

# HOTAIR Launches IncRNAs

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We are pleased to present a series of Annotated Classics celebrating 40 years of exciting biology in the pages of *Cell*. This installment revisits "Functional Demarcation of Active and Silent Chromatin Domains in Human *HOX* Loci by Noncoding RNAs" from Howard Chang's lab. Here, Irene Bozzoni comments on how Rinn et al. ignited our appreciation for the ability of noncoding RNAs to regulate key cellular and developmental processes in *trans*.

Each Annotated Classic offers a personal perspective on a groundbreaking *Cell* paper from leader in the field with notes on what stood out at the time of first reading and retrospective comments regarding the longer term influence of the work.

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# Functional Demarcation of Active and Silent Chromatin Domains in Human *HOX* Loci by Noncoding RNAs

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DOI 10.1016/j.cell.2007.05.022

## SUMMARY

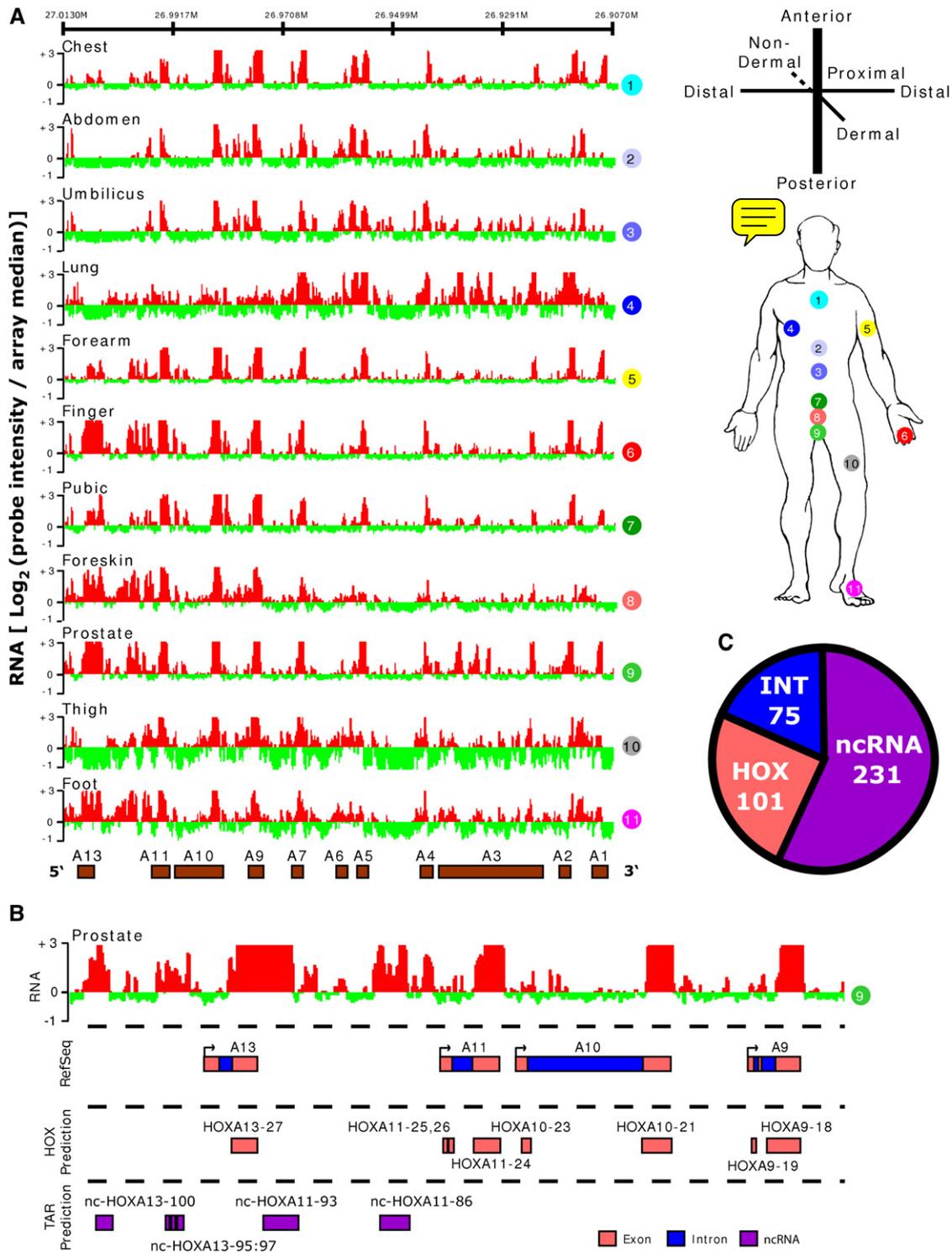
Noncoding RNAs (ncRNA) participate in epigenetic regulation but are poorly understood. Here we characterize the transcriptional landscape of the four human *HOX* loci at five base pair resolution in 11 anatomic sites and identify 231 *HOX* ncRNAs that extend known transcribed regions by more than 30 kilobases. *HOX* ncRNAs are spatially expressed along developmental axes and possess unique sequence motifs, and their expression demarcates broad chromosomal domains of differential histone methylation and RNA polymerase accessibility. We identified a 2.2 kilobase ncRNA residing in the *HOXC* locus, termed HOTAIR, which represses transcription in trans across 40 kilobases of the *HOXD* locus. HOTAIR interacts with Polycomb Repressive Complex 2 (PRC2) and is required for PRC2 occupancy and histone H3 lysine-27 trimethylation of *HOXD* locus. Thus, transcription of ncRNA may demarcate chromosomal domains of gene silencing at a distance; these results have broad implications for gene regulation in development and disease states.

## INTRODUCTION

A distinguishing feature of metazoan genomes is the abundance of noncoding RNA (ncRNAs), which function by means other than directing the production of proteins. In addition to small regulatory RNAs such as miRNAs, recent studies have predicted the existence of long ncRNAs—ranging from 300 nucleotides (nt) to over

10 kb—that are spliced, polyadenylated, and roughly as diverse in a given cell type as protein-coding mRNAs (Bertone et al., 2004; Carninci et al., 2005; Kapranov et al., 2005; Rinn et al., 2003). Long ncRNAs may have diverse roles in gene regulation, especially in epigenetic control of chromatin (Bernstein and Allis, 2005). Perhaps the most prominent example is silencing of the inactive X chromosome by the ncRNA XIST. To normalize the copy number of X chromosomes between male and female cells, transcription of XIST RNA from one of the two female X chromosome is involved in recruiting Polycomb group proteins (PcG) to trimethylate histone H3 on lysine 27 (H3K27me3), rendering the chromosome transcriptionally silent (Plath et al., 2003). It is believed that Polycomb Repressive Complex 2 (PRC2), comprised of H3K27 histone methyl transferase (HMTase) EZH2 and core components Suz12 and EED, initiates this histone modification and that, subsequently, Polycomb Repressive Complex 1 (PRC1) maintains this modification and promotes chromatin compaction (reviewed by Sparmann and van Lohuizen, 2006). Presently, the mechanism by which XIST ncRNA guides Polycomb activity is unclear. Several PcG proteins possess RNA-binding activity, and RNA is required for PcG binding to DNA, suggesting that specific ncRNAs may be critical interfaces between chromatin-remodeling complexes and the genome (Bernstein et al., 2006; Zhang et al., 2004).

In addition to dosage compensation, long ncRNAs may also play critical roles in pattern formation and differentiation. In mammals, 39 *HOX* transcription factors clustered on four chromosomal loci, termed *HOXA* through *HOXD*, are essential for specifying the positional identities of cells. The temporal and spatial pattern of *HOX* gene expression is often correlated to their genomic location within each loci, a property termed colinearity (Kmita and Duboule, 2003; Lemons and McGinnis, 2006). Maintenance of *HOX* expression patterns is under complex epigenetic regulation. Two opposing groups of histone-modifying



**Figure 1. The Human HOX Transcriptome**

(A) Site-specific transcription of the *HOXA* locus. Left: The hybridization intensity of 50,532 probes that tile the human *HOXA* locus for each of the 11 samples (numbered in circles). The intensity of each probe is displayed as the  $\log_2$  of the ratio of the individual probe intensity divided by the average intensity of all 301,027 probes on the array. The  $\log_2$  ratio of each probe was averaged over a 100 bp window; red and green bars indicate expression above or below the array mean, respectively. Genomic locations of protein-coding *HOX* genes are displayed as brown boxes. Right: Anatomic origins of the 11 fibroblast samples with respect to the developmental axes.

complexes, the trithorax group (TrxG) of H3K4 HMTase and the PcG H3K27 HMTase, maintain open and closed chromatin domains in the *HOX* loci, respectively, over successive cell divisions (Ringrose and Paro, 2007). Transcription of many ncRNAs has been observed in fly, mouse, and human *HOX* loci (Bae et al., 2002; Bernstein et al., 2005; Carninci et al., 2005; Drewell et al., 2002; Sessa et al., 2006), and three models have been proposed to account for their action based on experiments in *Drosophila*. First, elegant genetic studies suggested that transcription of ncRNAs altered the accessibility of DNA sequences important for TrxG and PcG binding; the act of intergenic transcription enabled TrxG activation of downstream *HOX* genes and prevented PcG-mediated silencing (Ringrose and Paro, 2007; Schmitt et al., 2005). Second, the above model has been extended by a recent report that several ncRNAs transcribed 5' of the *Drosophila HOX* gene *Ubx* bind to and recruit the TrxG protein Ash1 to the *Ubx* promoter, thereby inducing *Ubx* transcription (Sanchez-Elsner et al., 2006). However, these results have been challenged by the third model of “transcriptional interference,” where transcription of 5' ncRNAs into the promoters of downstream *HOX* genes prevents *HOX* gene expression, leading to transcriptional silencing in *cis* (Petruk et al., 2006). The extent to which any of these models and alternative mechanisms explain the copious amount of transcription in mammalian *HOX* loci remains to be discovered. Nonetheless, the large number of *HOX* ncRNAs, their complex clustering on the chromosomes, and their potentially diverse modes of action suggest ncRNAs play a significant role in *HOX* regulation. By profiling the entire transcriptional and epigenetic landscapes of ~500 kilobase *HOX* loci at near nucleotide resolution, we will begin to discern competing models of ncRNA action in humans and reveal potentially new mechanisms of ncRNA function.

Transcriptomic and proteomic analysis of the *HOX* loci require pure cell populations with distinct positional identities. Rather than study whole animals where cells of many histologic types and positional identities are intermixed, we and others have observed that primary adult human fibroblasts retain many features of the embryonic pattern of *HOX* gene expression both in vitro and in vivo (Bernstein et al., 2005; Chang et al., 2002; Rinn et al., 2006). Differential and colinear expression of *HOX* genes in adult fibroblasts faithfully reflects their position along the anterior-posterior and proximal-distal axes of the developing body (Rinn et al., 2006) and is believed to be important for maintenance of regional identities of skin throughout the lifetime of the animal (Chuong, 2003). The remarkable persistence—over decades—of the embryonic patterns of *HOX* gene expression in these human cells suggests the action of a powerful epigenetic machin-

ery operative over the *HOX* loci. In this study, we create an ultrahigh-resolution tiling microarray to interrogate the transcriptional and epigenetic landscape of the *HOX* loci in a unique collection of primary human fibroblasts with 11 distinct positional identities. Our results identify numerous novel human *HOX* ncRNAs, clarify potential mechanisms of their regulation, and reveal a novel mechanism of ncRNA-assisted transcriptional silencing via the PcG proteins in *trans*.

## RESULTS

### Noncoding RNAs of the Human *HOX* Loci: Identity, Conservation, Expression Pattern, and Sequence Motifs

To systematically investigate the transcriptional activity of the human *HOX* loci, we designed a DNA microarray for all four human *HOX* loci at five base pair (bp) resolution along with two megabases of control regions (Table S1). Computational and experimental analysis confirmed the specificity of the tiling array to distinguish highly related *HOX* sequences (Figures S1 and S2).

Because adult primary fibroblasts are differentiated based on their anatomic site of origin and retain canonical features of the embryonic *HOX* code (Chang et al., 2002; Rinn et al., 2006), we used *HOX* tiling arrays to profile polyadenylated transcripts from fibroblasts representing 11 distinct positional identities (Figure 1A). Previously, analytic methods for tiling arrays have allowed present/absent calls of transcripts and binding events but were less successful in quantification of signal intensity (Bernstein et al., 2005; Bertone et al., 2004). We addressed this challenge by adapting a signal processing algorithm used in computer vision termed Otsu's method (Otsu, 1979). The method dynamically searches for statistically significant cutoffs between signal and background and detects contiguous regions of at least 100 bp (20 probes) with signal intensity significantly above background. Averaging the signal intensity over all probes in the called region thus produces a quantitative measure of transcript abundance. Using this algorithm, we identified a total of 407 discrete transcribed regions in the four *HOX* loci (Table S2). We used current genome annotations to partition them into known *HOX* gene exons, introns, and intergenic transcripts (Figure 1B). As expected, we detected many transcribed regions that corresponded to known *HOX* exons and introns (101 and 75, respectively), including exonic transcription for 34 of the 39 *HOX* genes, thus indicating that these 11 samples encompass the majority of *HOX* transcriptional activity. In all cases examined, the expression of *HOX* genes as determined by the tiling array matched that previously determined by cDNA microarray

(B) Transcribed regions were identified by contiguous signals on tiling array, then compared with Refseq sequence to define genic (exonic, pink color, and intronic, blue) and intergenic transcribed regions (purple). Each predicted *HOX* exon or intron was named HOXn or int-HOXn, respectively. Intergenic transcribed regions were named as nc-HOXn, where n is the *HOX* paralog located 3' to the ncRNA on the *HOX*-coding strand.

(C) Summary of transcribed regions in all four *HOX* loci defining the number of *HOX* genic, intronic, and ncRNA-transcribed regions.

and RT-PCR for these same samples (Chang et al., 2002; Rinn et al., 2006).

Interestingly, the majority of the transcribed regions (231 of 407) arise from intergenic regions (Figure 1C). By comparison to databases of all known amino acid sequences, we found that only 13% (29 of 231) of these intergenic transcripts showed any coding potential in all six possible translational frames (Experimental Procedures; Table S3). In contrast, 88% (84 of 96) of the *HOX* exon transcripts had coding potential, where the 12% non-coding exonic transcripts corresponded to untranslated exonic regions. While these results do not completely rule out the possibility of new protein-coding genes interspersed throughout the *HOX* loci, these intergenic transcripts are more likely candidate noncoding RNAs. We therefore refer to these intergenic transcripts as *HOX* ncRNAs. We named each ncRNA by its genomic location, affixing the name of the *HOX* gene located 3' to the ncRNA on the *HOX*-coding strand (Figure 1C). As previously suggested (Sessa et al., 2006), the majority of ncRNAs (74%) demonstrate evidence for opposite-strand transcription from the *HOX* genes (Table S4). Fifteen percent of the ncRNAs we identified are novel, while the majority of ncRNAs (85%) have been independently observed by EST sequencing or other means (Experimental Procedures). Even for the known ncRNAs, our data suggest that almost all ncRNAs are longer than previously believed (Table S4). The average extension for previously observed ncRNAs is 202 bases; in total we discovered over 30 kilobases of new transcribed bases in the human *HOX* loci. Thus, in just 11 hybridizations, we have substantially expanded the number and length of known transcribed regions in the human *HOX* loci as well as defined their expression patterns throughout the human body.

We found several lines of evidence that confirm the biological importance of the *HOX* ncRNAs. First, comparative analysis with seven vertebrate genomes revealed that some ncRNAs are preferentially conserved in evolution over nontranscribed or intronic *HOX* sequences. For instance, more than one-third of the top 100 conserved transcribed regions in the *HOX* loci are ncRNAs (Figure S3A). Second, RT-PCR analysis of 40 predictions of ncRNA expression levels from the tiling array confirmed a high level of agreement (85%) between array signal intensity and transcript abundance as measured by RT-PCR (Figure S3B).

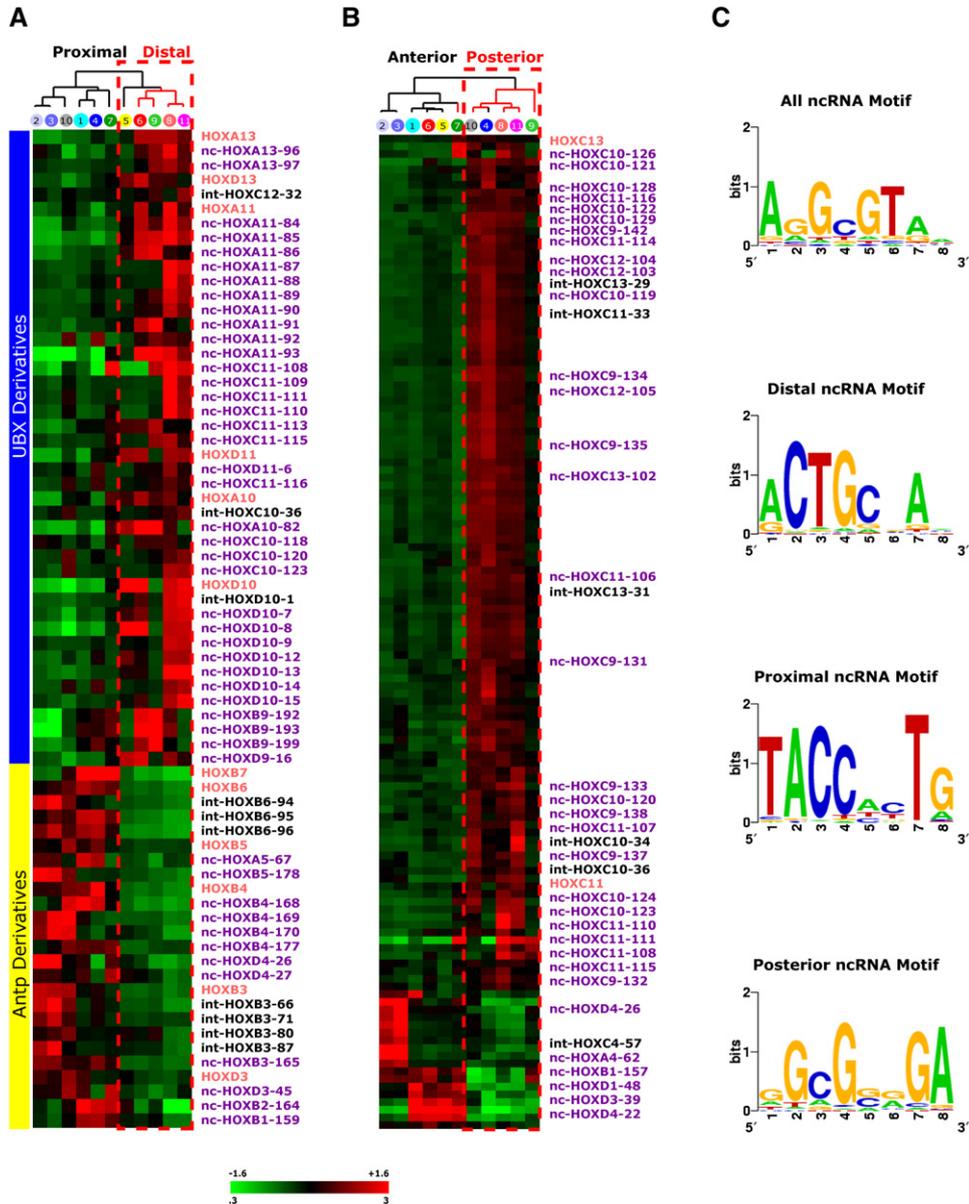
Third, we found that, like canonical *HOX* genes, ncRNAs also systematically vary their expression along developmental axes of the body in a manner coordinated with their physical location on the chromosome (Figure 2; Table S5). One hundred forty-seven of 231 *HOX* ncRNAs (64%) are differentially expressed along a developmental axis of the body ( $p < 0.05$ ). For instance, 48 *HOX* ncRNAs are differentially expressed with their neighboring *HOX* genes along the proximal-distal axis (close or far from the trunk of the body;  $p < 0.05$ , Figure 2A). Strikingly, all 41 transcribed regions (both *HOX* genes and ncRNAs) that are induced in distal sites belonged to *HOX* paralogous groups 9–13, and all 30 transcribed regions that are repressed in

distal sites belonged to paralogous groups 1–6, precisely recapitulating the evolutionary origin of the two domains from *Drosophila ultrabithorax* and *antennapedia* complexes, respectively ( $p < 10^{-19}$ , two-way chi-square test; Carroll, 1995). Similarly, 87 *HOX* ncRNAs are differentially expressed along the anterior-posterior axis (top to bottom of the body), this time with ncRNAs from *HOXC9–13* preferentially induced in posterior sites ( $p < 0.05$ , Figure 2B). Additionally, we observed 7 *HOX* genes and 12 ncRNAs that are either expressed in dermal (outside the body) or nondermal (inside the body) fibroblasts (Figure S4; Table S5). Systematic comparison of the expression pattern of every ncRNA with its immediate 5' and 3' *HOX* gene neighbor showed that the vast majority of ncRNAs (90%) are coordinately induced with their 3' *HOX* genes, while only 10% of instances are ncRNA expression anticorrelated with 3' *HOX* gene expression (Figure S5).

Fourth, in addition to their distinctive expression patterns, we found that the ncRNAs also possess specific sequence motifs. Using a discriminative motif finder that we previously developed (Segal et al., 2003), we found that ncRNAs are enriched for specific DNA sequence motifs based on their site-specific expression patterns ( $p < 10^{-9}$ , Figure 3C). We identified a sequence motif enriched in ncRNAs over exonic, intronic, or nontranscribed sequences, and we further identified sequence motifs for ncRNAs that are expressed in distal, proximal, or posterior sites. These sequence motifs may represent DNA- or RNA-binding sites for regulatory factors to regulate gene expression in *cis*. Together, these results establish that the majority of site-specific transcriptional output of the *HOX* loci consists of ncRNAs. Their evolutionary conservation, differential expression along developmental axes, and distinct primary sequence motifs suggest important and possibly widespread roles for these ncRNA transcripts in *HOX* gene regulation.

### Diametrical Domains of Chromatin Modifications Demarcated by *HOX* ncRNAs

The coordinate expression of *HOX* genes and neighboring ncRNAs raised the possibility that their expression may be regulated by chromatin domains, large contiguous regions of differential chromatin modifications that enable transcriptional accessibility or cause silencing. Such domains, first observed by Bernstein and colleagues for histone H3 lysine 4 dimethylation (H3K4me<sub>2</sub>; Bernstein et al., 2005), are a notable and unique feature of *HOX* loci chromatin (Bracken et al., 2006; Lee et al., 2006; Papp and Muller, 2006; Squazzo et al., 2006). We tested this idea by loci-wide chromatin immunoprecipitation followed by tiling array analysis (ChIP-chip). We found that both *HOX* and ncRNA transcription fell within broad domains occupied by RNA polymerase II, whereas the transcriptionally silent regions were broadly occupied by the PRC2 component Suz12 and its cognate histone mark, histone H3 trimethylated at lysine 27 (H3K27me<sub>3</sub>;  $p < 10^{-15}$ , chi-square test, Experimental Procedures; Figure 3). Comparison of cells from different anatomic



**Figure 2. Site-Specific Expression and Primary Sequence Motifs of HOX ncRNAs**

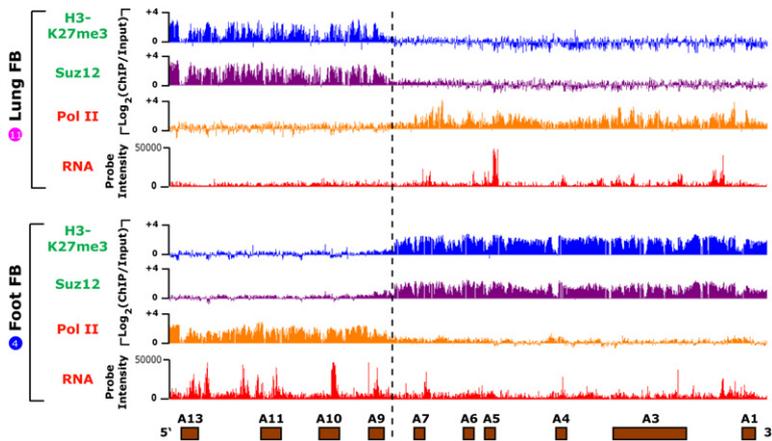
(A) HOX-encoded transcripts differentially expressed along the proximal-distal axis. Sixty transcribed regions (12 HOX genes and 48 ncRNAs) were differentially expressed ( $p < 0.05$ , Student's t test) between distal fibroblast samples (foot, finger, foreskin, and prostate) and all other cells. Expression level of each transcribed region above or below the global median is denoted by the color scale (3-fold to 0.3-fold on linear scale or +1.6 to -1.6 on  $\log_2$  scale). Transcribed regions were ordered by their position along the chromosome, and samples were hierarchically clustered by similarity of expression of these 60 transcripts. The evolutionary origin of HOX paralogs to fly *ultrathorax* (UBX) or *antennapedia* (Antp) are indicated by blue and yellow boxes, respectively.

(B) HOX-encoded transcripts differentially expressed along the anterior-posterior anatomic division. A total of 92 transcripts (6 HOX genes, 86 ncRNAs) were differentially expressed ( $p < 0.05$ , Student's t test) in anterior or posterior primary fibroblast cultures (above or below the umbilicus). Expression of each ncRNA is represented as in (A).

(C) Enriched sequence motifs in HOX ncRNA based on their pattern of expression ( $p < 10^{-9}$ ). Logograms of sequence motifs enriched in the primary sequences of ncRNAs over nontranscribed HOX sequences or in ncRNAs with distal, proximal, or posterior patterns of expression are shown. ncRNAs expressed in anterior anatomic sites did not share a primary sequence motif more than expected by chance.

origins showed that the primary DNA sequence can be programmed with precisely the same boundary but in the opposite configuration. For example, in lung fibroblasts

the 5' HOXA locus is occupied by Suz12 but not PolII, whereas in foreskin fibroblasts this exact same chromatin domain is occupied by PolII but not Suz12. Thus,



**Figure 3. Diametrically Opposed Chromatin Modifications and Transcriptional Accessibility in the *HOXA* Locus**

Occupancy of Suz12, H3K27me3, and PolII versus transcriptional activity over ~100 kb of the *HOXA* locus for primary lung (top) or foot (bottom) fibroblasts (Fb). For ChIP data, the  $log_2$  ratio of ChIP/Input is plotted on the y axis. For RNA data, the hybridization intensity on a linear scale is shown. Dashed line highlights the boundary of opposite configurations of chromatin modifications and intergenic transcription.

positional identity in differentiated cells may be marked by diametric or mutually exclusive domains of chromatin modifications, which switch their configurations around a center of inversion in a site-specific manner.

Interestingly, the boundary of the diametric chromatin domains defined by ChIP-chip is precisely the same as that suggested by our transcriptional analysis. In the *HOXA* locus, the chromatin boundary and switch between proximal versus distal expression patterns occurs between *HOXA7* and *HOXA9*. Additional ChIP-chip analysis showed the domain of PolII occupancy precisely overlaps the domain of H3K4 dimethylation, but H3K9 trimethylation, a histone modification characteristic of constitutive heterochromatin, is not present on any *HOX* loci in these cells (Figure S6). These results suggest that *HOX* loci transcription in adult fibroblasts is governed by opposing epigenetic modifications over large chromosomal regions and further define the locations of specific boundary elements that delimit chromatin domains.

**HOTAIR: A ncRNA that Regulates Chromatin Silencing In trans**

We next asked whether the coordinate transcription of *HOX* ncRNAs is merely a consequence of the broad chromatin domains or whether the ncRNAs are actively involved in establishing such domains. To address this question, we analyzed in depth the function of a long ncRNA situated at the boundary of two diametrical chromatin domains in the *HOXC* locus (Figure 4A). This ncRNA is transcribed in an antisense manner with respect to the canonical *HOXC* genes; we therefore named it HOTAIR for *HOX* Antisense Intergenic RNA. Molecular cloning and northern blot analysis confirmed that HOTAIR is a 2158 nucleotide, spliced, and polyadenylated transcript; strand-specific RT-PCR analysis confirmed that only one strand of HOTAIR that is antisense to *HOXC* genes is transcribed (Figures 4B and 4C). Computational analysis of HOTAIR secondary structure did not reveal obvious stem loops suggestive of pre-miRNAs. Northern blot analysis of size-fractionated RNA showed no evidence of small RNA products suggestive of micro- or siRNA

production, while we readily detected the ubiquitous miRNA let7 in parallel experiments (Figure 4D).

Our tiling array data suggested that HOTAIR is preferentially expressed in posterior and distal sites, and indeed this expression pattern is confirmed by additional RT-PCR experiments (Figure 4E). In situ hybridization of developing mouse embryos confirmed that HOTAIR is expressed in posterior and distal sites, indicating the conservation of anatomic expression pattern from development to adulthood (Figure 4F). Interestingly, this transcript has very high nucleotide conservation in vertebrates (99.5%, 95%, 90%, and 85% sequence identity in chimp, macaque, mouse, and dog genomes, respectively), yet is riddled with stop codons with little amino acid sequence conservation amongst vertebrates (Supplemental Experimental Procedures). These results suggest that HOTAIR may function as a long ncRNA.

HOTAIR ncRNA may regulate gene expression in *HOX* loci in *cis* or *trans*; alternatively, it may be the act of antisense transcription in the *HOXC* locus rather than the ncRNA itself that has a functional role in gene regulation. To distinguish between these possibilities, we depleted HOTAIR ncRNA by RNA interference in primary human fibroblasts and determined the consequences on the transcriptional landscape of the *HOX* loci. Strikingly, while siRNA-mediated depletion of HOTAIR had little effect on transcription of the *HOXC* locus on chromosome 12 compared to wild-type and control siRNA targeting GFP, depletion of HOTAIR led to dramatic transcriptional activation of the *HOXD* locus on chromosome 2 spanning over 40 kb, including *HOXD8*, *HOXD9*, *HOXD10*, *HOXD11*, and multiple ncRNAs (Figures 5A, 5B, and S7). To ensure that this was not an off-target effect of RNA interference, we employed four independent siRNA sequences targeting HOTAIR. Each siRNA depleted HOTAIR ncRNA and led to concomitant *HOXD10* activation as determined by quantitative RT-PCR (Figures 5C and 5D). These observations indicate that HOTAIR ncRNA is required to maintain a transcriptionally silent chromosomal domain in *trans* on the *HOXD* locus.

### HOTAIR ncRNA Enhances PRC2 Activity at the *HOXD* Locus

To investigate the molecular mechanisms involved in the HOTAIR-dependent silencing of the *HOXD* locus, we used chromatin immunoprecipitation to interrogate changes to the *HOXD* chromatin structure upon depletion of HOTAIR. Our previous ChIP-chip experiments indicated that in primary foreskin fibroblasts, the entire *HOXD* locus was occupied by both Suz12 and H3K27me3. Depletion of HOTAIR followed by ChIP-chip revealed substantial and global loss of H3K27me3 occupancy over the *HOXD* locus, with the greatest loss residing in the intergenic region between *HOXD4* and *HOXD8* (Figure 6A). HOTAIR depletion also led to a modest but consistent loss of Suz12 occupancy of the *HOXD* locus (Figures 6B and S8). Importantly, occupancy of H3K27me3 and Suz12 across the silent *HOXB* locus was not affected by HOTAIR depletion in these cells. These results suggest that HOTAIR is selectively required to target PRC2 occupancy and activity to silence transcription of the *HOXD* locus.

Because PcG protein binding to chromatin can involve RNA and HOTAIR ncRNA is required for PRC2 function (i.e., H3K27 trimethylation), we reasoned that HOTAIR may bind to PRC2 and directly regulate Polycomb function. Indeed, native immunoprecipitation of Suz12 from nuclear extracts of two types of primary fibroblasts retrieved associated endogenous HOTAIR ncRNA as detected by RT-PCR, but not nonspecific U1 RNA or DNA (Figure 7A). HOTAIR ncRNA was not retrieved by immunoprecipitation of YY1, which has been suggested to be a component of PRC1 (Sparmann and van Lohuizen, 2006). Suz12 also did not associate with the neighboring *HOXC10* mRNA, indicating that PRC2 binds selectively to *HOXC*-derived transcripts (Figure S9). In the reciprocal experiment, we prepared purified biotinylated sense or antisense HOTAIR RNA by *in vitro* transcription and probed nuclear extracts of HeLa cells to identify HOTAIR-binding proteins. HOTAIR ncRNA retrieved PRC2 components Suz12 and EZH2 but not YY1 (Figure 7B). Antisense HOTAIR RNA did not retrieve any of the above proteins, indicating that the binding conditions are highly specific. Collectively, these experiments indicate that HOTAIR is physically associated with PRC2 either directly or indirectly; loss of this interaction may reduce the ability of PRC2 to methylate histone tails and silence transcription at the *HOXD* locus.

## DISCUSSION

### Panoramic Views of the *HOX* Loci by Ultrahigh-Resolution Tiling Arrays

By analyzing the transcriptional and epigenetic landscape of the *HOX* loci at high resolution in cells with many distinct positional identities, we were afforded a panoramic view of multiple layers of regulation involved in maintenance of site-specific gene expression. The *HOX* loci are demarcated by broad chromosomal domains of transcriptional accessibility, marked by extensive occupancy of

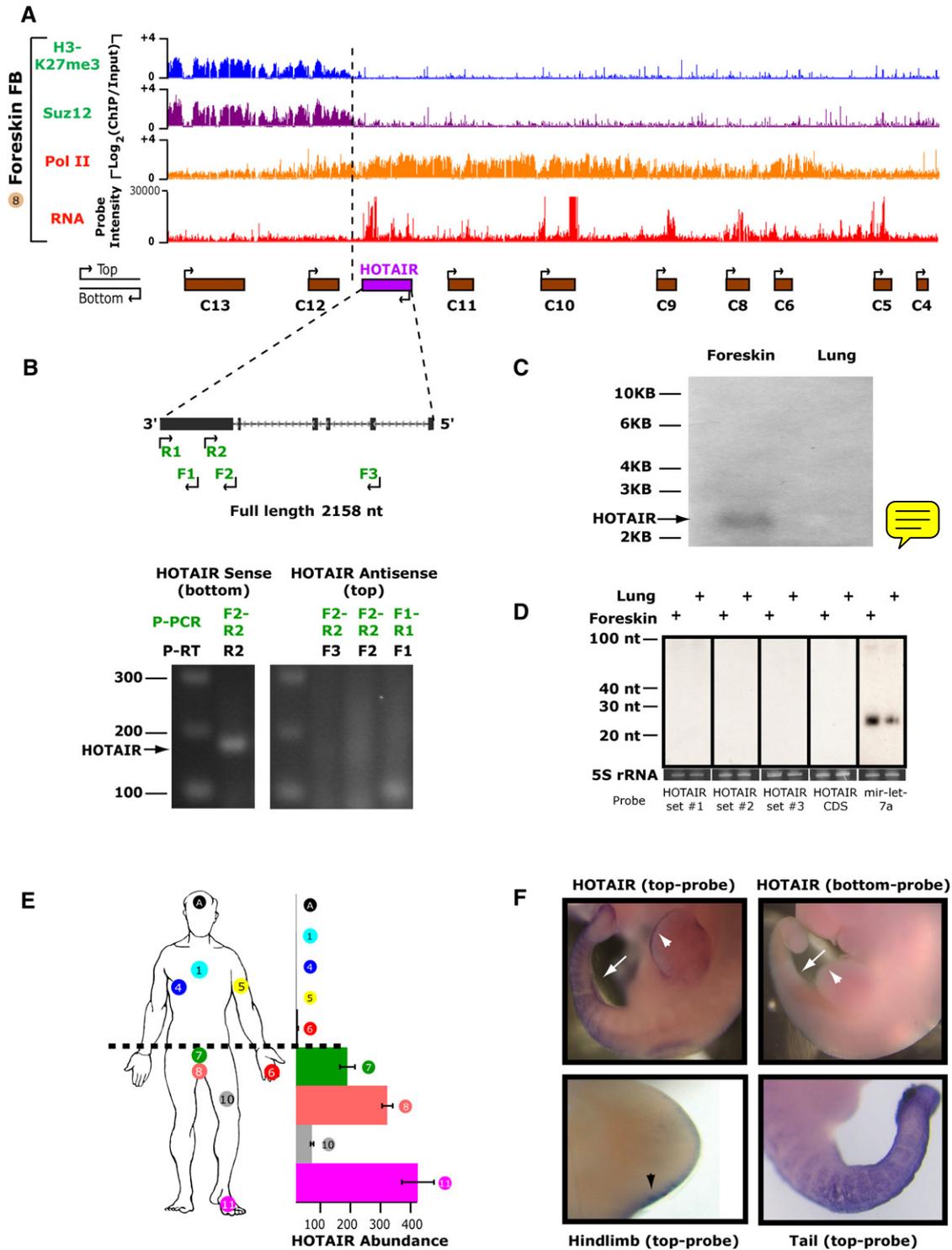
RNA polymerase II and H3K4 dimethylation and, in a mutually exclusive fashion, by occupancy of PRC2 and H3K27me3. The active, PolII-occupied chromosomal domains are further punctuated by discrete regions of transcription of protein-coding *HOX* genes and a large number of long ncRNAs. Our results confirm the existence of broad chromosomal domains of histone modifications and the occupancy of HMTases over the *Hox* loci observed by previous investigators (Bernstein et al., 2005; Boyer et al., 2006; Guenther et al., 2005; Lee et al., 2006; Squazzo et al., 2006) and extend on these observations in several important ways.

First, by comparing the epigenetic landscape of cells with distinct positional identities, we showed that the broad chromatin domains can be programmed with precisely the same boundary but with diametrically opposite histone modifications and consequences on gene expression. Our data thus functionally pinpoint the locations of chromatin boundary elements in the *HOX* loci, the existence of some of which has been predicted by genetic experiments (Kmita et al., 2000). One such boundary element appears to reside between *HOXA7* and *HOXA9*. This genomic location is also the switching point in the expression of *HOXA* genes between anatomically proximal versus distal patterns and is the boundary of different ancestral origins of *HOX* genes, raising the possibility that boundary elements are features demarcating the ends of ancient transcribed regions. Second, the ability to monitor 11 different *HOX* transcriptomes in the context of the same cell type conferred the unique ability to characterize changes in ncRNA regulation that reflect their position in the human body. This unbiased analysis identified more than 30 kb of new transcriptional activity, revealed ncRNAs conserved in evolution, mapped their anatomic patterns of expression, and uncovered enriched ncRNA sequence motifs correlated with their expression pattern—insights which could not be gleaned from examination of EST sequences alone (Sessa et al., 2006). Our finding of a long ncRNA that acts *in trans* to repress *HOX* genes in a distant locus is mainly due to the ability afforded by the tiling array to comprehensively examine the consequence of any perturbation over all *HOX* loci. The expansion of a handful of *Hox*-encoded ncRNAs in *Drosophila* to hundreds of ncRNAs in human *HOX* loci suggests increasingly important and diverse roles for these regulatory RNAs.

An important limitation of the tiling array approach is that while we have improved identification of transcribed regions, the data do not address the *connectivity* of these regions. The precise start, end, patterns of splicing, and regions of double-stranded overlap between ncRNAs will need to be addressed by detailed molecular studies in the future.

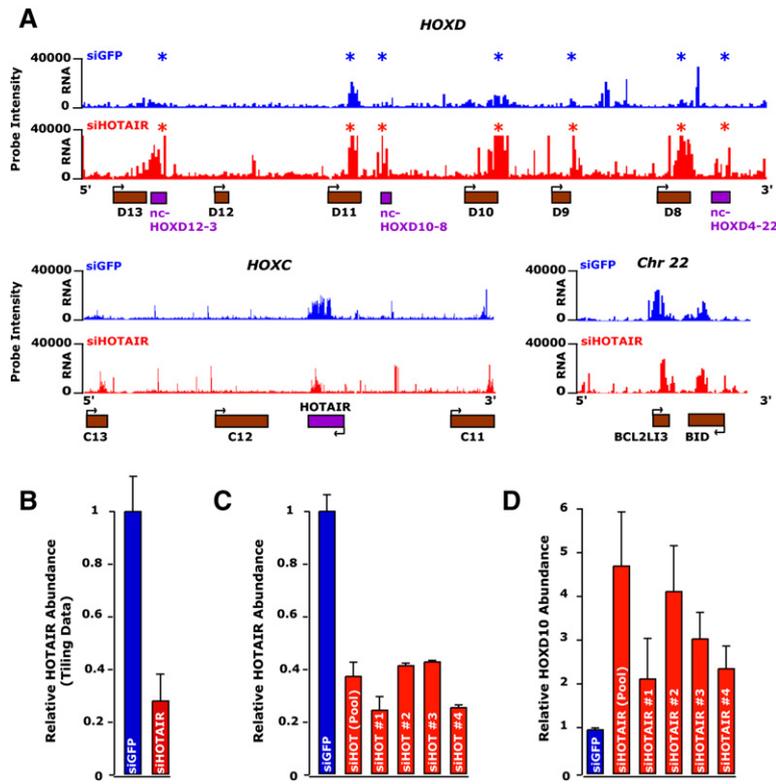
### ncRNA Transcription and *HOX* Gene Expression

Noncoding RNAs are emerging as regulatory molecules in specifying specialized chromatin domains (Bernstein and Allis, 2005), but the prevalence of a different



**Figure 4. HOTAIR, an Antisense Intergenic Long ncRNA of the HOXC Locus**

(A) Genomic location of HOTAIR at the boundary of two chromatin domains. ChIP-chip and RNA expression on tiling array are as shown in Figure 3. (B) Strand-specific RT-PCR shows exclusive expression of HOTAIR from the strand opposite to *HOXC* genes (bottom). Primers for reverse transcription (P-RT) and PCR (P-PCR) were designed to specifically target either the top (primers F1–F3) or bottom strand (primer R1) of HOTAIR. (C) Northern blot analysis of HOTAIR in lung and foreskin fibroblast RNA. (D) Size-fractionated small RNA was probed with pools of oligonucleotides spanning HOTAIR (sets #1–3), full-length antisense HOTAIR (CDS), or a probe against miRNA let7a.



**Figure 5. Loss of HOTAIR Results in Transcriptional Induction of *HOXD* Locus**

(A) RNA expression profiles of *HOXD* locus (top), *HOXC* locus surrounding HOTAIR (bottom left), and a control region on chromosome 22 (bottom right) following transfection of siRNA targeting GFP (siGFP) or a pool of four siRNAs targeting HOTAIR (siHOTAIR). Intensities of RNA hybridized to the tiling array from the siGFP and the siHOTAIR transfections are plotted on a linear scale in blue and red, respectively. \* indicates genes with significant increased transcription.

(B) qRT-PCR measuring the relative abundance of the HOTAIR transcript in the primary foreskin samples shown in (A). Mean  $\pm$  standard deviation are shown.

(C and D) qRT-PCR measuring the relative abundance of the HOTAIR (C) and *HOXD10* (D) transcripts after depletion of four individual siRNAs to HOTAIR and the pool. Mean  $\pm$  standard deviation are shown.

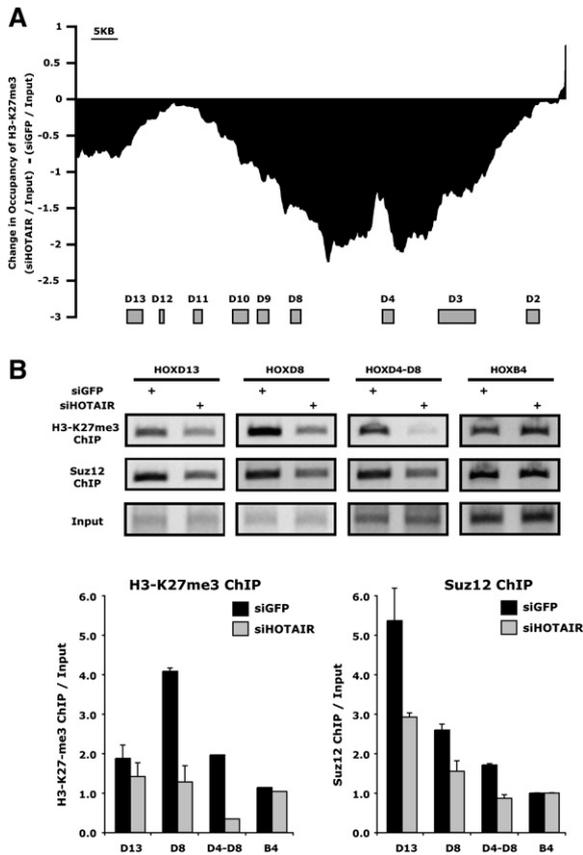
mechanism by which they act is not known. In *Drosophila*, transcription of ncRNAs was proposed to induce *HOX* gene expression by activation of *cis*-regulatory elements (Schmitt et al., 2005) or by ncRNA-mediated recruitment of the TrxG protein Ash1 (Sanchez-Elsner et al., 2006). However, an alternative model, termed “transcriptional interference,” argues that ncRNA transcription prevents the expression of 3' located *Hox* genes (Petruk et al., 2006). These two classes of models make opposite predictions on the correlation between expression of 5' ncRNA and the 3' *HOX* gene. Our finding of widespread position-specific ncRNAs that flank and are coordinately induced with neighboring human *HOX* genes is consistent with models of *cis* activation by ncRNA transcription. Only 10% of *HOX* ncRNAs demonstrate anticorrelated expression pattern with their cognate 3' *HOX* genes (Figure S5), suggesting that transcriptional interference is not the main mode of ncRNA action, at least in the cell types that we studied. Our results are also consistent with a recent analysis of *HOX* gene activation during teratocarcinoma cell differentiation, where transcription of certain 5' ncRNAs immediately preceded *HOX* gene

activation (Sessa et al., 2006). Transcriptional interference may be a more prominent mechanism during embryonic development, where its role in *Hox* gene expression was documented in *Drosophila* (Petruk et al., 2006).

Our results uncovered a new mechanism whereby transcription of ncRNA dictates transcriptional silencing of a distant chromosomal domain. The four *HOX* loci demonstrate complex cross regulation and compensation during development (Kmita and Duboule, 2003; Lemons and McGinnis, 2006). For instance, deletion of the entire *HOXC* locus exhibits a milder phenotype than deletion of individual *HOXC* genes, suggesting that there is negative feedback within the locus (Suemori and Noguchi, 2000). Multiple 5' *HOX* genes, including *HOXC* genes, are expressed in developing limbs (Nelson et al., 1996), and deletion of multiple *HOXA* and *HOXD* genes is required to unveil limb patterning defects (Zakany et al., 1997). Our results suggest that deletion of the 5' *HOXC* locus, which encompasses HOTAIR, may lead to transcriptional induction of the homologous 5' *HOXD* genes, thereby restoring the total dosage of *HOX* transcription factors.

(E) Posterior and distal expression of HOTAIR in human fibroblasts as measured by qRT-PCR. The site of origin of each fibroblast sample is indicated by the sample number on the anatomic cartoon. “A” is derived from the scalp. The relative abundance of HOTAIR in each position, relative to scalp (most anterior), is shown on the x axis.

(F) Whole mount in situ hybridization using HOTAIR sense (bottom strand) or antisense (top strand) probes in embryonic day 10.5 whole-mount embryos (top panels) and the hind limb and tail (bottom left and right panels, respectively). Expression of HOTAIR in posterior hindlimb (arrowhead) and tail (arrow) are highlighted.



**Figure 6. HOTAIR Is Required for H3K27 Trimethylation and Suz12 Occupancy of the *HOXD* Locus**

(A) Change in H3K27me3 ChIP-chip signal over the *HOXD* locus caused by depletion of HOTAIR compared to control siRNA against GFP. The location of *HOXD* genes are indicated by boxes. (B) ChIP of H3K27me3 and Suz12 of select promoters across the *HOXD* locus after siRNA treatment targeting GFP or HOTAIR. Bottom: Quantitation of ChIP assays (mean ± standard error).

How *HOX* ncRNAs may contribute to cross regulation among *HOX* genes should be addressed in future studies.

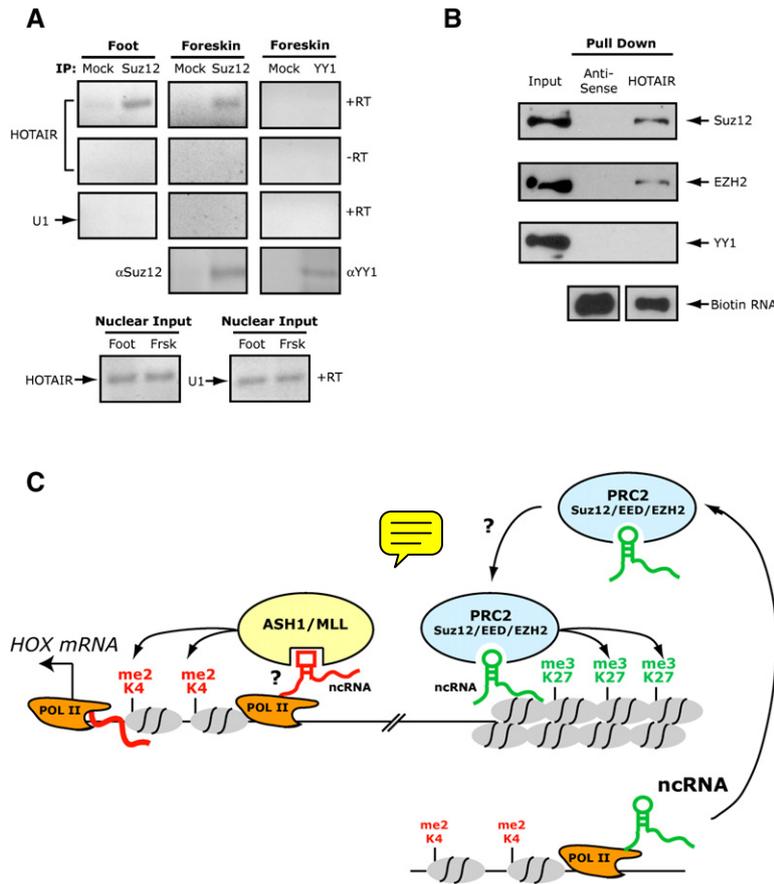
**HOTAIR ncRNA Is Involved in PRC2-Mediated Silencing of Chromatin**

Because many HMTase complexes lack DNA-binding domains but possess RNA-binding motifs, it has been postulated that ncRNAs may guide specific histone modification activities to discrete chromatin loci (Bernstein and Allis, 2005; Sun and Zhang, 2005). We have shown that HOTAIR ncRNA binds PRC2 and is required for robust H3K27 trimethylation and transcriptional silencing of the *HOXD* locus. HOTAIR may therefore be one of the long-sought-after RNAs that interface the Polycomb complex with target chromatin. A potentially attractive model of epigenetic control is the programming of active or silencing histone modifications by specific noncoding RNAs (Figure 7C). Just as transcription of certain ncRNA can facilitate H3K4 methylation and activate transcription of

the downstream *Hox* genes (Sanchez-Elsner et al., 2006; Schmitt et al., 2005), distant transcription of other ncRNAs may target the H3K27 HMTase PRC2 to specific genomic sites, leading to silencing of transcription and establishment of facultative heterochromatin. In this view, extensive transcription of ncRNAs is both functionally involved in the demarcation of active and silent domains of chromatin as well as a consequence of such chromatin domains.

Several lines of evidence suggest that HOTAIR functions as a bona fide long ncRNA to mediate transcriptional silencing. First, we detected full-length HOTAIR in vivo and in primary cells but not small RNAs derived from HOTAIR indicative of miRNA or siRNA production. Second, depletion of full-length HOTAIR led to loss of *HOXD* silencing and H3K27 trimethylation by PRC2, and third, endogenous or in-vitro-transcribed full-length HOTAIR ncRNA physically associated with PRC2. While these results do not rule out the possibility that RNA interference pathways may be subsequently involved in PcG function (Grimaud et al., 2006; Kim et al., 2006), they support the notion that the long ncRNA form of HOTAIR is functional. The role of HOTAIR is reminiscent of XIST, another long ncRNA shown to be involved in transcriptional silencing of the inactive X chromosome (Plath et al., 2003). An important difference between HOTAIR and XIST is the strictly *cis*-acting nature of XIST. To our knowledge, HOTAIR is the first example of a long ncRNA that can act in *trans* to regulate a chromatin domain. While we have observed a *trans*-repressive role for HOTAIR, our data do not permit us to rule out a *cis*-repressive role in the *HOXC* locus. Our siRNA-mediated depletion of HOTAIR was substantial but incomplete; further, the proximity between the site of HOTAIR transcription and the neighboring *HOXC* locus may ensure significant exposure to HOTAIR even if the total pool of HOTAIR in the cell were depleted. The precise location of HOTAIR at the boundary of a silent chromatin domain in the *HOXC* locus makes a *cis*-repressive role a tantalizing possibility. Judicious gene targeting of HOTAIR may be required to address its role in *cis*-regulation of chromatin.

The discovery of a long ncRNA that can mediate epigenetic silencing of a chromosomal domain in *trans* has several important implications. First, ncRNA guidance of PRC2-mediated epigenetic silencing may operate more globally than just in the *HOX* loci, and it is possible that other ncRNAs may interact with chromatin-modification enzymes to regulate gene expression in *trans*. Second, PcG proteins are important for stem cell pluripotency and cancer development (Sparmann and van Lohuizen, 2006); these PcG activities may also be guided by stem cell- or cancer-specific ncRNAs. Third, Suz12 contains a zinc finger domain, a structural motif that can bind RNA (Hall, 2005), and EZH2 and EED both have in vitro RNA-binding activity (Denisenko et al., 1998). The interaction between HOTAIR and PRC2 may also be indirect and mediated by additional factors. Detailed studies of HOTAIR and PRC2 subunits are required to elucidate the structural features that establish the PRC2 interaction



**Figure 7. HOTAIR ncRNA Binds PRC2**

(A) Immunoprecipitation of Suz12 retrieves endogenous HOTAIR. Nuclear extracts of foot or foreskin fibroblasts were immunoprecipitated by IgG (lanes 1, 3, and 5), anti-Suz12 (lanes 2 and 4), or anti-YY1 (lane 6). Coprecipitated RNAs were detected by RT-PCR using primers for HOTAIR (rows 1 and 2) or U1 small nuclear RNA (row 3). To demonstrate that the HOTAIR band was not due to DNA contamination, each RT-PCR was repeated without reverse transcriptase (–RT, row 2). Immunoprecipitation of Suz12 and YY1 were successful as demonstrated by IP-western using the cognate antibodies (row 4). RT-PCR of nuclear extracts demonstrated equal input RNAs (row 5).

(B) In vitro-transcribed HOTAIR retrieves PRC2 subunits. Immunoblot analysis of the indicated proteins is shown; five percent of input extract (5 μg) was loaded as input control.

(C) Model of long ncRNA regulation of chromatin domains via histone-modification enzymes. Transcription of ncRNAs in *cis* may increase the accessibility of TrxG proteins such as ASH1 or MLL or directly recruit them, leading to H3K4 methylation and transcriptional activation of the downstream *HOX* gene(s). In contrast, recruitment of PRC2 is programmed by ncRNAs produced in *trans*, which targets PRC2 activity by as-yet-incompletely-defined mechanisms to target loci. PRC2 recruitment leads to H3K27 methylation and transcriptional silencing of neighboring *HOX* genes.

with HOTAIR. As we illustrated here, high-throughput approaches for the discovery and characterization of ncRNAs may aid in dissecting the functional roles of ncRNAs in these diverse and important biological processes.

## EXPERIMENTAL PROCEDURES

Tiling array design, hybridization, signal processing, RT-PCR validation of ncRNAs, and motif analysis are described in [Supplemental Data](#).

### Chromatin Immunoprecipitation

Conventional ChIP and ChIP-chip were performed using anti-H3K27me3 (Upstate Cell Signaling cat# 07-449), anti-Suz12 (Abcam cat# 12,201), anti-PolII (Covance cat# MMS-126R), anti-H3K4me2 (Abcam cat# ab7766), anti-H3K9me3 (Abcam cat# ab1186), and Whole Genome Amplification kit (Sigma) as previously described ([Squazzo et al., 2006](#)).

### HOTAIR Cloning and Sequence Analysis

5' and 3' RACE were performed using the RLM Race kit (Ambion) as recommended by the manufacturer.

### HOTAIR Expression Analysis

In situ hybridizations of C57BL/6 mouse embryo using human HOTAIR sequence 164–666 (clone 7T; [Albrecht et al., 1997](#)), northern blot using full-length HOTAIR, qRT-PCR with SYBR Green (forward HOTAIR, GGGGCTTCCTTCTTCTTATC; reverse, GGTAAGAAAAGCAAC

CACGAAGC), and Taqman analysis of *HOXD10* expression (Applied Biosystems, cat# Hs00157974\_m1) were as described ([Rinn et al., 2004](#)). Small RNA northern blotting was as described ([Lau et al., 2001](#)) with the following modifications: 15 μg of small RNA retained total RNA (mirVana miRNA isolation kit, Ambion) was denatured in Novex sample loading buffer and loaded onto 15% TBE-urea gel in Novex running buffer (Invitrogen). RNA was transferred onto Hybond-XL membrane (Amersham) and probed with pools of <sup>32</sup>P-γ-ATP end-labeled 40 mer oligos spanning HOTAIR sequence 1–400 (set 1), 401–800 (set 2), 801–1200 (set 3), full-length HOTAIR probe, or a probe for microRNA let-7a (AACTATACAACCTACT ACCTCA) as positive control.

### RNA Interference

Foreskin fibroblasts were transfected with 50 nM of siRNAs targeting HOTAIR (#1 GAACGGGAGUACAGAGAGAUU; #2 CCACAUGAACGCCAGAGAUU; #3 UAACAAGACCAGAGAGCUGUU; and #4 GAGGAAAAGGGAAAUCUAUU) or siGFP (CUACAACAGCCACAAC GUCdTdT) using Dharmafect 3 (Dharmacon, Lafayette, CO, USA) per the manufacturer's direction. Total RNA was harvested for total RNA 72 hr later for microarray analysis as previously described ([Rinn et al., 2006](#)).

### RNA Immunoprecipitation

Foreskin and foot fibroblasts were grown as previously described ([Rinn et al., 2006](#)). 10<sup>7</sup> cells were harvested by trypsinization and resuspended in 2 ml PBS, 2 ml nuclear isolation buffer (1.28 M sucrose; 40 mM Tris-HCl pH 7.5; 20 mM MgCl<sub>2</sub>; 4% Triton X-100), and 6 ml water on ice for 20 min (with frequent mixing). Nuclei were pelleted by centrifugation at 2,500 G for 15 min. Nuclear pellet was

resuspended in 1 ml RIP buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 9 µg/ml leupeptin, 9 µg/ml pepstatin, 10 µg/ml chymostatin, 3 µg/ml aprotinin, 1 mM PMSF, 100 U/ml SUPERASin; Ambion). Resuspended nuclei were split into two fractions of 500 µl each (for Mock and IP) and were mechanically sheared using a dounce homogenizer with 15–20 strokes. Nuclear membrane and debris were pelleted by centrifugation at 13,000 RPM for 10 min. Antibody to Suz12 (Abcam cat# 12,201), YY1 (Santa Cruz Biotechnology cat# sc1703), or FLAG epitope (Mock IP, Sigma) was added to supernatant (Suz12: 6 µg, YY1: 10 µg) and incubated for 2 hr at 4°C with gentle rotation. Forty microliters of protein A/G beads were added and incubated for 1 hr at 4°C with gentle rotation. Beads were pelleted at 2,500 RPM for 30 s, the supernatant was removed, and beads were resuspended in 500 µl RIP buffer and repeated for a total of three RIP washes, followed by one wash in PBS. Beads were resuspended in 1 ml of Trizol. Coprecipitated RNAs were isolated, and RT-PCR for HOTAIR (Forward, GGGGCTTCTTGCTCTTCTTATC; reverse GGTA GAAAAAGCAACCACGAAGC) or U1 (forward, ATACTTACCTGG CAGGGGAG; reverse, CAGGGGGAAAGCGCGAACGCA) was performed as described (Rinn et al., 2006). Protein isolated by the beads was detected by western blot analysis.

#### HOTAIR RNA Pull-Down of PcG Proteins

Biotin-labeled, full-length HOTAIR RNA and antisense HOTAIR fragment (clone 7T) were prepared with the Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Stratagene). Biotinylated RNAs were treated with RNase-free DNase I and purified on G-50 Sephadex Quick Spin columns (Roche). Ten picomole biotinylated RNA was heated to 60°C for 10 min and slow-cooled to 4°C. RNA was mixed with 100 µg of precleared transcription and splicing-competent HeLa nuclear extract (Gozani et al., 1994) in RIP buffer supplemented with tRNA (0.1 µg/ul) and incubated at 4°C for 1 hr. Sixty microliters washed Streptavidin agarose beads (Invitrogen) were added to each binding reaction and further incubated at 4°C for 1 hr. Beads were washed briefly five times in Handee spin columns (Pierce) and boiled in SDS buffer, and the retrieved protein was visualized by immunoblotting.

#### URLs

All primary data are available at the Stanford Microarray Database (<http://genome-www5.stanford.edu/>) and Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>). Full-length sequence of human HOTAIR ncRNA has been deposited in Genebank (Accession # DQ926657 (bankit841140)).

#### Supplemental Data

Supplemental Data include Experimental Procedures, nine figures, five tables, and References and can be found with this article online at <http://www.cell.com/cgi/content/full/129/7/1311/DC1/>.

#### ACKNOWLEDGMENTS

We thank A.S. Adler, M.L. Cleary, O. Gozani, D. Herschlag, D. Hogan, P.A. Khavari, A.E. Oro, O.J. Rando, and C. Woo for discussion and critical review of the manuscript. Supported by grants from the National Institutes of Health (J.A.H., P.J.F., E.S., and H.Y.C.), Israel Science Foundation (M.K. and E.S.), and National Science Foundation (J.K.W.). E.S. is the incumbent of the Soretta and Henry Shapiro Career Development Chair; J.L.R. is a Fellow, and H.Y.C. is the Kenneth G. and Elaine A. Langone Scholar of the Damon Runyon Cancer Research Foundation. H.Y.C. is also supported by the Emerald Foundation.

Received: January 19, 2007

Revised: March 28, 2007

Accepted: May 9, 2007

Published: June 28, 2007

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#### Accession Numbers

Full-length sequence of human HOTAIR ncRNA has been deposited in Genebank (Accession # DQ926657 (bankit841140)).