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Interaction of Positive Coactivator 4 with Histone 3.3 Protein is Essential for Transcriptional Activation of the Luteinizing Hormone Receptor Gene

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Abstract

The luteinizing hormone receptor (LHR) is essential for sexual development and reproduction in mammals. We have established that Sp1 has a central role in derepression of LHR gene transcription induced by Trichostatin A (TSA) in MCF7 cells. Moreover, the co-activator PC4 which associates directly with Sp1 at the LHR promoter is essential for TSA-mediated LHR transcription. This study explores interactions of PC4 with histone proteins, which presumably triggers chromatin modifications during LHR transcriptional activation. TSA treatment of MCF7 cells expressing PC4-Flag protein induces acetylation of histone 3 (H3) and immunoprecipitation (IP) studies revealed its interaction with PC4-Flag protein. MS/MS analysis of the protein complex obtained after IP from TSA treated samples detected H3.3 acetylated at K9, K14, K18, K23 and K27 as a PC4 interacting protein. The association of PC4 with H3.3 was corroborated by IP and re-ChIP using H3.3 antibody. Similarly, IP and reChIP showed association of PC4 with H3 acetylated protein. Knockdown of PC4 in MCF7 cells reduced H3.3 enrichment, H3 acetylation at the Lys sites and LHR promoter activity in TSA treated cells despite an increase in H3 and H3.3 protein induced by TSA, linking PC4 to H3 acetylation and LHR transcription. Depletion of H3.3 A/B in MCF7 cells impair chromatin accessibility and enrichment of Pol II and TFIIB at the LHR promoter and its activation, resulting in marked reduction of LHR gene expression. Together, these findings point to the critical role of PC4 and its association with acetylated H3.3 in TSA-induced LHR gene transcription.

Keywords

LHR; PC4; H3.3; histone acetylation; transcriptional regulation

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1. Introduction

The luteinizing hormone receptor (LHR) is a member of the G-protein-coupled receptor family, which is primarily expressed on the surface of gonadal cells. This receptor mediates responses to luteinizing hormone (LH) signals, which regulate cyclic changes in the structure/function of the ovary, and the development and function of Leydig cells in the testis [1-4]. In both types of reproductive organs, cyclic AMP-mediated activity of LH/LHR supports regulation of steroid hormone production by steroidogenic enzymes, which is essential to maintenance of reproductive functions, including ovulation and spermatogenesis [3, 4]. LHR is also expressed in non-gonadal cells, tumor tissue, and cancer cells [5, 6].

Stable cultures of cancer cells provide major advantages over transient cultures of gonadal cells and have permitted in-depth exploration of basic aspects of transcription and the identification of complex regulatory systems [7]. Early studies in JAR choriocarcinoma and MCF7 cancer cells, demonstrated that *LHR* transcriptional expression is repressed under basal conditions because of histone hypoacetylation [6]. Subsequent studies using the histone deacetylase inhibitor, TSA, which promotes histone acetylation, derepression of the *LHR* gene, and activation of its transcription in these cells, revealed its regulation by complex and diverse networks, in which coordination and interactions between regulatory effectors are essential for silencing and activation of *LHR* expression [6, 8-11].

The proximal Sp1 site in the 176 bp *LHR* promoter recruits histone (H) deacetylases and the Sin3A corepressor complex, which contribute to the silencing of *LHR* transcription [8]. Site-specific acetylation/methylation-induced phosphatase release serve as an on-switch for Sp1 phosphorylation at Ser641 by PI3K/PKCζ [8, 9]. These processes cause release of the repressor, pRb homologue 107, from Sp1, enrichment of TFIIB and Pol II, and transcriptional activation [10]. Maximal derepression of the gene is dependent on DNA demethylation of the promoter, H3/H4 acetylation, and HDAC/Sin3 A release [6, 8]. In further studies, our laboratory demonstrated that positive cofactor 4 (PC4) has an important role in assembly of the preinitiation complex in trichostatin A (TSA)–mediated *LHR* transcription [11]; PC4 is recruited by Sp1 following TSA treatment and acts as its coactivator. The coactivator domain of PC4 (amino acids (aa) 22–91) and the DNA-binding domain of Sp1 are required for PC4/Sp1 interaction [11]. Only the non-phosphorylated form of PC4 interacts directly with Sp1, and the interaction is inhibited by PC4 phosphorylation. PC4 does not participate in TSA-meditated release of phosphatases from Sp1, Sp1 phosphorylation, or release of repressor complexes. Although TFIIB enrichment is dependent on PC4, we ruled out TFIIB as its direct target in the activation process [11].

In this study, we demonstrate that TSA induces acetylation of a PC4-interacting protein, identified as acetylated H3.3. We also identified the sites of acetylation of the H3.3 histone variant by MS/MS analysis, its interaction with PC4, and its presence in a complex associated with chromatin in the promoter region. The H3.3-PC4 interaction is essential for TSA-induced transcriptional activation and expression of the *LHR* gene.

2. Materials and methods

2.1. Reagents and antibodies

Trichostatin A (TSA) was purchased from Calbiochem. The antibody against PC4 (#PA-117-01) was obtained from ProteinOne, Rockville, MD, and those for β-actin (#SC69879) and Pan-acetyl C2 (#SC8649) from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against H3 (#4499S), H3K9-Ac (#9649S), H3K14-Ac (#7227S), H3K18-Ac (#13998S), H3K23-Ac (#8848S), H3K27-Ac (#8173S), H3K36-Ac (#11885), H2A (#2778S), Histone H2A-Ac, Histone H2B (#8135S), Histone H2B-Ac, Histone H4 (#2592), Histone H4-Ac -Ser1/Lys5/Lys/Lys12 (#SC377520), and GST (#2625S) were purchase from Cell Signaling Technology Inc, Danvers, MA. H3.3 antibody (monoclonal) was obtained from Abcam (Cambridge, MA) (#ab176840). Antibodies against TFIIB (#SC271736) and Pol II (#05-623) were from Santa Cruz Biotechnology and Millipore (Burlington, MA), respectively. FLAG antibody (#8146S), anti-FLAG M2 Affinity gel, and Mouse IgG agarose were purchased from Sigma-Aldrich (St. Louis, MO) (#A2220). Recombinant H3, H4, H3.3, and H3-H4 tetramer were obtained from New England Biolabs (Ipswich, MA) and recombinant H3.3-H4 tetramer was from Sigma-Aldrich.

2.2. Expression vectors and cell culture

The reporter gene construct containing the *LHR* promoter was generated by cloning the human *LHR* gene promoter region (−176 to +1) into the SacI/BglII sites of the pGL2 basic vector [12]. pCMV6-PC4 was purchased from Origene (Rockville, MD) and used as PCR template for generation of constructs expressing PC4-Flag protein in MCF7 cells. p3XFLAG-PC4 vector was created by inserting PCR-amplified *PC4* cDNA into the EcoRI and KpnI sites of the p3XFLAG-CMV-7.1 vector (Sigma) [11]. MCF7-A2 cells, kindly provided by Dr. Erica Berleth (C. Roswell Park Cancer Institute, Buffalo, NY) [13], were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Invitrogen). All studies were conducted using cells of passages 6–8. Cells plated in 10-mm culture dishes, were transfected with p3XFLAG-PC4 vector (10 μg) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Transfection of an equal amount of empty p3XFLAG vector was used as a negative control.

2.3. siRNA analysis

Validated siRNAs designed to knock-down the endogenous expression of PC4 and scrambled negative control siRNA were purchased from Ambion (Austin, TX). Transfections of siRNA into MCF7 cells were performed using siPORT Neo FX reagent (Invitrogen), as previously described [11]. Validated siRNAs from Ambion were used to knock-down H3.3A and H3.3B. The siRNA sequences were as follows: 5′-UUAAAGUCCUGAGCAAUUUCT-3′ (H3.3A siRNA ID# S6422) and 5′-UAUGAGAACAAGUGCAGUCAG-3′ (H3.3B siRNA ID# S6424). After 24 h of transfection, cells were grown in RPMI 1640 medium for additional 48 h before harvesting. For reporter gene assays, the *LHR* gene promoter and reporter gene were introduced into cells using Lipofectamine and Plus Reagent at 24 h post-transfection of siRNA, and

luciferase activity was determined 40 h later. TSA (500 ng/ml) or vehicle were added to cells 16 h prior to harvest.

2.4. RNA isolation and real-time RT-PCR

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA) followed by treatment with DNase I (Invitrogen), as previously described (11). Total RNA was reverse-transcribed with random primers for synthesis of first strand cDNA using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA). Relative levels of *LHR* mRNA were determined by real-time PCR using SYBR-Green Master Mix in an ABI 7500 sequence detection system, as previously described [14]. Relative *LHR* mRNA levels were calculated using the comparative C_T method, with human β-actin as an internal control. The *LHR* primer sequences were: 5′-TCTACACCCTCACCGTCATCACTC-3′ (*LHR* forward) and 5′-AGCCATCCTCCAAGCATAATCA-3′ (reverse).

2.5. Immunoprecipitation (IP)

MCF7 cells transfected with p3XFLAG-PC4 or empty vector were treated with or without TSA (500 ng/ml) for 16 h. Cell lysates were then extracted using IP lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100; pH 7.4) in the presence of protease inhibitor. The Bradford protein assay was used to determine protein concentration. Proteins (1 mg per reaction) were then pre-cleared by incubation with 40 μl mouse-IgG agarose for 3 h with end-to-end rotation at 4°C. Supernatants were collected and immunoprecipitated using 40 μl anti-FLAG M2 affinity gel overnight. Agarose/protein complexes were washed four times with IP lysis buffer and once with TE buffer. Sample buffer (2 × 40 μl) was used to elute protein complexes from affinity agarose. After heating at 70°C for 10 min, samples were subjected to western blot analyses, which were performed as described previously [11]. In experiments where immunoprecipitated proteins were examined using gel staining, IP samples eluted from several IP reactions were concentrated using an Amicon Ultra filter (Millipore) prior to loading on tris-glycine gels.

2.6. GST-pull down assay

The GST-PC4 vector was generated by inserting PCR amplified *PC4* cDNA into the EcoRI and XhoI sites of the pET-41a (+) vector (Novagen). GST-tagged protein and GST-PC4 protein were expressed in *Escherichia coli* strain Rosetta™ 2 (DE3) (ThermoFisher Scientific). After culture for 16 h at 37°C, cells were diluted 1:50 and cultured for 3 h at 37°C, followed by incubation with 0.5 mM IPTG for 6 h at 37°C. Cultures were harvested and lysed using GST-pull-down lysis buffer (ThermoFisher Scientific), supernatants collected, and the expression of GST-PC4 confirmed by western blotting using GST antibody. GST-pull down assays were performed using a GST Protein Interaction Pull-Down kit (ThermoFisher Scientific), according to the manufacturer's protocol. Briefly, bacterially expressed GST-tagged or GST-PC4 proteins immobilized on GST-agarose resin were used as bait proteins. The resin with immobilized GST-PC4 was incubated overnight with total protein extracts (as prey) isolated from MCF7 cells treated with and without TSA for 16 h. Beads were washed five times with wash buffer to remove unbound proteins and eluted with elution buffer containing 10 mM glutathione to collect bound proteins. The eluted samples were resolved by SDS-PAGE for western blot analysis using H3, H3.3, and H4 antibodies.

Additional GST-pull down experiments were carried out using GST-PC4 as bait and recombinant H3, H3.3, H4, H3-H4 tetramer, or H3.3-H4 tetramer (50 μg/each) as prey. Eluted samples were resolved by SDS-PAGE and stained with Coomassie Blue stain (ThermoFisher Scientific).

2.7. Chromatin immunoprecipitation (ChIP) and re-ChIP assays

ChIP was performed using the Magnify Chromatin Immunoprecipitation System (Invitrogen), according to the manufacturer's instructions. Cells transfected with PC4-Flag or Flag empty vector, treated with or without TSA, were collected and washed with 1x PBS, and then fixed with 1% formaldehyde for 10 min. Chromatin was then prepared by sonication shearing, optimized according to the manufacturer's instructions. ChIP was performed on sheared chromatin from approximately 2×10^5 cells, using IgG antibody as a negative control or antibodies recognizing Flag, TFIIB, Pol II, H3K9-Ac, or H3.3. Chromatin purified from an equivalent number of cells not subjected to the immunoprecipitation step was used as the input control. Real-time PCR was performed on DNA isolated from each of the ChIP reactions using primer pairs specific for *LHR* 5′-ACTGGGCACTGTCGCAGGTC-3′ (sense) and 5′-CATGGCCGGCGAACTGGGCT-3′ (anti-sense) [6]. The amplified region of the 189 bp *LHR* promoter contains the relevant proximal Sp1 response element. For ReChIP analyses, complexes resulting from the primary immunoprecipitation were eluted from protein A-agarose beads by incubation of samples with 5 mM dithiothreitol at 37°C for 20 min. Supernatants, containing protein complexes eluted with PC4-Flag or Flag (control), were recovered by centrifugation and diluted in ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl; pH 8.1). A second round of immunoprecipitation was performed with the specified antibodies, including those against H3 acetylated at various individual Lys residues or H3.3, using the Magnify Chromatin Immunoprecipitation System (Invitrogen). Realtime PCR was performed on DNA isolated from each of the ChIP reactions using a primer pair specific for *LHR* described above. The 2^{-dCt} method was used to calculate relative enrichment values for each antibody with respect to input control [15].

2.8. MS/MS analysis

Initial characterization of PAGE-resolved proteins by mass spectrometry was performed using bands digested "in-gel" with mass spectrometry-grade trypsin (Promega) overnight at 37°C. In subsequent experiments, samples eluted following immunoprecipitation with anti-Flag M2 affinity gel and subjected to histone-extraction using a histone purification mini kit (Active Motif) were analyzed by digestion with a mixture of endoproteinases [trypsin, chymotrypsin, and GluC (V8 protease)]. The resulting reaction mixture was analyzed by LC-MS/MS, using an Easy nanoLC II HPLC system (Thermo) interfaced via electrospray ionization (ESI) to a dual pressure linear ion trap mass spectrometer (LTQ velos, Thermo Fisher). In some experiments, to characterize non-acetylated lysines in H3 samples, protein samples were treated with propionic anhydride (Sigma Aldrich) before digestion with trypsin [16]. Mass spectrometry data were searched using the Proteome Cluster program, provided by Bioproximity Inc. (Springfield, VA). The output of the database search was manually analyzed using the Global Proteome Machine to obtain spectral images.

2.9. Chromatin accessibility

The effect of H3.3 knock-down on chromatin accessibility in the *LHR* gene promoter region was determined using an EpiQuik analysis kit (BioRad). After siRNA (control scrambled or H3.3 A and H3.3B siRNAs) transfection, MCF7 cells (approximately 1 χ 10^7 cells per group) were divided equally into nuclease and non-nuclease groups. Chromatin was extracted and subjected to nuclease/non-nuclease digestion (37°C for 2 min) followed by DNA clean up using EpiQuik spin columns. The resulting eluted DNA was subjected to real-time PCR (SYBR), using the primers (sense) 5′-CACTCAGAGGCCGTCCAA-3′ or 5′-GTGTGTGGAAGGGCAGCCAAG-3′, and anti-sense: 5′-GGCGAACTGGGCTCCTG-3′ designed to amplify the −55 to +1 and −99 to +1 regions of the *LHR* promoter, respectively. The latter region contains a relevant Sp1 response element at −71/-79. The results were normalized to the reference *PC4* gene (sense:5′-GCTCTTCTGGCAGTGATTCT-3′ and anti-sense: 5′-CTGCTGCTGCTCTGTTTAGA-3′), whose expression is not changed by treatment with TSA.

2.10. Statistical analysis

The significance of differences between groups was determined by Tukey's or Dunn's multiple-comparison tests (one-way ANOVA analysis) or two-tailed unpaired student *t*-tests, using the Prism software program (GraphPad Prism 7.02 Software, San Diego, CA).

3. Results

3.1. TSA induced acetylation of PC4 interacting proteins

PC4 participates in TSA-induced derepression of the *LHR* gene by linking Sp1 to assembly of the preinitiation complex at the *LHR* promoter. In this context, TSA does not alter PC4 expression, its cellular distribution, or its phosphorylation levels [11], suggesting its participation in other mechanisms, such as PC4 acetylation or modulation of protein/protein interactions. To explore this hypothesis, we initially performed Flag antibody IP using lysates from MCF7 cells transfected with a plasmid expressing Flag-tagged PC4 protein, followed by western blot detection with pan-acetyl antibody (Fig. 1 A). PC4 was not acetylated under basal conditions or after treatment with TSA, since no acetylated signal corresponding to the PC4-Flag protein (approximately 22 kDa) was detected using the pan acetyl-antibody (Fig. 1A), while the Flag antibody revealed two bands of approximately 22 kDa, representing the phosphorylated and non-phosphorylated forms of PC4 (Fig. 1B). These findings exclude a role for PC4 acetylation in TSA-induced *LHR* expression. IP with Flag antibody revealed that TSA treatment specifically induced acetylation of two unknown PC4-interacting proteins, a more prominent band of approximately 16 kDa and a minor band of approximately 10 kDa (Fig. 1A, right), while the input revealed several acetylated bands (Fig. 1A, left).

3.2. Identification of PC4-interacting protein using Co-IP and MS/MS analysis

The finding that TSA treatment can induce acetylation of two PC4-interacting proteins suggested that those proteins may participate in *LHR* activation in response to TSA. We next attempted to identify these two proteins using IP assays combined with mass spectrometry.

For these studies, either a Flag-tagged PC4 expression construct or an empty vector expressing Flag peptide was transfected into MCF7 cells, which were then lysed, and the lysates immunoprecipitated using Anti-FLAG M2 affinity agarose. Precipitated protein complexes were analyzed by SDS-PAGE. The silver stained gel revealed the presence of four protein species in the region of 10–25 kDa (Fig. 1C). One of those proteins is presumed to be non-specific, because it was also observed in the negative control sample. Bands, S1 (which appears as doublet), S2, and S3 were precipitated only in the PC4-FLAG sample; therefore, these were assumed to be proteins of interest. The S1 band may correspond to the phosphor (upper band) and non-phospho (lower band) forms of PC4-Flag (Fig 1B and 1C).

To identify the PC4-interacting proteins, the two bands were excised from the gels and subjected to LS-MS/MS tandem mass spectrometry. MS/MS analysis of band S1 identified PC4-Flag protein. In this case, the 5′ sequence was missing (Table 1); however, the sequence, the molecular weight of the stained S1 band in the gel (21 kDa; Fig. 1C), and confirmation by western blotting using FLAG antibody (Fig. 1B), led us to conclude that the band corresponded to PC4-Flag. PC4 (endogenous) and H3 proteins were detected in the S2 band (Table 1). These data indicate that endogenous PC4 may dimerize with PC4-FLAG, which would be consistent with early studies on the crystal structure of the PC4 carboxy terminal domain, that revealed PC4 dimer formation, in which kinked α-helices abut the β-sheets of their dimeric partner [17].

The detection of dimerization of PC4-Flag with endogenous PC4 in our study indicated that endogenous PC4 could be pulled down using Flag antibody in our experiments. Despite the presence of endogenous PC4 in the S2 sample, we excluded that this form of PC4 was the protein acetylated by TSA because the tagged form of PC4 (PC4-FLAG) was not observed as an acetylated protein after treatment with TSA (Fig. 1 A). Therefore, we deduced that H3 is the protein acetylated by TSA detected in our IP experiment. Because we were unable to obtain a sequence from the minor acetylated band (S3), our further studies focused on H3.

Two specific H3 peptides were identified in the S2 band (Table 1). In initial studies, the peptide comprising aa 18–36, which contains two lysines, K27 and K36, was found to be acetylated (Table 1), while the other peptide (aa 73–83), containing K79, was not acetylated. These results, based on limited sequence information, suggest that PC4 interacts with acetylated H3 on induction with TSA. We also used a method involving protection of free lysine residues using a propionylation reagent, which has been widely adopted for characterization of free lysines in histone proteins, by treating the excised S2 band with propionylation reagent (propionic anhydride) prior to trypsin digestion. MS identified three H3 peptides and propionylation was observed at lysines K56, K79, and K122 (Table 1), indicating that those lysines were not acetylated.

3.3. PC4 interactions with H3 and acetylated H3

In this study, we treated cultures with 500 ng/ml TSA for 16 h, which was shown in our previous investigation to induce maximal de-repression of *LHR,* through a complex mechanism, and to stimulate gene transcription and expression (6). As shown in Fig. 2A, a dose-dependent increase in H3 and H3 acetylated at K9 (H3K9-Ac) was observed in MCF7

cells following treatment with TSA for 16 h. All subsequent studies were performed in cells cultured with or without TSA treatment (500 ng/ml).

To verify the interaction of PC4 with H3, we carried out co-IP followed by western blot analyses. PC4 was immunoprecipitated in cells transfected with PC4-Flag, but not in negative control cells transfected with empty Flag vector. The level of PC4 was not changed in response to TSA treatment, as determined using Flag antibody (Fig. 2B IP: Flag Ab). Using H3 antibody, which recognizes H3 and its variants, H3 was significantly immunoprecipitated from PC4-Flag samples, regardless of TSA treatment; however, the amount pulled down was significantly increased by exposure to TSA and no H3 was detected in the control Flag-tag samples.

To further verify that H3 is one of the two PC4 interacting proteins that are acetylated by TSA treatment (Fig. 1A), acetylated H3 antibody was used to probe the blot. While H3 was acetylated in response to TSA treatment in both PC4- Flag and Flag -tag transfected cells (Fig. 2B, Input), acetylated H3 was only immunoprecipitated by FLAG antibody from cells expressing PC4- Flag (Fig. 2B).

In a related experiment, inputs were normalized for H3 prior to IP (Fig. 2C), resulting in a similar association of PC4 and H3 in the presence or absence of TSA, while PC4 interaction with H3K9-Ac was observed only in the TSA-treated sample. These results support a direct interaction between PC4 and H3, and a TSA-induced interaction of PC4 with acetylated H3, consistent with a report that PC4 is a chromatin-associated protein [18].

In other study, cells transfected with PC4-Flag or Flag-tag vectors and incubated with or without TSA for 16 h were subject to IP using FLAG M2 affinity gel, followed by histone extraction. Using this approach, we demonstrated that, while H3 interacts with PC4 in both the presence and absence of TSA, no associations were observed with H2A, H2B, or H4 (Fig. 2D). In addition, a TSA-induced association of PC4-FLAG with H3K9-Ac was observed, while no interaction with H4Ac was detected. Furthermore, no interaction with H2A/B-Ac was observed (Fig. 2D).

In GST-pull down assays using GST-PC4 as bait and lysates from MCF7 cells incubated with or without TSA as prey, we demonstrated direct interaction between H3 and H3.3 *in vitro,* while no pull-down of H4 was observed (Fig. 2E). *In vitro* pull-down assays using recombinant H3 demonstrated an interaction of H3.3 with GST-PC4. In contrast, no interaction was observed between GST-PC4 and H4. Interaction of GST-PC4 with the H3-4 tetramer revealed two bands, corresponding to H3 and H4; similarly, with the H3.3-H4 tetramer, bands corresponding to H3.3 and H4 were resolved. These data indicate *in vitro* interaction of GST-PC4 with the tetramer via the H3 protein (Fig. 2F)

3.4. MS/MS analysis of H3.3 acetylation sites

MCF7 cells were transfected with a construct expressing tagged PC4 and IP assays of cell lysates conducted using anti-FLAG M2 affinity agarose. Precipitated protein complexes were then subjected to histone purification using a histone extraction kit and the purified samples subjected to peptide digestion using mixture of enzymes (trypsin, chymotrypsin,

and GluC) before LS-MS/MS tandem mass spectrometry. We obtained several specific peptides corresponding to H3.3 with 100% protein sequence coverage (Fig. 3A) and detected acetylation of this histone variant at lysine residues K9, K14, K18, K23 and K27 (Fig. 3B–E).

3.5. Association of H3.3 with PC4-Flag

To confirm association of H3.3 with PC4, cells were transfected with 3XFlag-PC4 or 3XFlag (control) and further incubated in the presence or absence of TSA. IP with Anti-Flag M2 affinity gel, followed by western blotting using an anti-H3.3 antibody revealed association of PC4 with H3.3, which was significantly elevated in cells treated with TSA relative to controls (Fig. 4A). In contrast, no association was observed in cells transfected with control vector (Fig. 4A). We assumed that the observed increase was related to H3.3 acetylation in response to TSA treatment, as indicated from our results using antibodies recognizing acetylated H3 (Fig. 2A, 2B, 5A, and 5C–D) and the findings of MS/MS analyses (Fig. 3B–D). This *in vitro* PC4-H3.3 interaction was also observed at the chromatin level, as ReChiP from samples subjected to ChIP with an anti-FLAG antibody using an anti-H3.3 antibody generated similar results, with basal levels of PC4-H3.3 enrichment significantly increased by TSA treatment. These findings further indicate an association between PC4 and H3.3 that is significantly increased in the presence of TSA (Fig. 4B). Combined treatment of cells with siRNAs specific for H3.3 (A and B) effectively knocked down H3.3 levels and, while only minor changes in H3 levels were observed in TSA-treated H3.3 depleted cells, this was not the case in untreated samples (Fig. 4C), demonstrating that the knock-down was highly specific for H3.3. A lack of available antibodies specific for acetylated H3.3 prevented direct evaluation of H3.3 acetylation; therefore, we proceeded using antibodies recognizing acetylated H3 for this and subsequent experiments.

3.6. IP and ChIP demonstrating interaction of PC4 with specific acetylated H3 proteins

To identify the specific H3 acetylation responsible for mediating interaction with PC4, immunoprecipitated complexes and total cell lysates were subjected to western blotting using antibodies recognizing various acetylated epitopes. H3 acetylated at lysine residues K9, K14, K18, K23, K27, and K36 were significantly pulled down using the anti-FLAG antibody in MCF7 cells treated with TSA (Fig. 5A). To determine whether PC4 localizes to the *LHR* promoter in association with acetylated H3, we performed ChIP, followed by Re-Chip. The initial ChIP was performed using lysates from cells transfected with 3XFLAG-PC4 or 3XFLAG in the presence or absence of TSA, using a Flag antibody (Fig. 5B). Significant binding of PC4 was detected under basal conditions, which is consistent with our previous localization of 3XFLAG-PC4 to the promoter. Non-immune IgG controls (in the presence or absence of TSA) showed undetectable or barely detectable levels of enrichment (Fig. 5B). The second ChIP (Re-ChIP) was performed using immunoprecipitated complexes from the first ChIP and antibodies specific for H3 acetylated at individual Lys sites previously identified by sequencing analyses and verified by western blotting. The presence of complexes of PC4 and acetylated H3 associated with chromatin at the *LHR* promoter site was demonstrated (Fig. 5C above, below).

3.7. Effect of PC4 knock-down on association with acetylated H3 and *LHR* promoter activity

In cells with endogenous PC4 effectively knocked down using PC4 siRNA, endogenous levels of H3 were significantly increased in response to treatment with TSA (Fig. 6A, above). In contrast, the levels of H3 protein acetylated at different lysine residues (K9, K14, K18, K23, K27, and K36) were markedly reduced in cells with PC4 knocked down compared with those in cells transfected with control scrambled siRNA, while no changes were observed in the absence of TSA (Fig. 6A, above). These data indicate that acetylation of H3 at the indicated sites is favored in the presence of PC4 and independent of endogenous H3 levels. Moreover, on PC4 knock-down, endogenous H3.3 levels were significantly increased by TSA treatment (Fig. 6A, below). A major reduction in TSA-induced *LHR* promoter activity was observed in cells depleted of PC4 (Fig. 6B). This is consistent with the substantial reduction of H3K9 enrichment induced by TSA (scrambled) in PC4 depleted cells (Fig. 6C). This marked reduction of *LHR* promoter activity caused by PC4 depletion (Fig. 6B) may reflect a lack of, or low, association of PC4 coactivator, owing to the marked reduction of acetylation of the relevant histone (Fig. 6A and 6C.) Importantly, knock-down of PC4 resulted in a marked decrease in H3.3 enrichment at the *LHR* promoter (Fig. 6D). This finding links reduction of PC4 with the association of H3.3 to chromatin at the *LHR* promoter and reduced acetylation of H3 in the absence of the coactivator.

3.8. Effect of H3.3 depletion on *LHR* promoter activity and *LHR* mRNA levels

Knock-down of H3.3 caused a significant decrease in TSA-induced Pol II and TFIIB enrichment (Fig. 7A and 7B) at the *LHR* promoter, and a consequent substantial reduction of *LHR* promoter activity and mRNA levels (Fig. 7C and D) relative to cells treated with control scrambled siRNA. This resulted in a decrease in the accessibility of chromatin at the *LHR* promoter region, as indicated by the relative increase of DNase protection in cells with H3.3 knocked down (Fig. 7E). Taken together, these findings indicate the relevance of PC4-H3.3 complexes to *LHR* transcription. The basal levels of H3.3 observed in MCF7 cells were comparable with those detected in CHO cells and moderately higher than those in normal mouse tissues, including liver and heart (Supp. Fig. S1).

4. Discussion

Our results demonstrate an association between PC4 and acetylated H3 in TSA-induced *LHR* derepression in MCF7 cells. MS/MS analysis revealed that PC4 associates with the H3.3 variant acetylated at several lysine residues. The presence of these modifications was further confirmed by immunoprecipitation of a PC4-H3 complex using specific antibodies and detection of acetylated H3 sites by western blotting. ChIP analysis also demonstrated increased enrichment for complexes containing PC4 and H3 acetylated at these sites at the *LHR* promoter on TSA treatment in MCF7 cells. Depletion of endogenous PC4 or H3.3 by siRNA reduced TSA-induced formation of the pre-initiation complex, its enrichment at the *LHR* promoter, and transcriptional activation of the *LHR* gene. Taken together, these findings indicate a critical role for PC4 associated with acetylated H3.3 in TSA-mediated activation of *LHR* gene expression.

Our previous and present studies demonstrate that PC4 has a significant role in the assembly of the general transcriptional machinery in TSA-induced *LHR* transcription. PC4 enrichment at the *LHR* promoter was enhanced by its interaction with Sp1 following TSA treatment [11]. Knock-down of PC4 blocked TSA-induced enrichment of both Pol II and TFIIB, which correlated with a reduction in *LHR* promoter activity. Immunoprecipitation of lysates from MCF7 cells expressing PC4-Flag protein using Flag antibody, followed by western blot detection using a pan-acetyl antibody revealed that TSA specifically induced acetylation of two unknown PC4-interacting proteins (of approximately 16 and 10 kDa; Fig. 1A). Our results demonstrate that PC4 is not acetylated by TSA, and the FLAG-antibody detected two closely migrating bands (~22 kDa), representing phosphorylated and non-phosphorylated forms of PC4-Flag. Analysis of the two PC4-interacting proteins induced by TSA revealed peptides corresponding to endogenous PC4 and H3 protein (16 kDa). The presence of endogenous PC4 in the complexes following immunoprecipitation indicates the formation of dimers comprising PC4-Flag and endogenous PC4 (Fig. 1; [16]). Although we were unable to obtain a sequence for the 10 kDa species, our data indicate the existence of at least one additional PC4-interacting species.

MS/MS analysis detected peptides covering the entire sequence corresponding to the H3.3 variant, acetylated at various lysine residues (Fig. 3). The histone variant, H3.3, is highly conserved evolutionarily and has important and specific roles in the regulation of chromatin dynamics and transcription. With only five amino-acid differences that have defined specific functions relative to canonical H3, H3.3 has a marked inhibitory effect on the folding of chromatin fibers at enhancer and promoter regions, and promotes gene activation [18]. This histone variant is the predominant H3 species expressed throughout the cell cycle [18, 19], and is incorporated into actively transcribed genes and has an important role in active gene transcription. H3.3 undergoes modifications at lysine residues (K9, K14, and K27) and is essential for transcriptional activation of genes during chromatin remodeling [19, 20, 21, 22]. Our studies revealed an association of acetylated H3.3 with PC4 at the promoter in TSA-induced gene activation, and that H3.3 is acetylated at Lys K9, K14, K18, K23, K27, and K36 in the derepressed state. Previous studies from other groups have shown interaction of H3.3 with chaperone proteins, including FACT (facilitates chromatin transcription), ASF1 (anti-silencing function 1), and DAXX (death domain-associated protein), and the chromatin remodeler, ATRX (alpha thalassemia mental retardation syndrome X-linked) [23, 24, 25, 26]. The DAXX/ATRX complex is essential for deposition of H3.3 at telomeres in murine embryonic stem cells [26] and at heterochomatin regions [25]. Another H3.3 specific chaperone, HIRA, deposits H3.3 at the promoters of actively transcribing genes throughout the cell cycle [24]. In vertebrate cells, H3.3 is incorporated at promoters and enhancers, in gene bodies, and at transcription termination sites, as well as in telomeres. H3.3 turnover is lowest at telomeres, intermediate across gene bodies, and highest at promoters and enhancers [24]. Recent studies have shown that the H3.3 variant almost completely replaces H3.1/2 in mouse tissues with advanced age and in normal adult hepatocytes, and this appears to occur in concert with marked changes in post-translational modifications, including combined methylation of H3.3 at K27me2 /K36me2 [27]. Similarly, replacement of canonical histones with variants has been observed during *in vitro* aging of human diploid fibroblasts [28]. All our work was carried out using competent, non-senescent dividing

MCF7 cells in culture, at early passages (not beyond 6–8) and maintained for brief periods in culture for the indicated treatments. In addition, methylation of H3.3 at K27me2 and K36me2, a signature of H3.3 replacement in senescence, was not detected by MS/MS analysis in our study. In *Drosophila*, widespread transcriptional defects are observed in H3.3 mutants, including male sterility as a consequence of chromatin-remodeling failure before meiosis, indicating that H3.3 is essential for male fertility [29]. In our study, we observed a significant reduction in TSA-induced *LHR* promoter activity when H3.3 was knocked down using siRNA. We also observed that enrichment of Pol II and TFIIB was significantly blocked as a consequence of H3.3 loss, which correlated with a marked reduction in TSA-mediated *LHR* promoter activation, and gene expression. These results are clearly attributable to reduced chromatin accessibility at the promoter region (Fig. 7).

PC4 is a component of chromatin and a multifunctional transcriptional coactivator in RNA polymerase II-mediated transcription through direct interactions with general transcriptional machinery and transcriptional activators, including GAL4-BRCA1, GAL4-VP16, GAL4-CTF1, GAL4-Sp1, and AP2 [30]. Moreover, PC4 can impede AP2 self-expression in a Ras-transformed cell line and thereby act as a putative tumor suppressor [31]. The PC4 protein can also act as a coactivator of MyoD and MEF2C by relieving the inhibitory effect of HDAC4, thus playing a pivotal role during myoblast differentiation [32]. PC4 is also a unique activator of p53 function and can stimulate sequence-specific DNA binding of p53 [33]. PC4 interacts directly with the core histones, H3 and H2B, and induces chromatin folding/condensation, indicating that it has an important role in chromatin organization [34]. Both PC4 and p53 undergo post-translational modification (phosphorylation and acetylation) with various functional consequences [35]. Acetylation of PC4 enhances p53 binding to p53 responsive promoters, while phosphorylation of PC4 negatively regulates its acetylation and coactivator function [35]. In our previous studies, we found that PC4 phosphorylation negatively regulates *LHR* transcription [11]. Data from the present studies cannot determine whether H3.3 associates with the phospho and/or non-phospho form of PC4, since all forms were immunoprecipitated by PC4-Flag, including endogenous PC4, because of its tendency to form dimers; however, given the deleterious effect of phosphorylation of PC4 on *LHR* transcription [11], we propose that non-phospho PC4 is the most likely form to associate with acetylated H3.3.

GST pull-down studies demonstrated an association of H3/H3.3 with GST-PC4 in MCF7 cell extracts, while no direct association with H4 was clearly established (Fig. 2E). Similarly, association with recombinant H3 and H3.3 proteins was observed in GST-PC4 pull-down assays, indicating direct association of PC4 with H3 and H3.3, but not H4 (Fig. 2F). Since addition of H3-H4 or H3.3-H4 tetramers revealed H3 and H4 bands or H3.3 and H4 bands, respectively, in GST pull-down assays, we conclude that PC4 associates with these tetramers via H3 or H3.3. This is in contrast with findings in PC4-Flag transfected cells, where PC4 associated with endogenous histone proteins, but not with the tetramer, as evidenced by the presence of only H3 and absence of H4 in an IP using Flag antibody. This could indicate dissociation of the tetramer, resulting from association with the PC4-Sp1 complex. The recruitment of PC4 to Sp1 and formation of the PC4-Sp1 complex has previously been shown to be essential for *LHR* transcription [11]. The present data indicate that PC4 expression is not affected by TSA; therefore, PC4 levels are not associated with

changes in H3/H3.3-Ac. In contrast, PC4-H3.3 interaction could favor histone acetylation. Notably, knock-down of endogenous PC4 in MCF7 cells resulted in a significant reduction in H3K-acetylated protein expression, TSA-induced enrichment of H3K9-Ac and H3.3 at the *LHR* promoter, and consequent inhibition of *LHR* transcription. Our findings also link acetylation of H3 to PC4, because its absence abolished H3 acetylation induced by TSA, despite major increases in expression of both H3 and H3.3 proteins induced by TSA.

5. Conclusion

Together, these findings demonstrate the critical role of PC4 in the formation and assembly of transcriptional machinery in TSA-induced *LHR* gene activation, involving interaction of PC4 with acetylated H3.3, a histone variant known to be deposited at promoter regions of actively transcribing genes. Depletion of either H3.3 or PC4 impaired TSA-induced enrichment of Pol II and TFIIB at the *LHR* promoter, leading to repression/silencing of *LHR* gene expression. H3.3 acetylated at several lysine residues interacted with PC4 recruited to the promoter region, leading to an increase in chromatin accessibility and transcription during *LHR* derepression induced by TSA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

1. Interaction of PC4 with the histone variant, H3.3, is required for *LHR* transcription.
2. Acetylation of H3.3 leads to increase in chromatin accessibility and gene transcription.
3. H3.3 knock-down impairs the enrichment of Pol II and TFIIB at the *LHR* promoter and its TSA-induced activation.

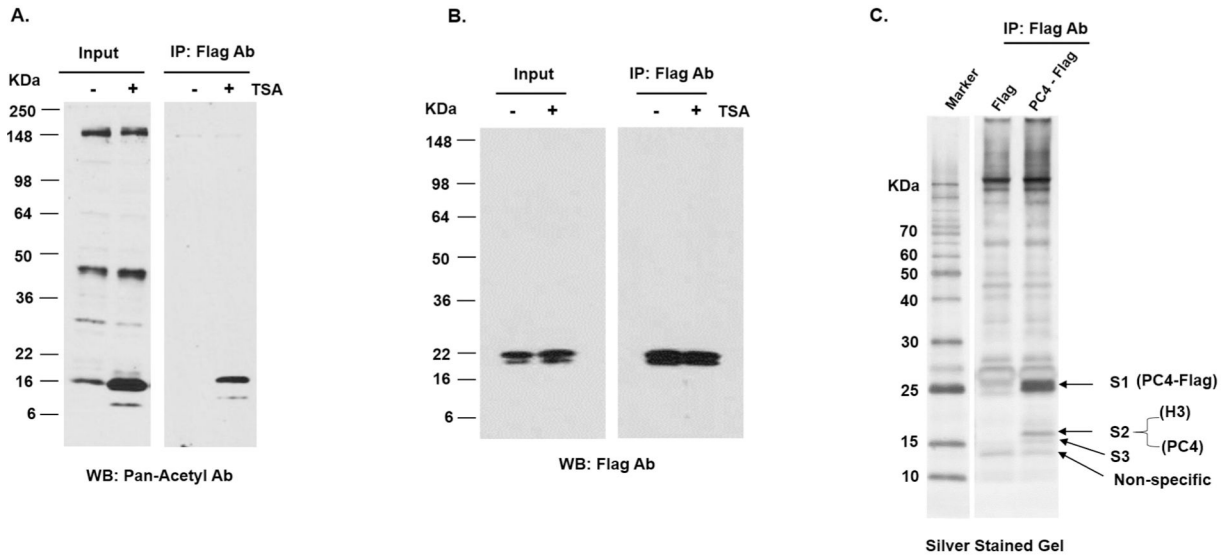

Fig. 1. TSA induced acetylation of PC4 interacting proteins.

Cell lysates from MCF7 cells transfected with 3XFlag PC4 expression vector in the presence and absence of TSA treatment (500ng/ml, 16h), were immunoprecipitated using Flag antibody. The precipitated complexes were subjected to western blot with pan-acetyl-antibody (A) and Flag antibody (B) On the left of (A) and (B) are shown the inputs probed by the corresponding antibodies. The molecular weight of 3XFlag PC4 is expected to be ~22 kDa, phospho (upper band) and non-phospho (lower band) forms. (C) A representative silver stained gel of immunoprecipitated proteins. Cells lysates from MCF7 cells transfected with 3XFlag PC4 expression vector (PC4-Flag) or empty vector (Tag-Flag) in the presence of TSA (500ng/ml, 16h), were immunoprecipitated with anti-Flag M2 affinity gel. The precipitated complexes were eluted with 2X SDS sample buffer and concentrated with an Amicon Ultra centrifugal filter. The samples were then resolved on 14% Tris-Glycine polyacrylamide gel followed by silver staining. Bands S1, S2, S3 are the bands cut from the gel and subjected to MS/MS analysis for protein characterization. Results are representative of three independent experiments.

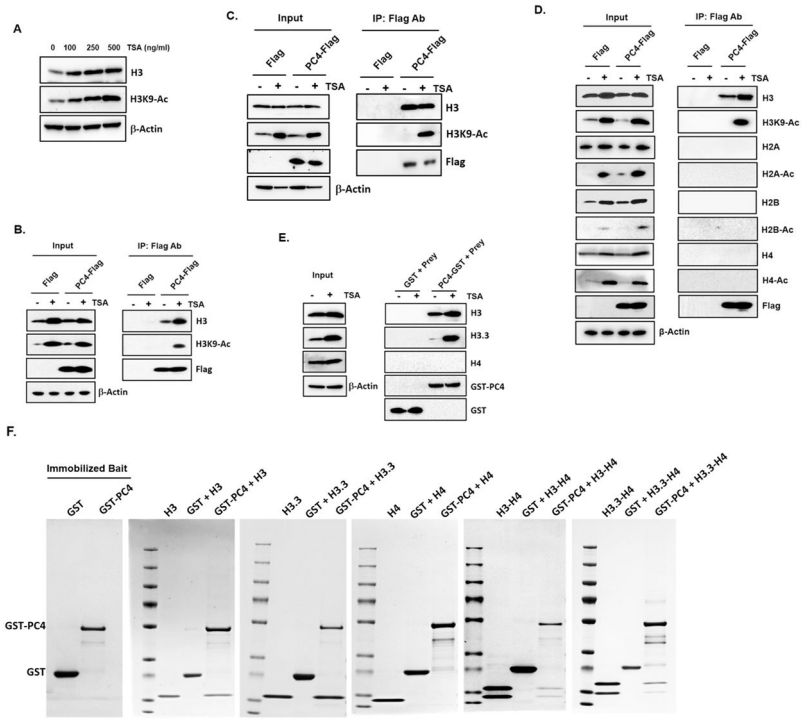

Fig. 2. Immunoprecipitation of PC4-with H3 and acetylated-H3.

(A) Western blots showing dose related increases in H3 and H3K9-ac following incubation of MCF7 cells with TSA for 16 h compared to untreated sample. β-actin was used as a loading control. (B) Western blots showing interaction between PC4-Flag protein and histones. Cells lysates from MCF7 cells transfected with 3XFlag PC4 expression vector or Flag-tag vector with/without TSA treatment (500ng/ml, 16h), were immunoprecipitated with anti-Flag M2 affinity gel. Total lysates in input and immunoprecipitated complexes were subjected to western blot using specific antibodies (H3, H3-Ac and Flag). (C) Identical IP protocol as mentioned above, where H3 levels were normalized (as shown in input) prior to IP reaction. Note that normalization of H3 caused reduction of Flag and β-actin in input compared to Fig. 2B. (D) Western blots showing input and immunoprecipitated complexes after IP with anti-Flag M2 affinity gel using specific antibodies for the detection of H3, H2A, H2B, H4, H3K9-Ac, H2A-Ac, H2B-Ac, H4-Ac and Flag. β-actin was used as a loading control. (E) GST pull-down assay: interaction of bacterial expressed GST-PC4 (bait) with H3, H3.3, H4 (prey) from MCF7 cell extracts detected by their specific antibodies in western blot. GST-PC4 after pull-down was revealed by GST antibody. H4 did not show interaction with GST-PC4. (F) GST pull-down assay showing interaction of GST-PC4 (bait) with recombinant H3, H3.3, H4, H3-H4 tetramer or H3.3-H4 tetramer (prey) in Coomassie blue stained gels. Results are representative of three independent experiments.

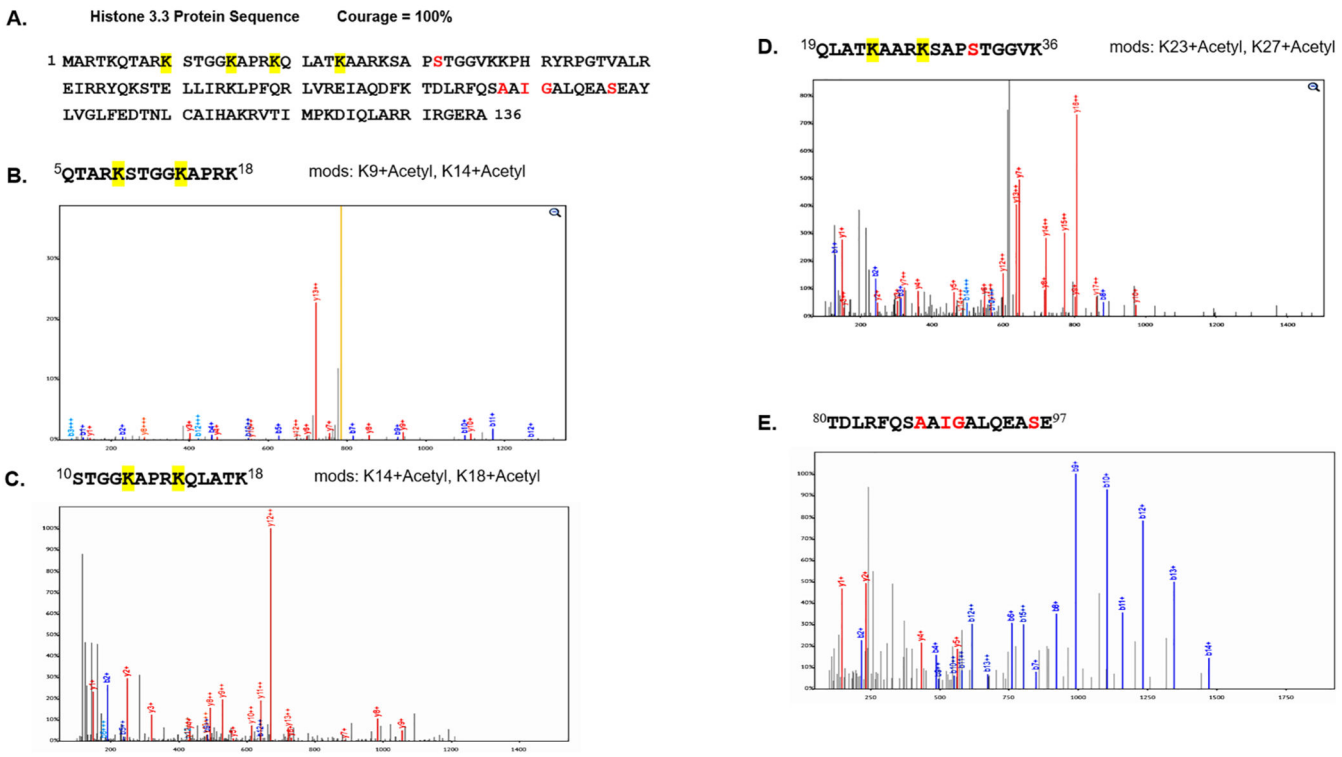

Fig. 3. ESI-MS/MS spectra of acetylated peptides of H3.3 protein obtained from MCF7 cells treated with TSA

(A) Peptide sequence of H3.3 showing acetylated lysine residues in red with 100% amino acid sequence coverage obtained from MS/MS. (B) MS/MS spectrum of acetylated peptide QTARK(Ac)STGGK(Ac)APRK. (C) MS/MS spectrum of the acetylated peptide STGGK(Ac)APRK(Ac)QLATK. (D) MS/MS spectrum of the acetylated peptide QLATK(Ac)AARK(Ac)SAPSTGGVK. (E) MS/MS spectrum of the peptide TDLRFQSAAIGALQEASE showing amino acids marked in red color unique to H3.3. Spectral images were obtained using Tandem data base where y-type immonium ions represented by red peaks. All the MS/MS data were acquired using Thermo Scientific Velos Pro ion trap mass spectrometer. Acetylation at K residues were highlighted in yellow. Amino acids which characterized H3.3 in the sequence are shown in red. Results are representative of three independent experiments.

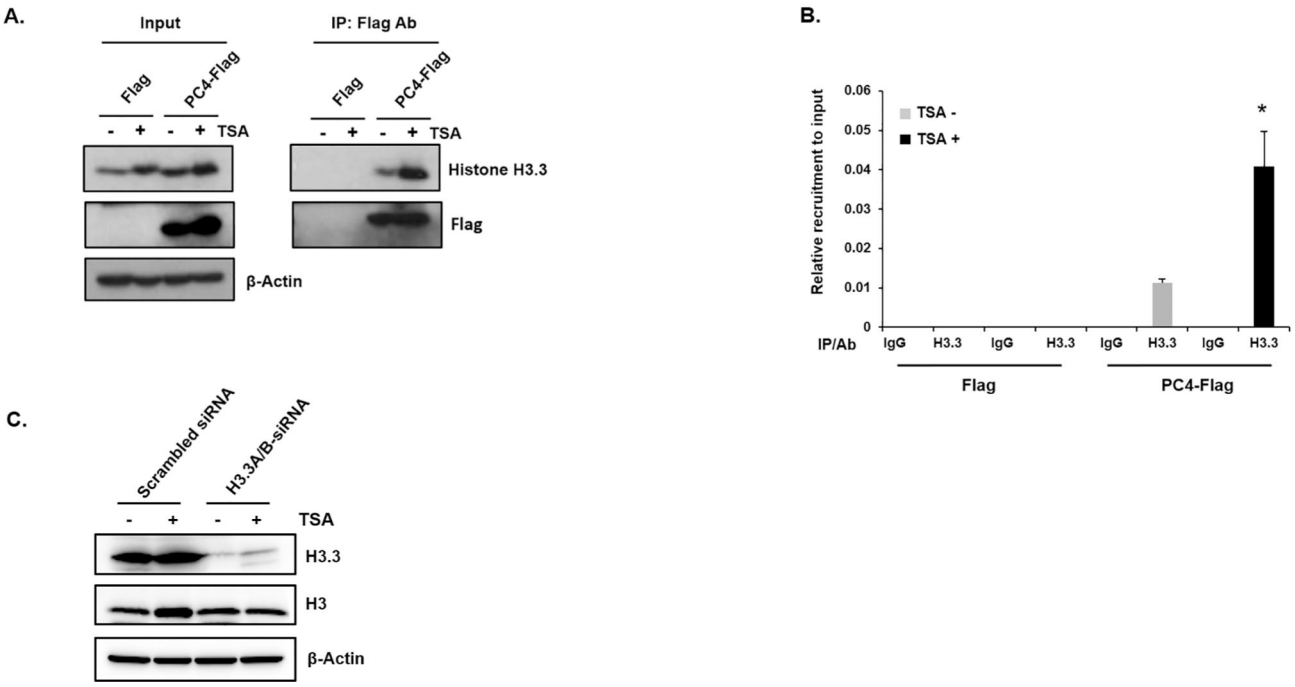

Fig. 4. Association of PC4 with H3.3.

(A) Western blot showing interaction between PC4 and H3.3 by IP in the presence or absence of TSA from cells transfected with PC4-Flag or Flag empty vector (control). Corresponding inputs are shown on the left. β-actin is shown as a loading control. Results are representative of three independent experiments. (B) Re-Chip analysis showing interaction of PC4-Flag with H3.3 in MCF7 cells transfected with PC4-Flag or Flag empty vector. After first ChIP using Flag or IgG antibody a Re-Chip was performed using IgG or H3.3 antibody. Asterisks (*) indicate statistically significant changes between TSA treated and untreated groups (t test; $P < 0.01$). Data represent mean ± SE of two independent experiments performed in triplicates. (C) Knock-down of endogenous H3.3 by H3.3 A/B siRNA versus scrambled siRNA. Western blot showing levels of H3.3 and H3K9 in control and TSA treated cells transfected with H3.3 A/B siRNA or scrambled siRNA. β-actin was used as loading control.

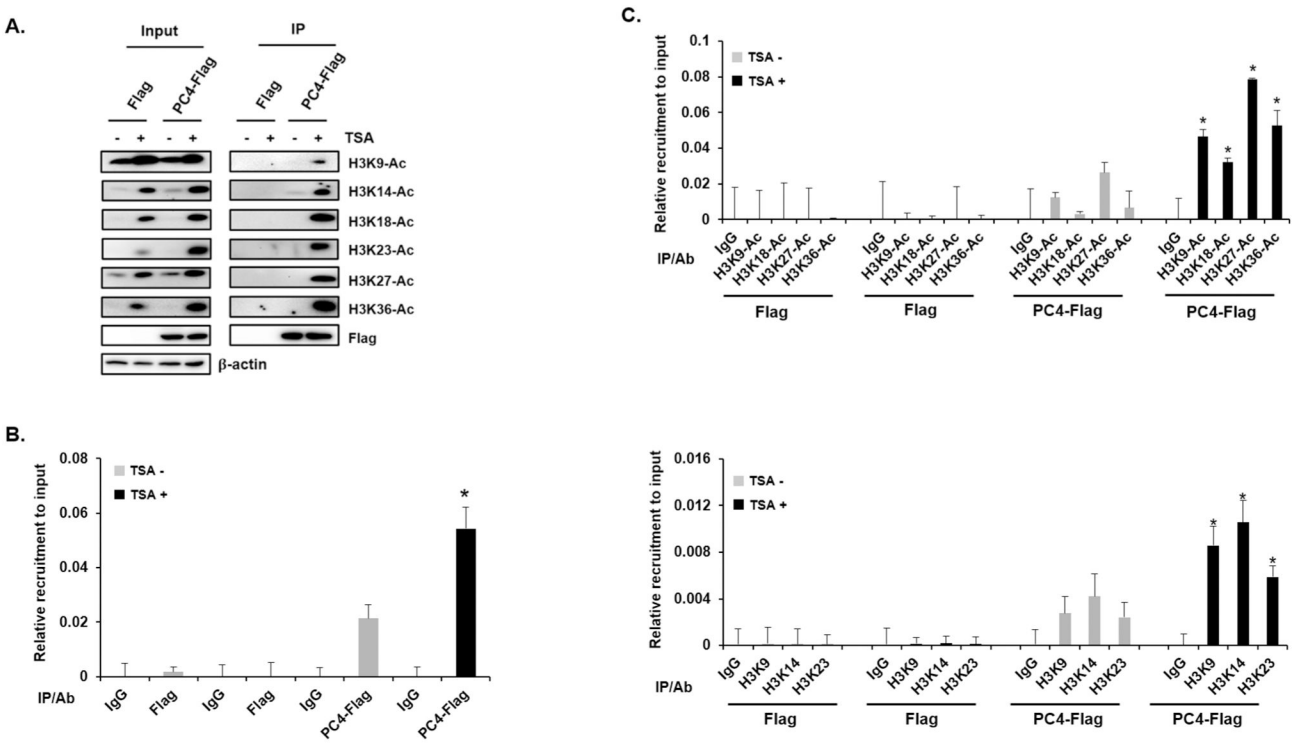

Fig. 5. Western blots showing interaction between PC4 and acetylated Histone 3 and at the chromatin level by ChIP and Re-ChIP in MCF7 cells.

(A) Lysates from MCF7 cells transfected with PC4-Flag or Flag-tag vector with/without TSA treatment (500ng/ml, 16h), were immunoprecipitated with anti-Flag-M2 affinity gel. The precipitated complexes and total lysates (Input) were subjected to western blotting for detection of H3 acetylated at different lysine residues (K9, K14, K18, K23, K27 and K36) and PC4-Flag. β-actin is shown as a loading control. Results are representative of three independent experiments. (B) ChIP showing relative enrichment of Flag protein and PC4-Flag expressed protein in MCF7 cells treated with/without TSA at the LHR promoter. Asterisks (*) indicate statistically significant changes between TSA treated and untreated groups (Student-t test; $P < 0.05$). (C) Above and Below - Enrichment of different acetylated histone proteins at K9, K14, K18, K23, K27 and K36 residues associated with Flag PC4 at the LHR promoter. Re-ChIP was performed with chromatin isolated from MCF7 cells expressing PC4-Flag followed by sequential IP with Flag antibody and subsequently with specific acetylated H3 antibodies. Asterisks (*) indicate statistically significant changes between TSA treated and untreated groups (t test; $P < 0.01$). Data represent mean ± SE of two independent experiments performed in triplicates.

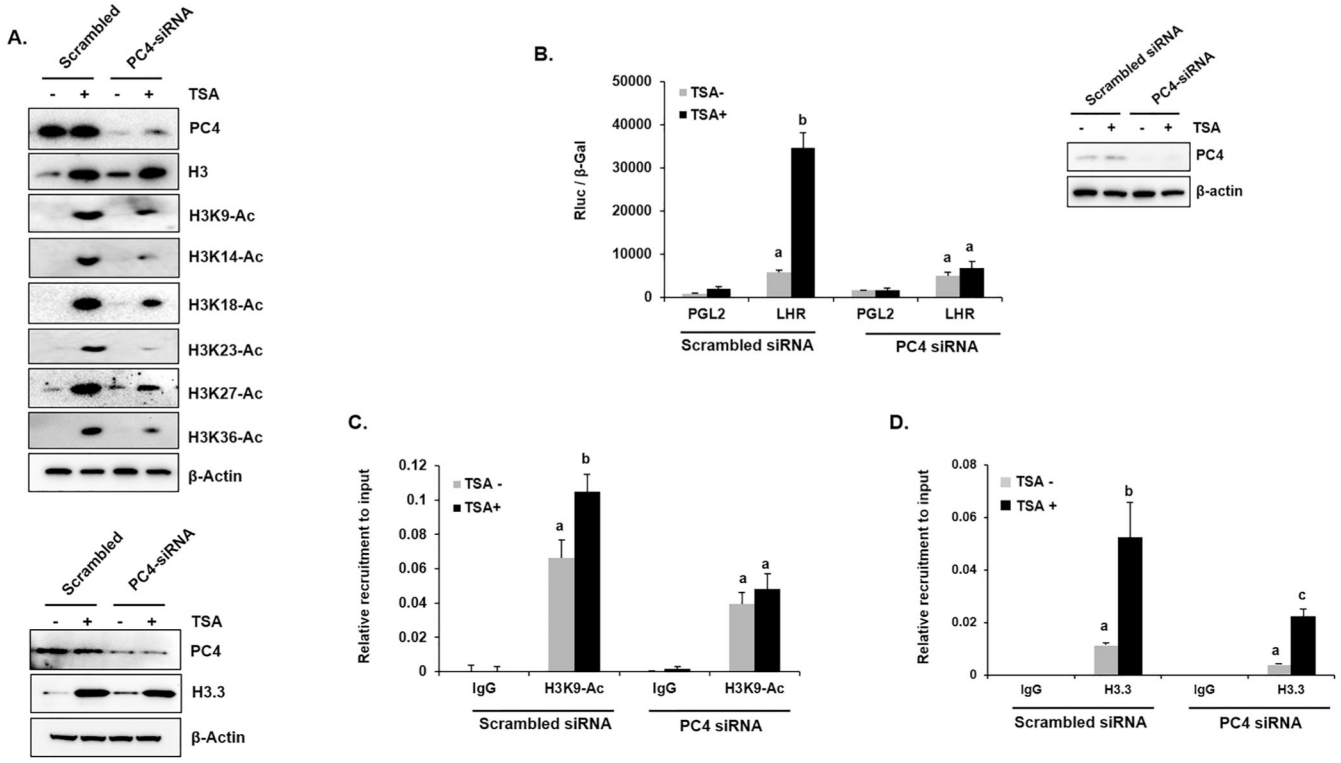

Fig. 6. Effect of endogenous PC4 knock-down on acetylated H3 association, and LHR promoter activity.

(A, above) Western blot showing PC4, H3 and acetylated H3 levels in MCF7 cells transfected with scrambled or PC4 siRNA followed by treatment with or without TSA (500ng/ml) for 16 h revealed by PC4, H3 and specific H3 acetylated antibodies to H3 Lys residues (K9, K14, K18, K23, K27 and K36). (A, below) Western blot showing PC4 and H3.3 levels in MCF7 cells treated with scrambled or PC4 siRNA followed by treatment with or without TSA. β-actin is shown as a loading control. Results are representative of three independent experiments. (B) Effect of endogenous PC4 knock-down by siRNA on LHR promoter activity. Cells transfected with scrambled (control) or PC4 siRNA followed by transfection of LHR promoter construct or PGL2 basic vector and later treated with or without TSA prior to measurement of promoter activity. Different superscript letters indicate significant differences (Dunn's multiple comparison test, $P < 0.001$). (C) ChIP analysis illustrates relative enrichment of H3K9-Ac onto the LHR promoter of cells transfected with scrambled or PC4 siRNA cultured in the presence or absence of TSA. Different superscript letters indicate significant differences (Dunn's multiple comparison test, $P < 0.05$). (D) H3.3 enrichment at the LHR promoter assessed in cells treated with and without TSA following PC4 knockdown. Different superscript letters indicate significant differences (Dunn's multiple comparison test, $P < 0.05$). Representative western blot showing knock-down of PC4 protein in MCF7 cells following PC4 siRNA treatment. Data represent mean ± SE of two independent experiments performed in triplicates.

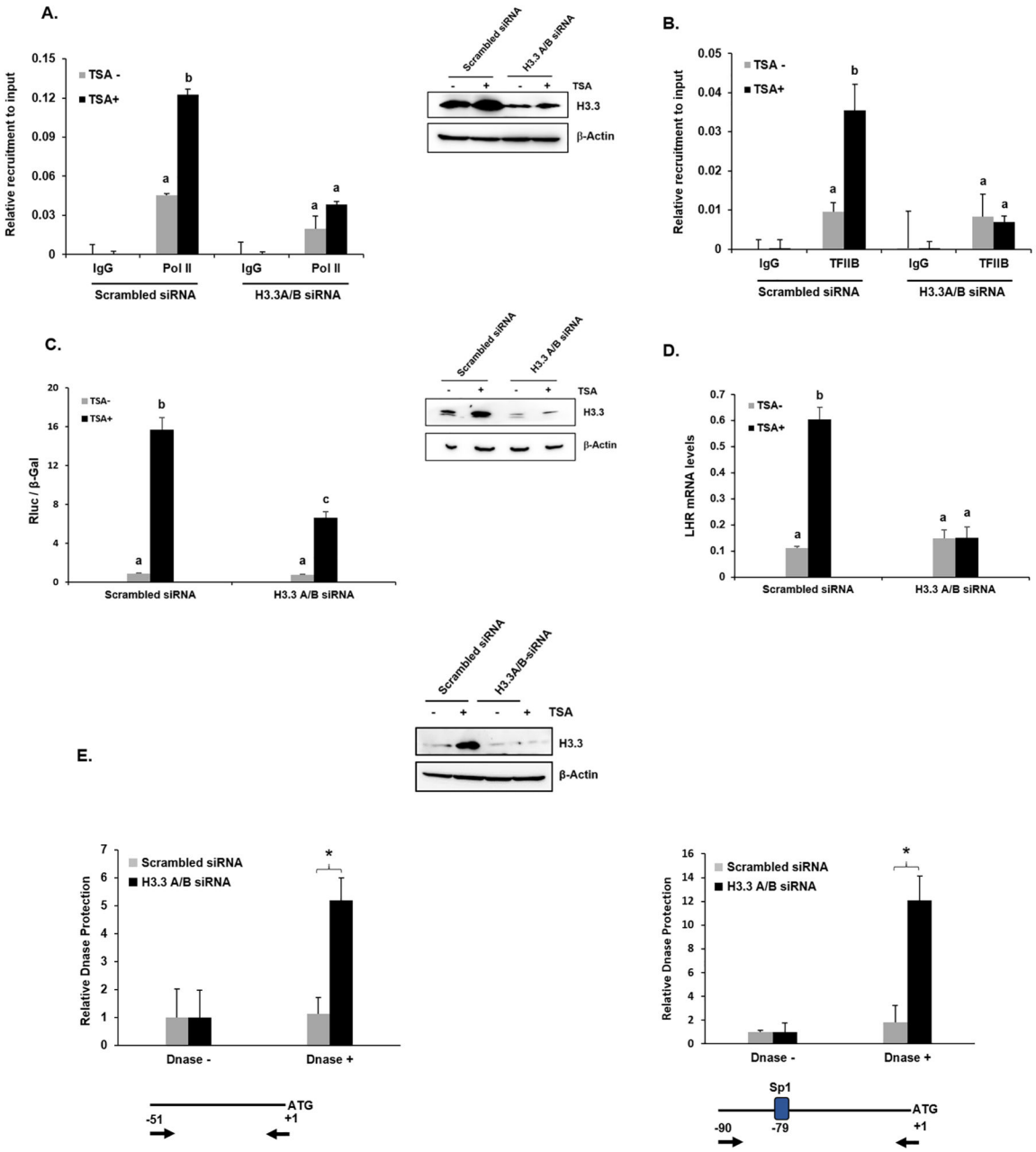

Fig. 7. Effect of H3.3 A and B knock-down on TFIIB and Pol II enrichment at the LHR promoter and promoter activity and chromatin accessibility.

A. ChIP analysis showing enrichment of Pol II at the LHR promoter in MCF7 cells transfected with scrambled or H3.3A/B siRNAs in the presence or absence of TSA. Data represent mean ± SE of two independent experiments performed in triplicates. (B) ChiP analysis showing enrichment of TFIIB at the LHR promoter in MCF7 cells transfected with scrambled or H3.3A/B SiRNAs in the presence or absence of TSA. Different superscript letters indicate significant differences (Tukey's multiple comparison test, $P < 0.05$). Data represent mean ± SE of two independent experiments performed in triplicates. Effect of H3.3 knockdown by H3.3 A/B siRNAs on LHR promoter activity (C) and LHR mRNA

levels (D). Cells transfected with LHR 176 bp promoter construct or empty PGL vector, followed by transfection with scrambled (control) or H3.3 A/B siRNAs and by treatment with or without TSA prior to measurement of promoter activity. β-actin is shown as a loading control. Different superscript letters indicate significant differences (Tukey's multiple comparison test, $P < 0.001$). Data represent mean ± SE of three independent experiments performed in triplicates. (E) EpiQ analysis of the accessibility of chromatin on the promoter region of the LHR gene after TSA induction in H3.3 knockdown MCF7 cells (left & right). Below EpiQ graphs are schematically represented regions amplified by primers are −55/ +1 and −99/+1, respectively (see also methods). The EpiQ results were normalized with PC4 gene as a reference. Asterisks (*) indicate statistically significant changes between scrambled siRNA and H3.3 siRNA groups (Student-t test; $P < 0.01$). Data represent mean ± SE of three independent experiments performed in triplicates. Representative western blot showing knock-down of H3.3 protein in MCF7 cells following transfection with H3.3A/B siRNAs.

Table 1

Peptides identified in MS/MS analysis of protein bands S1 and S2

Protein Band	Peptides Detected
S1 (PC4)	^{54}QSSSSSRDDNMFQIGK^{68}
	^{87}EYWMDPEGEMKPGRK^{101}
	^{102}EQISDIDDAVR^{114}
	^{115}GISLNPEQWSQLK^{125}
S2 (H3)	^{18}KQLATKAARKSAPATGGVK^{36} K27 and K36 Acetylated
	^{73}EIAQDFKTDLR^{83}
S2 (H3)	^{54}YQKSTELLIR^{63} K56 Propionylated
	^{73}EIAQDFKTDLR^{83} K79 Propionylated
	^{117}VTIMPKDIQLAR^{130} K122 Propionylated
S2 (PC4)	^{2}PKSKELVSSS SSGSDSDSEVDKK^{24}
	^{4}SKELVSSSSS GSDSDSEVDK KLK^{26}
	^{30}QVAPEKPVK^{38}
	^{54}QSSSSRDDNM FQIGK^{68}
	^{101}KGISLNPEQW SQLKEQISDI DDAVR^{125}