ChIP-3C was performed using p300/CBP ChIP-enriched DNA samples, which is about 200–500 bp in size (Supporting Information Fig. S3B). Interactions between the binding fragments on the 5′ end and 3′ end of *Tcf7l1* were recapitulated (Fig. 4C). To further determine whether p300 and CBP are required for the formation of the chromosomal loops, we performed 3C analysis on ESCs transfected with *p300/CBP* shRNA and *Luc* shRNA. To exclude the effect of comprehensive differentiation and loss of Oct4 protein, cells were harvested at early time point when the protein level of and binding intensity of master transcription factors were unchanged (Supporting Information Fig. S3A, S3C). Strikingly, depletion of p300/CBP led to the abolishment of chromosomal loops, while the loop retained in the control cells (Fig. 4D). Also we found that the looping structure changes before the transcription of *Tcf7l1* changes, as we only start to see the reduction of transcription about 24 hours after the cells lose the looping structure (Fig. 4E). Collectively, we identified ESC-specific higher order structures within *Tcf7l1* locus and these structures are dependent on the presence of p300 and CBP.

Having shown the presence of long-range intragenic chromosomal looping, we next extended our investigation further by looking for the presence of intergenic chromosomal looping. The *Dppa3-Nanog-Slc2a3* gene cluster was chosen due to the presence of several Nanog and p300 binding loci within the cluster containing three genes preferentially expressed in mouse ESCs (Fig. 4F, Supporting Information Fig. S3D). Interactions were detected between region A and regions E, F, I, J, M, N, and O, which are next to Nanog and p300 binding sites (Fig. 4G). Similarly, such long-range interaction is ESC-specific as the loops were abolished upon RA-induced differentiation (Supporting Information Fig. S4E) and were dependent on the presence of p300/CBP as shown by ChIP-3C and 3C on RNAi samples (Fig. 4H, 4I). In addition, the long-range looping structure is biologically meaningful as the lost of looping structure

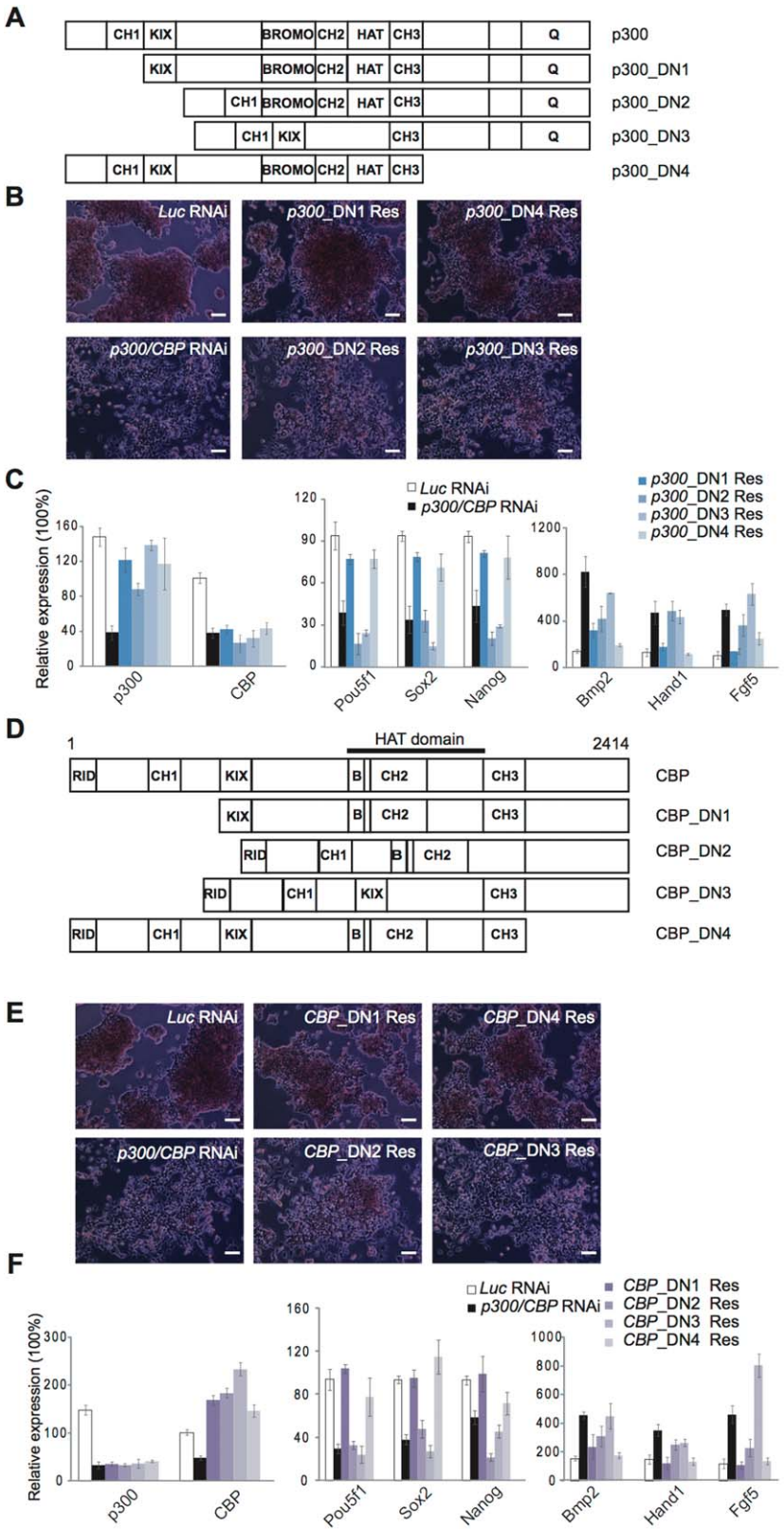
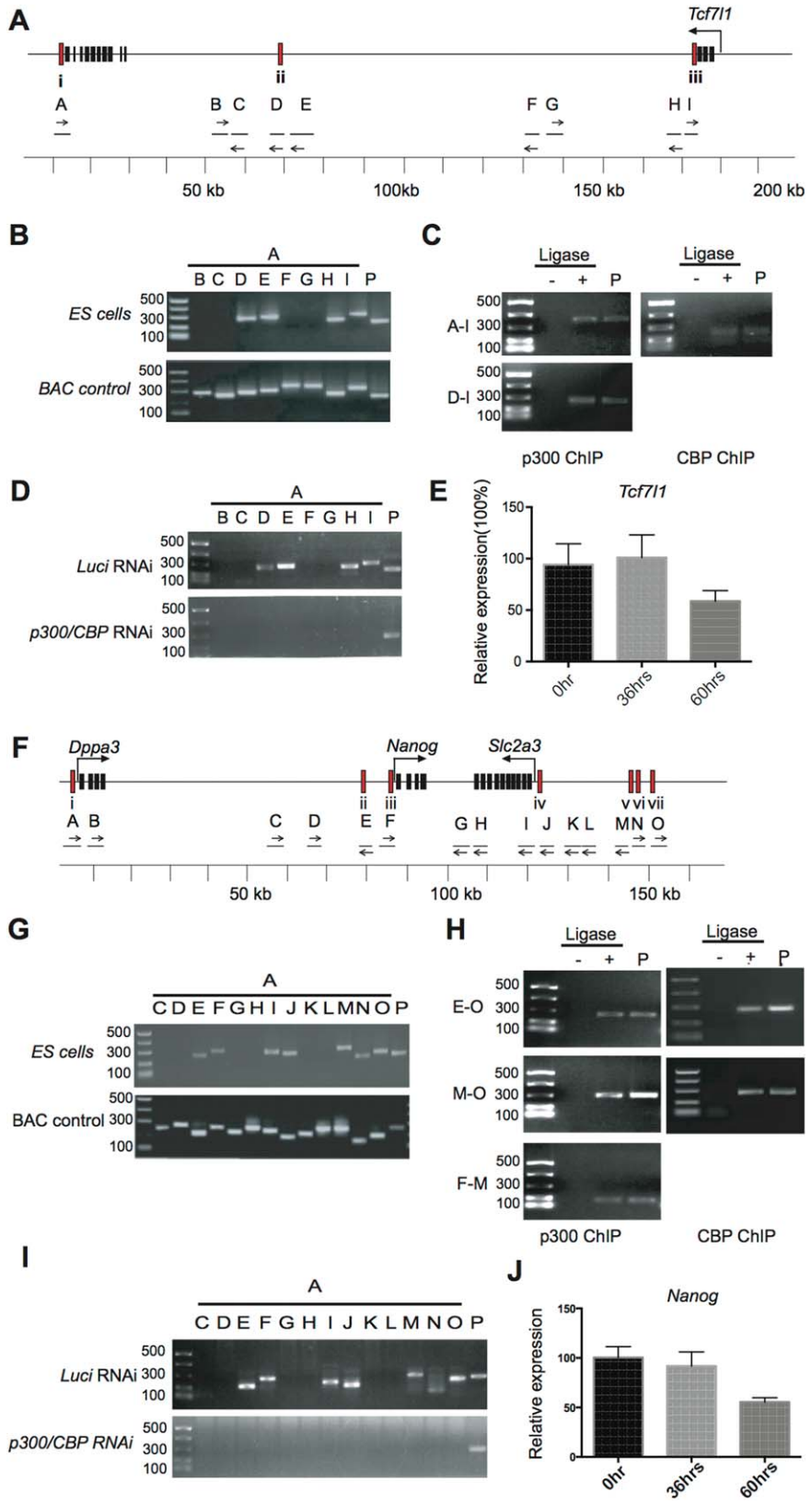


Figure 3. KIX and HAT domain of p300 and CBP are important for their function in the maintenance of embryonic stem cells (ESCs). **(A):** Panel of truncated *p300* expression constructs used for rescue. The deletion of domain is indicated for each construct. **(B):** Rescue of concurrent *p300/CBP* double knockdown phenotype by coexpression of RNAi-resistant truncated *p300* expression constructs. Alkaline phosphatase staining was performed to evaluate whether cells underwent differentiation. Scale bars = 100 μm. **(C):** Quantitative real-time PCR analysis of expression of *p300* and *CBP,* ESC-associated genes, and lineage-specific marker genes after rescue by coexpression of truncated RNAi-resistant *p300* expression constructs. Data are the mean ± SEM of three biological replicates. **(D):** Panel of truncated *CBP* expression constructs used for rescue. The deletion of domain is indicated for each construct. **(E):** Rescue of *p300/CBP* double knockdown phenotype by coexpression of RNAi-resistant truncated *CBP* expression constructs. Alkaline phosphatase staining was performed to evaluate whether cells underwent differentiation. Scale bars = 100 μm. **(F):** Quantitative real-time PCR analysis of expression of *p300* and *CBP,* ESC-associated genes, and lineage-specific marker genes. Data are the mean ± SEM of three biological replicates. Abbreviations: CBP, CREB-binding protein; HAT, histone acetylation.



affects the transcription of surrounding genes as indicated by *Nanog* expression (Fig. 4J).

Chromatin Looping Structure Is Evolutionarily Conserved in Human ESCs

To access whether similar looping interactions occur in human ESCs, we then performed ChIP and 3C assays on human ESCs on *TCF7L1* and DPPA3-NANOG-SLC2A14 loci, the orthologs of mouse *Tcf7l1* and *Dppa3-Nanog-Slc2a3* loci, respectively (Fig. 5A, 5D). We confirmed the binding of NANOG and P300 (Fig. 5B, 5E) and found that the regions encompassing the binding loci formed chromosomal loops (Fig. 5C, 5F). This data indicate that the intragenic loop structures are evolutionarily conserved in mouse and human. These looping structures in human ESCs are also ESC-specific as they were not present in HEK293T cells, a differentiated human cell line (Fig. 5C, 5F, bottom panel).

The DNA Fragments Involved in the Looping Interactions Have Enhancer Activities

To test whether the DNA involved in the looping structure has enhancer activity, we cloned fragments of about 400 bp encompassing each binding locus downstream of a luciferase reporter driven by *Pou5f1* minimal promoter. Interestingly, we found that fragment *Tcf7l1-ii*, *Tcf7l1-iii*, *Nanog-ii*, *Nanog-iii*, and *Nanog-vii* have strong enhancer activities, as shown by a higher luciferase activity relative to a luciferase reporter containing the *Pou5f1* minimal promoter only (Fig. 6A). Based on published ChIP-Seq data, we find that H3K4me1 and H3K27ac, two chromatin signatures of enhancers, also mark these regions (Supporting Information Fig. S5A).

To further investigate the functions of chromatin looping interactions in transcription activation in vivo, we took advantage of bacterial artificial chromosome (BAC), which encompasses the sequences involved in the formation of chromatin looping structures. Human BAC clone RP11–277J24, which contains the genomic fragment from human chromosome12 spanning the *DPPA3-NANOG* loci, was modified by *galK* positive and counter selection system. Three deletions, Del1, 2, and 3, were introduced toRP11–277J24, respectively. Del1 was made in the middle of *DPPA3* and *NANOG*, which showed no interaction with NANOG/P300 binding sites. Del2 and Del3 were made upstream of *NANOG* gene and correspond to region E and F, which were involved in formation of chromatin loops (Fig. 6B). The modification was confirmed by detailed restriction mapping (Supporting Information Fig. S5B) and sequencing. We then transfected the three modified human BAC clones into mouse ESCs, respectively, and established stable cell lines. The expression of mouse self-renewal and differentiation-associated genes was not affected in the BAC transfected stable cell lines (Supporting Information Fig. S5C). However, the expression of human *NANOG* and *DPPA3* expression was significantly reduced in the cells transfected with Del2 and Del3 BAC clones compared with cells transfected with Del1 BAC clone (Fig. 6C). These results indicate that deletion of interacting fragments that are involved in chromatin looping reduces the gene expression of related genes in vivo.

Discussion

In this article, we report the functional study of two coactivators, p300/CBP, in ESCs. Although p300 has been shown to be important for early embryo development [7–10] and ESC differentiation [11], our work is the first to identify that p300 and CBP play redundant roles in maintaining ESCs self-renewal. Our data demonstrate that introducing either p300 or CBP into p300/CBP double knockdown ESCs allows the cells to retain their self-renewal capacity, a result that supports the notion that total level of p300 and CBP is essential for ESC self-renewal.

Furthermore, our data show that p300/CBP interact with Nanog through the KIX domain. In the hematopoietic system, mice homozygous for point mutations in the KIX domain of p300 designed to disrupt the binding surface for c-Myb and CREB7–9 exhibited multilineage defects in hematopoiesis [30]. Further functional characterization identifies that the HAT domain is indispensable for p300/CBP functions, suggesting that p300/CBP may regulate transcription through their histone acetyltransferase activity. p300 and CBP are found to be able to acetylate all four core histones in vitro, including acetylation of histone H3 on lysine 56 recently [22], which is a histone modification marker that overlaps with the binding of the master transcription factors of ESCs [23].

In addition to the study of transcriptional machinery, understanding higher order chromatin organization in ESCs is of interest due to the unusual chromatin structure that has been found in ESCs [24–26]. The most widely accepted model of long-range regulatory interactions is the looping model, which proposes that distant enhancers and promoters are in physical contact, while the intervening regions are looped out. The first evidence to support this model is from the study of the chicken beta-globin gene cluster [16, 27]. In mouse ESCs, long-range chromosomal structure has been reported within *Nanog*

Figure 4. p300 and CBP mediate intragenic and intergenic looping interactions. **(A):** Schematic representation of the mouse *Tcf7l1* locus. Dark boxes represent exons and red boxes represent ChIP-Seq identified Nanog binding loci, named by the Roman numerals indicated below them. Relevant *Bgl*II restriction fragments are indicated by short horizontal bars. Primers are named by Roman alphabets and their orientations are indicated by arrows. **(B):** Upper panel, chromosome conformation capture (3C) analyses were carried out on *Tcf7l1* locus. Presence or absence of amplicons is detected in a 1.5% agarose gel using the primer combinations indicated on the top of each lane. Bottom panel, PCR controls done using BAC DNA harboring the *Tcf7l1* locus. Lane P is a BAC control and the leftmost lanes represent PCR markers to show sizes of the amplicons. **(C):** ChIP-3C analyses were done on *Tcf7l1* locus using p300 antibody (Left panel) and CBP antibody (right panel). Primer combinations are indicated on the left of each panel. DNA samples used in the PCR reactions were prepared with or without ligase added in the 3C assays to show that presence of amplicons is ligase-dependent. **(D):** 3C analyses of *Tcf7l1* locus on mouse ESCs transfected with control *Luc* RNAi construct (top panel), p300/CBP RNAi (bottom panel). **(E):** Quantitative real-time PCR analysis of expression of *Tcf7l1* at 0, 36, and 60 hours post-transfection of shRNA constructs. Data are the mean ± SEM of three biological replicates. **(F):** Schematic representation of the mouse *Dppa3-Nanog-Slc2a3* loci. Labels are the same as **(A)**. **(G):** 3C analysis on mouse *Dppa3-Nanog-Slc2a3* loci. Labels are the same as **(B)**. **(H):** ChIP-3C analyses were done on *Dppa3-Nanog-Slc2a3* loci. Labels are the same as **(C)**. **(I):** 3C analyses of *Dppa3-Nanog-Slc2a3* loci on mouse ESCs transfected with control *Luc* RNAi construct (top panel), p300/CBP RNAi (bottom panel). **(J):** Quantitative real-time PCR analysis of expression of *Nanog* at 0, 36, and 60 hours post-transfection of shRNA constructs. Data are the mean ± SEM of three biological replicates. Abbreviations: CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; ESCs, embryonic stem cells.

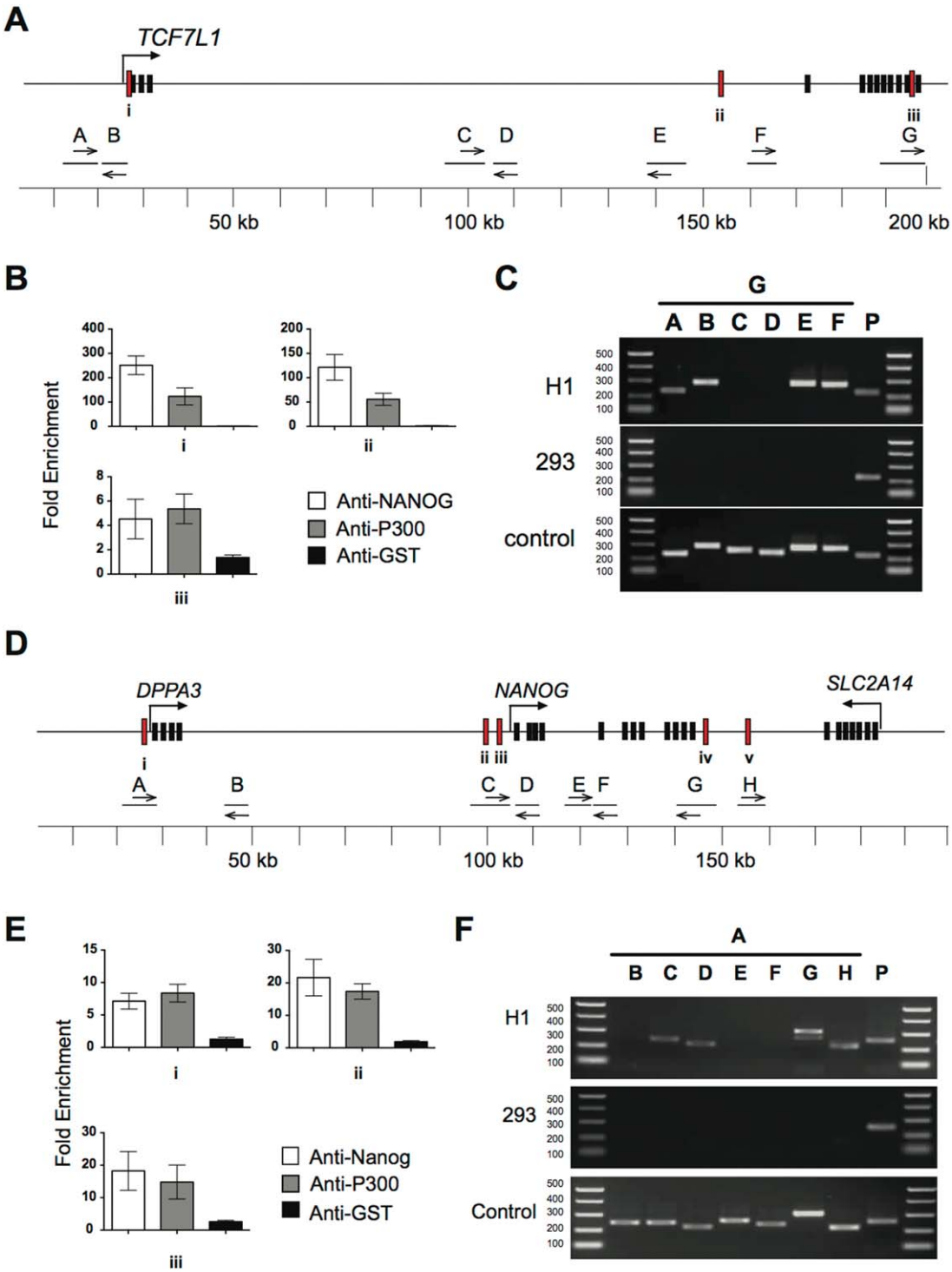


Figure 5. The intragenic and intergenic looping interactions are conserved in human embryonic stem cells (ESCs). **(A, D):** Schematic representation of the human **(A)** *TCF7L1* locus and **(D)** *DPPA3-NANOG-SLC2A14* loci. Labels are the same as in Figure 4A, 4F. **(B, E):** Chromatin immunoprecipitation (ChIP) analysis on *TCF7L1* locus **(B)** and *DPPA3-NANOG-SLC2A14* loci **(E)** using NANOG antibody (anti-Nanog, white) and P300 antibody (anti-P300, gray). ChIP analysis using GST antibody (anti-GST, black) is set as a mock control. **(C, F):** 3C analyses on the **(C)** *TCF7L1* locus and **(F)** *DPPA3-NANOG-SLC2A14* loci. Presence or absence of amplicons is detected in a 1.5% agarose gel using the variable primers indicated on top of each lane with the invariant primer G. Top panels, 3C analyses done on human ESCs. Middle panels, 3C analyses done on HEK293T cells (293 cells). Bottom panels (BAC control), 3C analyses done using BAC DNA harboring the relevant genomic regions to show that the all possible ligation products can be amplified using the indicated primer combinations.

locus by examining the DNaseI hypersensitive sites, and Oct4 has been shown essential for maintaining this higher order structure [21]. More recently, mediator is found to interact with cohesin to form a complex to stabilize DNA looping at specific loci [28]. Information about the proteins that are important for cell-type-specific higher order structure has only

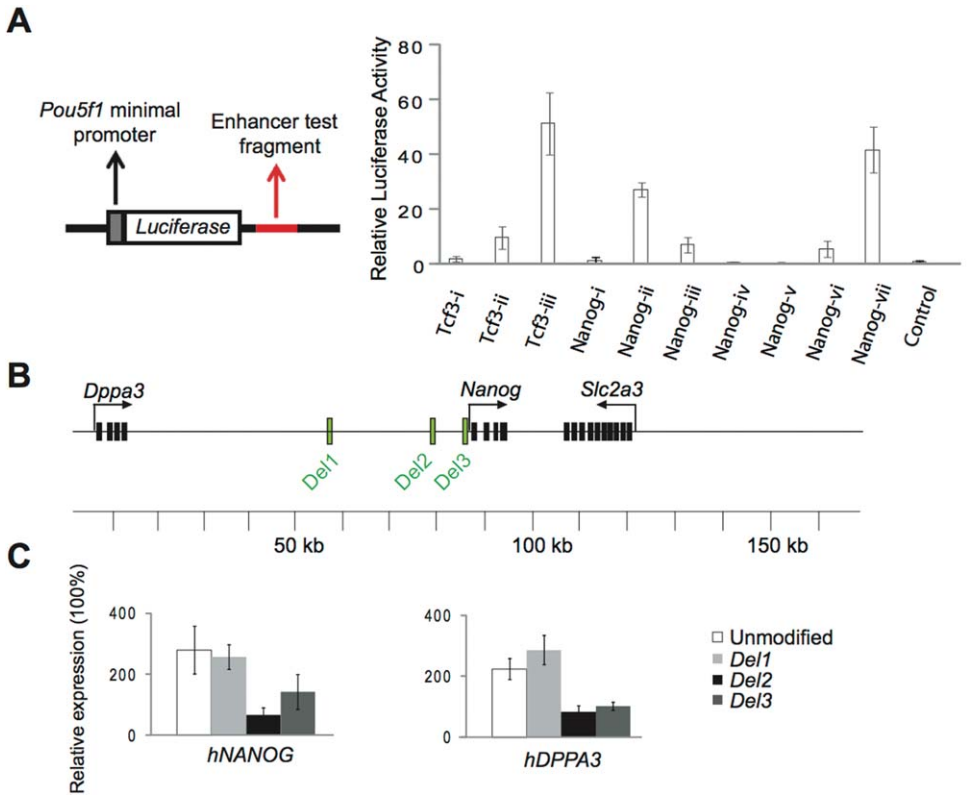


Figure 6. Characterization of the DNA fragments involved in looping interactions. **(A):** Reporter constructs used to assay for enhancer activity are shown. Genomic fragment of approximately 300 bp (in red) was inserted downstream of a *luciferase* gene driven by *Pou5f1* minimal promoter. The luciferase reporter was then transfected into mouse ESCs. *y*-Axis represents the fold enrichment of luciferase activity, calculated relative to a luciferase reporter containing the *Pou5f1* minimal promoter only and normalized over an internal transfection control. *x*-Axis represents the Nanog and p300 binding fragments (labeled as in Fig. 4A, 4F) cloned into the luciferase reporter plasmid. Data are the mean ± SEM of three biological replicates. **(B):** Schematic representation of the human *DPPA3-NANOG-SLC2A14* loci. Black boxes represent exons and green boxes represent deleted regions, as indicated below them. **(C):** Quantitative real-time PCR analysis of expression of human *NANOG* (*hNanog*) and *DPPA3* (*hDPPA3*) in stable mouse ESC lines with the insertion of specific modified BAC clones. Data are the mean ± SEM of three biological replicates.

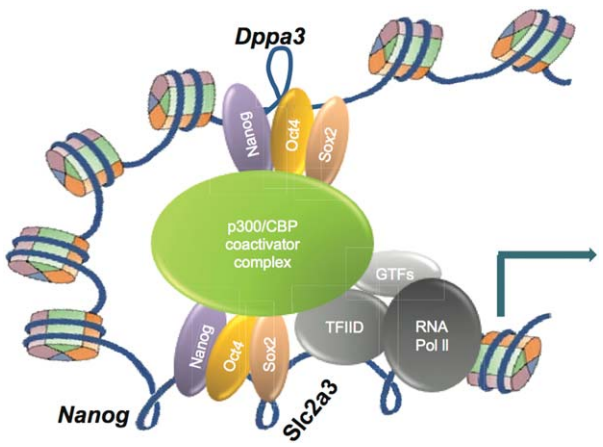


Figure 7. Model showing the three-dimensional organization of Dppa3-Nanog-Slc2a3 locus. Master regulators (e.g., Nanog, Oct4, and Sox2) recognize their specific cis-regulatory elements and bind to specific loci within the gene cluster of Dppa3-Nanog-Slc2a3. By recruiting coactivator p300/CBP, and together with other transcription factors and cofactors, they form a large nucleoprotein complex and draw DNA into a loop structure. p300/CBP act as the molecular tether to stabilize the protein complex with their intrinsic histone acetylation activity, they may acetylate local chromatin to facilitate transcription response. Abbreviation: CBP, CREB-binding protein.

started to be appreciated; it would be interesting to identify more proteins that have key roles in the formation and stability of such structures. Our data provide significant evidence to support that p300 and CBP are essential for stabilizing DNA loops at intragenic and intergenic sites in ESCs. In our study, Nanog and p300 binding loci on *Tcf7l1* (*TCF7L1*) and *Dppa3-Nanog-Slc2a3* (*DPPA3-NANOG-SLC2A14*) cluster are proven to be involved in long-range looping interactions and these interactions are pluripotent state-specific. Interestingly, the human *DPPA3-NANOG-SLC2A14* loci are located on human chromosome 12p, a region clustered with pluripotency genes, which has a distinctively central nuclear localization in ESCs but peripheral nuclear localization in differentiated cells, probably correlating with its unique looping structure described here. The presence of p300/CBP is crucial for the looping structure, as depletion of p300/CBP disrupts the structure, although the protein level and binding intensity of Oct4 and Nanog remained. Our finding extends on earlier studies of long-range chromatin structure in ESCs.

More importantly, we have presented the functional study of DNA loops in ESCs and showed that looping structure is essential for activating cell type-specific gene expression. We showed that several genomic elements involved in looping interaction have enhancer activity and deletion of them resulted in reduced gene expression. Taken together, the model

of long-range regulation we suggest here is that master transcription factors recognize and bind to *cis*-regulatory elements within a gene or gene cluster. They recruit other transcription factors and coregulators, including p300/CBP, to form a large nucleoprotein complex and draw DNA into a loop. In this way, they bring the self-renewal-associated genes, such as *Dppa3*, *Nanog*, and *Slc2a3*, together. They potentially share *cis*-regulating elements, activator and coactivator complexes, chromatin-remodeling complexes, and/or the transcription machinery for efficient expression [29]. p300/CBP and other cofactors act as molecular tether connecting different transcription factors to stabilize this higher order structure and facilitate the enhancement of gene expression (Fig. 7).

Conclusion

In summary, we have revealed that two coactivators, p300 and CBP, play redundant roles in maintaining the undifferentiated state of mouse ESC. They interact with Nanog and are recruited to ESC-specific gene locus. More importantly, we report that p300 and CBP regulate the formation of long-range chromatin looping structure in both mouse and human ESC and the looping is critical for specific gene enhancement. These findings will provide insights to future studies unraveling the unique higher-order chromatin structure in ESC.

Author Contributions

F.F.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; Y.X.: collection and/or assembly of data, data analysis and interpretation, and manuscript writing; K.-K.C.: collection and/or assembly of data and data analysis and interpretation; X.C.: data analysis and interpretation; P.M.: data analysis and interpretation and final approval of manuscript; H.-H.N.: conception and design, data analysis and interpretation, and final approval of manuscript. F.F. and Y.X. contributed equally to this article.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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