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PITX2 associates with PTIP-containing histone H3 lysine 4 methyltransferase complex

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ABSTRACT

Pituitary homeobox 2 (PITX2), a Paired-like homeodomain transcription factor and a downstream effector of Wnt/β-catenin signaling, plays substantial roles in embryonic development and human disorders. The mechanism of its functions, however, is not fully understood. In this study, we demonstrated that PITX2 associated with histone H3 lysine 4 (H3K4) methyltransferase (HKMT) mixed-lineage leukemia 4 (MLL4/KMT2D), Pax transactivation domain-interacting protein (PTIP), and other H3K4·HKMT core subunits. This association of PITX2 with H3K4·HKMT complex was dependent on PITX2's homeodomain. Consistently, the PITX2 protein complex was shown to possess H3K4·HKMT activity. Furthermore, the chromatin immunoprecipitation result revealed co-occupancy of PITX2 and PTIP on the promoter of the PITX2's transcriptional target. Taken together, our data provide new mechanistic perspectives on PITX2's functions and its related biological processes.

1. Introduction

Pituitary homeobox 2 (PITX2) is a transcription factor with a *Paired*-like homeodomain. It is evolutionarily conserved in many animal species including zebrafish and human. Previous studies have demonstrated that PITX2 plays significant roles in embryonic development and human disorders. *Pitx2* knockout mice die by embryonic day 15 due to severe developmental defects [1–3]. It is critical for the establishment of the left–right body axis, the asymmetrical development of the heart, lungs, liver, and spleen, twisting of the gut and stomach [1–3]. It is also responsible for the development of the eye [4,5], tooth [6] and cardiac outflow tract [3,7]. Mutations in the *PITX2* gene have been linked to several human disorders, including Axenfeld–Rieger syndrome, iridogoniodysgenesis syndrome, sporadic cases of Peters anomaly, and atrial fibrillation [4,8]. We and others recently reported an oncogenic role of PITX2 in human thyroid cancer [9], ovarian cancer [10], colon cancer [11], renal cancer [12] and esophageal squamous cell carcinoma [13].

Mechanistically, PITX2 has been shown to serve as a downstream effector of Nodal and Wnt signaling [3,14]. It can transcriptionally activate expression of genes required for cell proliferation (e.g., c-Myc, Cyclin Ds) [9,15] or cell differentiation (e.g., MyoD) [16]. Several PITX2-interacting transcriptional regulators, including β-catenin, LEF/TCF and YB-1, have been reported previously [17,18].

In our previous study, we demonstrated that transient overexpression of PITX2 in HEK293 cells resulted in 868 upregulated genes and 191 downregulated genes [18]. Among these regulated genes, 15 were imprinted genes including *H19*, *IGF2* and *CDKN1C*. This remarkable change of global gene expression prompted us to hypothesize that PITX2 might execute its effect through interacting with chromatin-remodeling molecules. In this study, we tested this hypothesis and demonstrated that PITX2 formed complex with histone lysine methyltransferase. This intriguing finding provides new insights on PITX2's functions and its related biological processes.

2. Materials and methods

2.1. Cell culture

HEK293 cells (CRL-1573™, ATCC, VA) were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, 10% fetal bovine serum, and penicillin–streptomycin (100 IU/ml). Cell

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culture media and supplements were purchased from ATCC. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Plasmid construction

Construction of the pEGFP-NFLAG-PITX2c plasmid (expressing N-terminal FLAG tagged full-length human PITX2C, amino acids 1-324) and the pEGFP-NFLAG-PITX2c-ΔHD plasmid (expressing N-terminal FLAG tagged, homeodomain-deleted PITX2c, amino acids 1-131) has been reported in our previous works [9,18]. The other truncated constructs of PITX2c were cloned from pEGFP-NFLAG-PITX2c and inserted into either the NheI/KpnI sites (for PITX2c amino acids 152-324) or the HindIII/KpnI sites (for others) of the pEGFP vector (Clontech Laboratories). Enhanced GFP (green fluorescent protein) was not fused with PITX2 in the abovementioned plasmids and its expression was solely served as an internal marker to monitor transfection efficiency.

2.3. Co-immunoprecipitation and immunoblotting

The plasmids with FLAG-tagged, full-length or truncated PITX2 were transfected into HEK293 cells that had been cultured overnight in 6-well plates by Fugene HD transfection reagent (Roche, IN). Transfection efficiency was monitored by green fluorescence 48 h later to ensure the consistency of experiments. Transfected cells were then lysed by the cell lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitors) and incubated with primary antibodies at 4 °C overnight before being precipitated by protein A beads (Pierce). Immunoprecipitated samples were boiled in the SDS loading buffer and subjected to immunoblotting analysis as described previously [9]. Antibodies used for immunoprecipitation were: FLAG M2 (Sigma, MO); PTIP, RBBP5 and ASH2L (Bethyl laboratory); MLL4/KMT2D (a gift from Dr. Kai Ge, NIDDK/NIH). Antibodies used for immunoblotting were: PITX2 (Abnova); PTIP, RBBP5 and ASH2L (Bethyl laboratory); hnRNP U (Abcam).

2.4. Histone methyltransferase assay

The nuclear extract (NE) of HEK293 cells was prepared by a NE-PER kit (Thermo Scientific Pierce) according to the manufacture's instruction. The PITX2, PTIP, or MLL4/KMT2D immunoprecipitated complex was prepared as described above. The NE or the immunoprecipitated complex was incubated with human histone H3.1 (New England Biolabs) and S-adenosylmethionine (SAM) (New England Biolabs) at room temperature for 1 h. After incubation, the reaction was subjected to immunoblotting by the antibody against H3K4 mono-methylation (H3K4me1) (Cell Signaling Technology).

2.5. Chromatin immunoprecipitation (ChIP) assay

HEK293 cells were transiently transfected with pEGFP-NFLAG-PITX2c or pEGFP-NFLAG-PITX2c-ΔHD. After 48 h of initial transfection, cells were cross-linked by 1% formaldehyde for 15 min. Cell genomic DNA was then sheared into fragments by sonication. Cell lysates were pre-cleared by protein A beads (Pierce, IL) for 2 h at 4 °C. Anti-FLAG M2 conjugated agarose beads (Sigma), anti-PTIP (Bethyl laboratory), anti-H3K4me2 (Cell Signaling Technology), anti-HDAC1 (Cell Signaling Technology) or IgG antibody was then incubated with pre-cleared cell lysates at 4 °C overnight. Beads were washed by the high salt and LiCl washing solution for eight times. Samples were reverse cross-linked in the high salt solution at 65 °C overnight. DNA was purified by phenol–chloroform extraction and precipitated by isopropanol. PCR was then used to analyze precipitated DNA samples. The PCR primers (ATGGAAACGCTCCCGCTAGGT and AGGACCAAGTGTCGAGGGATT) covered the promoter (−133 to −7) of the human Cyclin A1. PCR parameters are: 94 °C for 2 min; 94 °C for 20 s, 58 °C for 20 s, 72 °C for 50 s, 36 cycles; 72 °C for 6 min.

3. Results

3.1. PITX2 forms complex with MLL4, ASH2L, RBBP5 and PTIP

We previously reported that transiently overexpressed PITX2 significantly altered global gene expression patterns [18]. This finding prompted us to hypothesize that PITX2 might synergistically interact with chromatin-remodeling molecules. As a first attempt to test this hypothesis, we looked at histone H3 lysine 4 (H3K4) methyltransferases, which have been linked to transcriptional activation [19,20]. As shown in Fig. 1, PITX2 co-immunoprecipitated with H3K4 methyltransferase mixed-lineage leukemia 4 (MLL4/KMT2D). ASH2L and RBBP5, two core subunits of the MLL family of H3K4 methyltransferases, were also found to associate with PITX2. Consistently, we observed the association of PITX2 with PTIP, which is a unique subunit for the MLL3 and MLL4 methyltransferase complex [21]. On the other hand, hnRNP U, a PITX2 interacting protein identified in our previous study [18], did not form complex with MLL4 or other subunits. Collectively, our data demonstrate that PITX2 associates with MLL4, ASH2L, RBBP5 and PTIP in one complex.

3.2. The homeodomain of PITX2 is required for its association with the H3K4 methyltransferase complex

To determine which domain of PITX2 is required for its association with the H3K4 methyltransferase complex, we performed co-immunoprecipitation on various truncated forms of PITX2. RBBP5, a core subunit of the MLL family, was selected as a read-out for this experiment. The result revealed that PITX2 (FL, aa1-324) (full-length PITX2), PITX2 (aa1-180) (lacking C-terminal half) and PITX2 (aa91-324) associated with RBBP5, but PITX2 (aa1-131) (lacking C-terminal half and part of homeodomain) and PITX2 (aa152-324) (lacking N-terminal and homeodomain) did not (Fig. 2), indicating that the homeodomain of PITX2 is critical for this association.

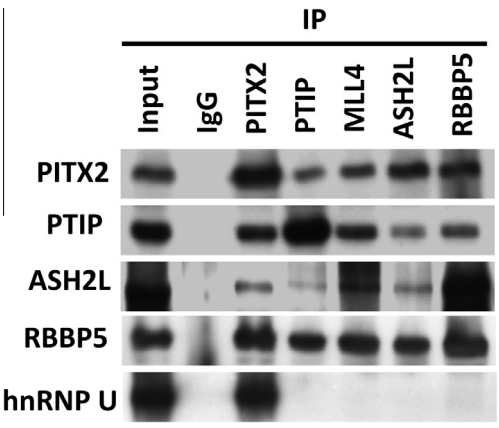

Fig. 1. PITX2 associates with MLL4 histone methyltransferase complex. The antibodies for FLAG (PITX2), PTIP, MLL4, ASH2L and RBBP5 were used to precipitate respective antigens and their associated proteins from HEK293 cells expressing FLAG-PITX2. Immunoblotting was used to examine the components of respective protein complex.

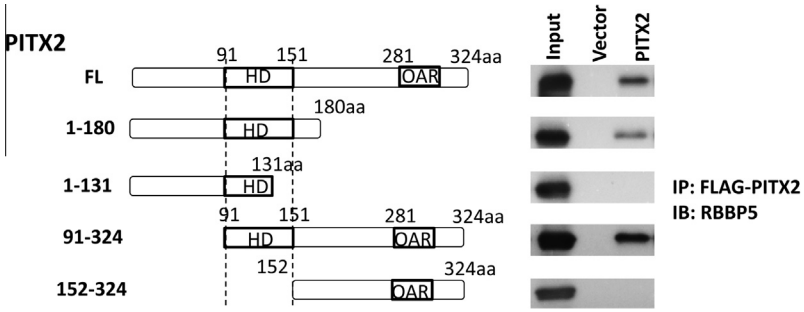

Fig. 2. The association of PITX2 with the H3K4 methyltransferase complex is dependent on its homeodomain. The plasmids containing full-length or truncated PITX2 were expressed in HEK293 cells. Anti-FLAG was used to co-immunoprecipitate PITX2 associated protein complex. RBBP5, a core subunit of the MLL family of H3K4 methyltransferases, was used as a read-out of the H3K4 methyltransferase complex. HD, homeodomain; OAR, Otp and Aristaless domain.

3.3. PITX2 complex methylates histone H3 on K4

The above results showed that PITX2 formed complex with MLL4/KMT2D. Thus, we asked whether the PITX2 complex possessed H3K4 histone lysine methyltransferase (HKMT) activity. As shown in Fig. 3, the immunoprecipitates of PITX2, PTIP and MLL4 all efficiently methylated histone H3 on K4. In contrast, the immunoprecipitates of IgG control and homeodomain-deleted PITX2 failed to do so. These data indicate that PITX2 *bona fide* associates with H3K4 methyltransferase complex. This previously uncharacterized finding suggests that PITX2 may transcriptionally activate expression of its target genes by linking H3K4·HKMT to the target's promoter, a point to be investigated next.

3.4. PITX2 and PTIP co-occupy the promoter of the PITX2's transcriptional target

To determine whether PITX2 and H3K4·HKMT co-occupy the promoter of PITX2's target genes, we performed chromatin immunoprecipitation (ChIP) assays. Unfortunately, the MLL4 antibody did not work in ChIP. Thus, we alternatively chose PTIP, a unique subunit for the MLL4 methyltransferase complex [21]. The gene tested in ChIP was Cyclin A1, which was newly identified as PITX2's transcriptional target in our recent works [18,22]. Consistent with our previous observation that Cyclin A1 transcription was turned on by full-length PITX2 but not by homeodomain-deleted PITX2 (PITX2ΔHD), our ChIP result revealed co-occupancy of PITX2 and PTIP on the Cyclin A1 promoter around the transcription start site (TSS) (Fig. 4). Similarly, we observed increased enrichment of H3K4 di-methylation (H3K4me2) on the Cyclin A1 promoter (Fig. 4). Conversely, the ChIP signal of histone deacetylase 1 (HADC1) was overtly decreased (Fig. 4). Taken together, these data suggest that

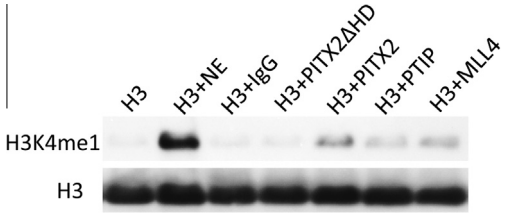

Fig. 3. PITX2 complex methylates histone H3 on K4. The nuclear extract (NE) of HEK293 cells, the IgG immunoprecipitate complex (prepared from HEK293 cells), the PITX2ΔHD immunoprecipitate complex (prepared from HEK293 cells expressing homeodomain-deleted PITX2), the PITX2 immunoprecipitate complex (prepared from HEK293 cells expressing full-length PITX2), the PTIP immunoprecipitate complex (prepared from HEK293 cells) or the MLL4 immunoprecipitate complex (prepared from HEK293 cells) was used as a potential source of H3K4 methyltransferases. H3K4 methylation assays were carried out as described in Section 2. Immunoblotting of H3 served as loading control.

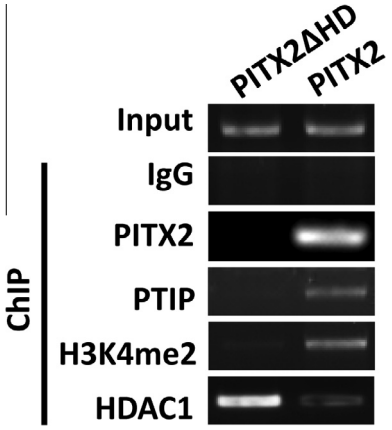

Fig. 4. PITX2 recruits PTIP-containing H3K4 methyltransferase to the Cyclin A1 promoter. Chromatin immunoprecipitation (ChIP) assays were performed on HEK293 cells transiently expressing homeodomain-deleted PITX2 (PITX2ΔHD) or full-length PITX2 (PITX2). The PCR primers covered the Cyclin A1 promoter (−133 to −7, with the transcription start site as position 1).

PITX2 can recruit H3K4·HKMT to the promoter of its transcriptional target and, consequently, activate gene transcription.

4. Discussion

Homeodomain transcription factor PITX2 plays substantial roles in embryonic development and human disorders. Thus, elucidating the mechanisms of its action is of great interest to many researchers. Herein, we report that PITX2 associates with PTIP-containing H3K4 methyltransferase complex. This finding is significant since it uncovers a novel, epigenetic mechanism for PITX2's functions and its related biological processes.

Although our data demonstrate the association of PITX2 and MLL4/KMT2D, by no means we can discount the possibility that PITX2 might form complex with other H3K4 methyltransferases or other chromatin-remodeling molecules in general. In fact, this study just opens a whole new avenue to investigate the physical and functional interaction between PITX2 and epigenetic regulators.

Our results indicate that the homeodomain of PITX2 is necessary for its association with H3K4 methyltransferase complex and associated HKMT activity. This is consistent with a previous report that demonstrated the requirement of homeodomain in the interaction between mouse Pitx2 and WDR5 (another core subunit of the MLL family of H3K4 methyltransferases) [23]. However, how PITX2 interacts with H3K4·HKMT complex, e.g., which HKMT subunit is the direct binding partner, is still unclear and should be

addressed next. The finding of PITX2–PTIP complex in this study deserves further attention. PTIP has been assigned three distinct functions, one for regulating gene transcription [24], one for promoting immunoglobulin class switch recombination [25,26], and the other for facilitating DNA damage repair (DDR) [27–30]. Although it is straightforward to line up PITX2–PTIP in gene transcription as previously reported PAX2–PTIP [31], it would be interesting to further ask whether PITX2 has a role in immunoglobulin class switch recombination and DDR through interacting with PTIP.

While we were preparing this manuscript, three recent reports have demonstrated that MLL4 is a major HKMT responsible for H3K4 mono- and di-methylation in mammalian cells and a majority of MLL4 binding sites are located in regions of potential enhancer elements [32–34]. With this regard, our finding of PITX2–MLL4 association might link the involvement of MLL4 in PITX2-depedent cell differentiation and development.

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