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Long noncoding RNA HOTTIP cooperates with CCCTC-binding factor to coordinate HOXA gene expression

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ABSTRACT

The spatiotemporal control of HOX gene expression is dependent on positional identity and often correlated to their genomic location within each loci. Maintenance of HOX expression patterns is under complex transcriptional and epigenetic regulation, which is not well understood. Here we demonstrate that HOTTIP, a lincRNA transcribed from the 5′ edge of the HOXA locus, physically associates with the CCCTC-binding factor (CTCF) that serves as an insulator by organizing HOXA cluster into disjoint domains, to cooperatively maintain the chromatin modifications of HOXA genes and thus coordinate the transcriptional activation of distal HOXA genes in human foreskin fibroblasts. Our results reveal the functional connection of HOTTIP and CTCF, and shed light on lincRNAs in gene activation and CTCF mediated chromatin organization.

1. Introduction

In vertebrates, homeotic genes (HOX genes) are clustered in four independent chromosomal loci called HOXA–D, encode homeodomain transcription factors that are critical for specifying the positional identity of cells, and express in a nested pattern along developmental axes [1,2]. Among which, the mammalian HOXA locus consists of a cluster of 11 HOX genes with a nested anterior-posterior or proximal-distal graded expression pattern along body appendages. This gradient is colinear with their genomic position from 3′ to 5′ of the cluster [3].

The HOX gene cluster functions as a classic model of gene regulation and plays a key role in proper patterning the axes during development [1]. The differential expression profile of HOX genes is established at the very early stage in the developing embryo and maintained even as the embryo continues through development [4]. Previous analyses have described the discovery and presence of many intergenic noncoding RNAs in the HOX gene cluster, and the majority of them are transcribed in the strand antisense to HOXA genes, moreover, like canonical HOX genes, these ncRNAs are also systematically vary their expression along a developmental axis of the body by coordinating with their physical location on the chromosome in different cell types [3,5,6].

Considering the great number of ncRNAs clustering in the HOX loci and their diverse modes of action, it has long been proposed that these ncRNAs play a significant role in transcriptional regulation of neighboring HOX genes, emerge as regulatory factors in specifying specialized chromatin domains and participate in epigenetic regulation [7,8,9]. Perhaps the most prominent example is a 2.2 kilobase ncRNA residing in the HOXC locus, termed HOTAIR, which is involved in Polycomb Repressive Complex 2 (PRC2) mediated silencing of chromatin, coordinate histone modifications by binding to multiple histone modification enzymes, and link the histone methylase and the demethylase by acting as a modular scaffold [3,10]. Interestingly, CTCF, a well-known insulator factor, has been found to partition the HOX clusters into antagonistic

Abbreviations: CTCF, CCCTC-binding factor; HOTTIP, HOXA transcript at the distal tip; ChIP, chromatin immunoprecipitation; RIP, RNA immunoprecipitation; CRISPR, clustered regularly interspaced short palindromic repeat; LincRNAs, large intergenic non-coding RNAs.

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functional chromatin domains to establish discontinuous HOX transcriptional programs [11]. However, whether CTCF has functionally connection with HOX ncRNAs remains to be investigated.

HOTTIP (HOXA transcript at the distal tip) is a lincRNA transcribed from the 5′ edge of the HOXA locus, and its transcription produces a 3764-nucleotide, spliced and polyadenylated lincRNA. Consistent with its genomic location 5′ to HOXA13, Wang *et al.* found that the expression of HOTTIP was predominantly observed in anatomically distal human fibroblasts, and it targets WDR5/MLL complexes across HOXA locus, driving histone H3 lysine 4 trimethylation and activation of distal HOXA genes [12]. In the present study, we demonstrate the physical association between CTCF and HOTTIP, and reveal their functional link in coordinating long-range HOXA gene activation in human foreskin fibroblasts.

2. Materials and methods

2.1. Antibodies

A list of antibodies that were used in this study is summarized in Supplementary Table S1.

2.2. Cell culture and RNAi transfection

Human foreskin fibroblasts BJ and HFF cell lines were obtained from ATCC and cultured as previously described [3,12]. The medium was prepared as DMEM supplemented with 10% heat-inactivated FBS. For RNA interference, 40% confluent cells were transfected with 50 nM of siRNAs targeting HOTTIP or siGFP control (siRNA sequences are shown in Supplementary Table S2) using Fugene6 (Promega) according to manufacturer`s instructions.

2.3. Quantitative real-time PCR

Total RNA was extracted using Trizol (Life Technologies) according to the manufacturer's protocol. cDNA was generated using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) according to manufacturer instructions. The abundance of RNA was detected using the Fast SYBR Green Master Mix (Applied Biosystems) following manufacturers' protocol on the LightCycler480 Real-Time PCR System (Roche). For ChIP-qPCR analysis, purified ChIP-DNA was quantified using Nanodrop ND1000 (Thermo Fisher) and 1 ng DNA was used for each PCR reaction. Data were normalized to input DNA. Values are expressed as mean ± s.d. of three independent experiments. All primers used in this study are shown in Supplementary Table S2.

2.4. Chromatin immunoprecipitation (ChIP)

ChIP assay was conducted as described previously [13]. Briefly, cells were chemically fixed with 1% (w/v) formaldehyde for 10 min at room temperature, the reaction was quenched by the addition of 125 mM glycine. After washing with PBS, cells were resuspended, lysed, and sonicated using a Bioruptor (Diagenode) to an average chromatin size of 200–500 bp and cleared by centrifugation. IPs were performed overnight with 5–10 μg of the appropriate antibody, after which Dynal Protein G magnetic beads (Life Technologies) were incubated for an additional 3 h. Beads were washed 5 times with RIPA buffer, once with TE and the complexes were eluted from beads in elution buffer by heating at 65 °C for 16 h. After reversal, DNA was treated with RNase A and proteinase K, and 10% of the input was also treated in parallel. The samples were further extracted by phenol-chloroform and one additional purification step using the DNA purification kit (Active Motif).

2.5. RNA immunoprecipitation (RIP)

RIP experiment was performed as described previously [10]. In brief, approximately 10^7 cells were treated with 0.5% formaldehyde for 10 min at 37 °C, the reaction was quenched with 125 mM glycine and washed twice in cold PBS and pelleted. The pellet was resuspended and lysed in 1 ml of RIP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% NP-40, 0.5% sodium deoxycholate, 0.5 mM DTT and 1 mM PMSF/cocktail, 9 μg/ml leupeptin, 9 μg/ml pepstatin,10 μg/ml chymostatin, 3 μg/ml aprotinin; Ambion). Antibodies were added and incubated for 4 h at 4 °C with gentle rotation. Beads were washed four times with wash buffer (50 mM Tris-Cl pH 7.9, 10% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% NP40), followed by one wash in PBS. The beads were then resuspended in RIP buffer and treated with proteinase K at 45 °C for 45 min. Co-immunoprecipitated RNAs were purified with RNeasy Mini Kit (QIAGEN), and detected by quantitative real-time PCR. Proteins isolated before proteinase K treatment by the beads were investigated by western blot analysis.

2.6. Biotin-labeled RNA pull-down assay

Biotin-labeled RNAs were *in vitro* transcribed and prepared with the Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Promega), then treated with RNase-free DNase I (Promega) and purified with RNeasy Mini Kit (QIAGEN) as previously described [10,12]. Biotin-HRP Northern blot was further performed to show that the RNAs are biotinylated and transcribed at the correct size according to manufacturer instructions (NorthernMax kit, Ambion). Biotinylated RNA was first heated to 90 °C for 2 min and slow-cooled to 4 °C, then used for the immunoprecipitation process. Sixty microliters washed Streptavidin agarose beads (Invitrogen) were mixed with each binding reaction and further incubated at 4 °C for 2 h. Beads were washed shortly five times in Handee spin columns (Pierce) and boiled in SDS loading buffer, and the retrieved proteins were visualized by western blot analysis.

2.7. GST pull-down

Like performed previously [12], HOTTIP and histone H2B1 mRNA were transcribed *in vitro* using T7 polymerase following manufacturers' protocol (Promega), denatured, and refolded in folding buffer (100 mM KCl, 10 mM MgCl2, Tris pH 7.0). GST-tagged CTCF was expressed in *Escherichia coli* and purified as described [13]. GST-CTCF was bound to glutathione beads (Amersham/GE Healthcare) and then mixed with either *in vitro* transcribed HOTTIP or histone H2B1 mRNA and incubated at room temperature for 1 h. After three washes with wash buffer (20 mM HEPES pH 7.6, 200 mM KCl, 0.05% NP40, 1 mM DTT, 1 mM PMSF), bound RNAs were extracted and detected by quantitative real-time PCR, as previously described [10,12].

2.8. CRISPR genome editing

Deletion of CTCF binding by CRISPR/Cas9 was performed as previously described [11]. Briefly, the optimal gRNA target sequence (Supplementary Table S2) was chosen using the http://crispr.mit.edu/ design tool and then cloned into the SpCas9-2AGFP vector (Addgene: PX458) via BbsI digestion and insertion [14]. To screen for deletion cell clones, BJ or HFF cells at about 80% confluence were transfected using Lipofectamine 2000 (Life Technologies) in a 6-well plate with plasmid DNA including Cas9 endonuclease and sgRNA constructs (2 μg each). After transfection, GFP positive cells were sorted and single clones were picked and plated in 96-well plates for genotyping. The resulting PCR products

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