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A myelopoiesis-associated regulatory intergenic non-coding RNA transcript within the human HOXA cluster

short title: ncRNA in HOXA associated with myelopoiesis

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We have identified an intergenic transcriptional activity that is located between the human *HOXA1* and *HOXA2* genes, shows myeloid-specific expression, and is up-regulated during granulocytic differentiation. The novel gene, termed *HOTAIRM1* (HOX Antisense Intergenic RNA Myeloid 1), is transcribed antisense to the HOXA genes and originates from the same CpG island that embeds the start site of *HOXA1*. The transcript appears to be a non-coding RNA containing no long open-reading frame, sucrose gradient analysis shows no association with polyribosomal fractions. *HOTAIRM1* is the most prominent intergenic transcript expressed and up-regulated during induced granulocytic differentiation of NB4 promyelocytic leukemia and normal human hematopoietic cells; its expression is specific to the myeloid lineage. Its induction during retinoic acid (RA)-driven granulocytic differentiation is through RA receptor and may depend on the expression of myeloid cell development factors targeted by RA signaling. Knock-down of HOTAIRM1 quantitatively blunted RA-induced expression of *HOXA1* and *HOXA4* during the myeloid differentiation of NB4 cells, and selectively attenuated induction of transcripts for the myeloid differentiation genes CD11b and CD18, but did not noticeably impact the more distal HOXA genes. These findings suggest that *HOTAIRM1* plays a role in the myelopoiesis through modulation of gene expression in the *HOXA* cluster.

Introduction

Human HOX gene clusters are known for the prevalence of intergenic transcription between coding genic members.[1] Similar activity has also been observed in other developmentally important or tissue-specific gene loci, such as those containing the human beta globin genes, cardiac myosin heavy chain (MHC) genes, and the IL-4/IL-13 gene cluster.[2-4] Extensive HOX gene cluster intergenic transcripts have been described largely as non-coding RNAs (ncRNAs) including both short microRNA (miRNA) species and long ncRNAs that are anti-sense to their canonical HOX neighbors. Well-defined HOX region ncRNAs include the mir-10 and mir-196 paralogs, bithoraxoid ncRNAs of the Drosophila bithorax complex, and human HOTAIR (HOX Antisense Intergenic RNA). [5-7]

Intergenic regions have been proposed as locations for novel radiational and reorganizing changes that have occurred in the evolution of HOX gene clusters, which are relatively constrained in structure in the higher vertebrates.[5,8] Several recent studies have focused on expression of intergenic ncRNAs in the human HOX regions, especially the HOXA cluster, in tumor cell lines, tissues, and fibroblasts from different anatomic origins. All reported unusually active transcription within the intergenic regions, occurring in patterns coordinated with their HOX neighbors.[7,9,10] Intergenic ncRNAs in the HOXA gene cluster were usually associated with CpG islands and their expression accompanied changes in either polycomb group repressive complex binding or methylation of histones, suggesting a pattern of *cis* modulation of the intergenic transcripts prior to the activation of adjacent HOX genes. However, the HOTAIR transcript, located between HOXC11 and HOXC12, was found to function in *trans* to repress a distal group of homologous HOXD genes by demarcating an extended silenced domain through interaction with the Polycomb group complex PRC2 histone methyltransferase [7,10,11]

De novo genomic transcription mapping has revealed that intergenic ncRNA is possibly the most abundant form of transcriptional output from the genomes of humans and other higher eukaryotic organisms.[12,13] Within the human genome, the majority of intergenic ncRNA are not highly conserved at

the sequence level, with long ncRNAs generally less conserved than short miRNAs. Nevertheless, their expression patterns may be conserved among tissues or along developmental axes.[14,15] More importantly, ncRNA function in gene regulation has emerged as an important mechanism in the control of many biological processes in development and carcinogenesis.[16]

In the present study, we have identified intergenic transcription of a unique long ncRNA, here termed HOTAIRM1 (HOX Antisense Intergenic RNA Myeloid 1) after the nomenclature of Rinn et al.,[7] located between the human HOXA1 and HOXA2 genes. Transcript levels are associated with retinoic acid (RA) mediated myeloid lineage differentiation and maturation. During differentiation induced by all trans retinoic acid (ATRA), shRNA-mediated knock-down of *HOTAIRM1* attenuated the transcriptional induction of HOXA genes from the 3' end of the cluster. The knockdown also interfered with the transcriptional induction of genes encoding beta2 integrins CD11b and CD18. These findings indicate that HOTAIRM1 is a myeloid lineage-specific ncRNA that may play a regulatory role in myelopoiesis.

Methods

Human cell lines

NB4, HL-60, K-562, THP1, Jurket, SuDHL6, HEK293, Hela, HepG2, and Saos2 cell lines were obtained from the American Type Culture Collection. For granulocytic differentiation, NB4 cells were seeded at $2x10^5$/mL in medium supplemented with 10% fetal bovine serum and 1uM ATRA or 9-cis-retinoic acid (9-cis-RA) and cultured for up to 96 hours. For monocytic differentiation, NB4 cells seeded at $2x10^5$/ml were primed with 100 uM Vitamin D3 for 8 hours, washed, and then cultured in 1uM tetradecanoyl phorbol acetate (TPA) for 72 hours. Total RNA was isolated with Tri-Reagent (Molecular Research Center, Inc.), and purified with a MegaClear spin column (Ambion) followed by DNaseI digestion. RNA quality was checked by agarose gel electrophoresis.

Human leukocyte isolation

Human circulating neutrophils, monocytes, and mononuclear cells were isolated from venous blood of healthy volunteers as previously described.[17] Human hematopoietic stem and progenitor cell populations were isolated from peripheral blood stem cells (mobilized by granulocyte colony-stimulating factor) and monocytes were prepared from peripheral blood, as previously described [18-20] The University of Massachusetts Medical School and Yale University human subjects committees approved procedures and consent forms at their respective sites. Volunteer informed consent was obtained in accordance with the Declaration of Helsinki.

All reagents and containers were certified as pyrogen-free by the manufacturers.

Polyribosome fractionation

From $5x10^7$ to $1x10^8$ NB4 cells differentiated in 1 uM ATRA for 24-48h were used for each gradient. Before harvesting, cells were incubated in media containing 100 μg/ml of cycloheximide for 5 minutes and washed twice with Hanks balanced salt solution containing 100 μg/ml cycloheximide. Cell pellets

were resuspended in 1 ml of lysis buffer containing 0.5% NonidetP40, 1 mM dithiothreitol, and10mM ribonucleoside-vanadyl complex (New England Biolabs), and incubated on ice for 10 min. After centrifugation at 10,000 x g for 10 min at 4ºC, the lysate was layered on top of a chilled 11 ml 15–50% (w/v) sucrose gradient and centrifuged at 35,000 rpm in a pre-chilled Beckman SW41Ti rotor for 180 min at 4°C with slow deceleration. Gradients were collected in 22 equal fractions, monitored for absorbance at 254/280 nm; total RNA was isolated as described above.

Microarray analysis

Transcription mapping was performed as previously described, using fragmented biotinylated ddATP end-labeled double-stranded cDNA samples analyzed on GeneChip® ENCODE01 1.0 Arrays (Affymetrix), as part of the pilot ENCODE (ENCyclopedia Of DNA Elements) project.[12] Hybridization, scanning, and signal scoring procedures have been reported previously. [21] For each target position, a mismatch probe was employed in background corrections for a perfectly matched probe; intensity estimates and significance of signal captured for each target were calculated within a sliding window centered on each probe pair and size-optimized for average exon size of investigated targets. Signal significance was ranked by p-value from upper-sided paired Wilcoxon signed rank sum tests, and corresponding signal intensity was computed as a pseudomedian within its window. To analyze across data sets from different sources, all samples were scaled to an array median intensity of 150 following a quantile normalization conducted within each sample. Results were mapped to genomic positions in human genome assembly hg 18 (NCBI Build 36.1) and subjected to visual inspection with a p-value threshold of positive signals that visualized signal stretches associated with known target structures.

Expression profiling with fragmented biotinylated NTP-labeled cRNA samples using HG_U133 GeneChip high density oligonucleotide arrays (Affymetrix) was performed with minor modifications of Affymetrix protocols as previously described.[22]. To analyze across multiple data sets from different cell types, pre-processing by within-sample quantile normalization and between-sample scaling was performed as described above.

Real-time and multiplex RT-PCR

All primers (Supplemental Table 1) were designed to span an exon-exon boundary when possible and searched by BLAST against GenBank or by *In-Silico* PCR in the UCSC Genome Browser. Due to high homology among HOX genes, primers for HOXA genes were further checked by multi-alignment to all clustered HOX gene sequences to verify specificity. First-strand cDNAs were reverse transcribed from total RNA using SuperScript III Reverse Transcriptase (Life Technologies). Real-time quantitative PCR assays using SYBR Green 1 chemistry were performed on an Applied Biosystems 7300 thermal cycler. Quantitation of the results on the basis of the threshold cycle for amplification was performed by the relative standard curve method according to the manufacturer's protocol. Relative expression levels of target genes were calculated from the results of at least triplicate experiments, normalized to either geometric means of a panel of endogenous reference genes including alpha 1b tubulin (*TUBA1B*), ribosomal protein S6 (*RPS6*), peptidylprolyl isomerase A (*PPIA*), and hydroxymethylbilane synthase (*HMBS*), or to single reference genes with appropriate expression levels. Multiplex PCR using the Multiplex PCR Kit (Qiagen) was employed for comparative analyses of expression in RNA from cell lines or from a multiple tissue cDNA panel (Human cDNA Panel II, ClonTech), and for analysis of pull-down DNA fractions in chromatin immunoprecipitation. The endpoint for multiplex PCR was optimized for the log phase of amplification, based on the available threshold cycle range of target genes established in real-time PCR.

Rapid amplification of cDNA ends (RACE) and northern blot analysis

RACE primers were targeted to regions of interest identified on tiling and expression arrays (Supplemental Table 1). 5' and 3' RACE were performed using the FirstChoice RLM-Race kit (Ambion), following manufacturer's protocols. PCR products were sequenced and aligned by BLAT to the latest human genome assembly in the UCSC genome browser. Northern blots were performed by standard techniques on 25 μg samples of glyoxal-denatured total RNA, as previously described.[23] Hybridization of radiolabeled probe (Supplemental Table 1) was detected on a Molecular Dynamics PhosphorImager.

Chromatin immunoprecipitation

Procedures were modified from the methods of Wang *et al*.[24] Briefly, $2x10^7$ NB4 cells were grown with or without 1uM ATRA. Formaldehyde was added directly to culture media to a final concentration of 1% and crosslinking proceeded at room temperature for 30 min before addition of glycine to a final concentration of 0.14 M. Cells were washed with ice cold PBS, then attached cells were scraped and collected in cold PBS. Lysed and micrococcal nuclease-digested samples were sonicated to shear the cross-linked chromatin to 0.5-1 kb fragments, then immunoprecipitated overnight at 4^{o}C with 2 µg of mouse monoclonal anti-RNA polymerase II (RNAP II) antibody A-10 (Santa Cruz Biotechnology). Subsequent steps of crosslinking reversal, proteinase K digestion and purification of immunoprecipitated DNA were performed as previously described.[24] For multiplex PCR reactions, 2.5–10% of the immunoprecipitated DNA and 0.0125% of total DNA (input) from a 10-cm plate served as templates.

shRNA Expressing Lentiviral Vector Construction and Transduction

siRNA targeting sites of HOTAIRM1 transcript were designed on the siRNA selection server at the Whitehead Institute for Biomedical Research[25] and further tested by BLAST for off-target homology within the HOXA cluster. Oligonucleotides for shRNA hairpin expression (Supplemental Figure 1) were inserted into the pLKO.1-TRC backbone plasmid (Addgene Plasmid #10878), according to the vendor's protocol, to construct the lentiviral pLKO.1-HOTAIRM1 vectors. The pLKO.1-HOTAIRM1 and pLKO.1 scramble shRNA (Addgene plasmid #1864) lentiviral vectors were produced and pseudotyped in the HEK293T cell line by co-transfecting with packaging plasmids pCMV-VSVG (Addgene plasmid #8454) and pCMV-dR8.2 (Addgene plasmid #8455), and titers of viral supernatants determined in NB4 cells. For long term silencing of HOTAIRM1, clones of stably transduced NB4 cells were generated. $2x10^5$ cells were exposed for 24 hours to pseudo-lentiviral particles with multiplicities of infection ranging from 1 to 4, in the presence of 8g/mL polybrene (Sigma), then washed and plated in soft agar medium containing 0.75 µg/ml puromycin for 6-9 days as previously described.[26] Colonies were identified by phase-contrast

microscope then transferred to 48-well plates and expanded in fresh medium. Clones with >70% knock-down of HOTAIRM1 during ATRA induction were identified by RT-PCR.

Results

Microarray analysis of intergenic transcription between human HOXA1 and HOXA2

To examine intergenic transcription in the HOXA cluster during myelopoiesis, we conducted a transcription mapping study of human peripheral blood neutrophils and in NB4 and HL-60 cells induced by ATRA to granulocytic differentiation, using data from the ENCODE project.[12] The analysis included data deposited in Gene Expression Omnibus (GEO) database (GSE2678, GSE2679, GSE2802) from our laboratories and others. The data were generated on GeneChip (Affymetrix) oligonucleotide tiling arrays designed to detect all transcripts from the pilot ENCODE regions, including the HOXA cluster.[12] Publicly available tiling array data on HeLa (GSE2800), GM06990 (GSE2800) and placental (GSE2671) transcription mapping with polyA+ RNA samples were included for reference. A paired Wilcoxon signed rank sum test was used for statistical identification of positive signal within a sliding window centered on each probe. Transcriptionally active regions (TARs) within genomic segments of the HOXA cluster, shown in Figure 1 by a p-value (color scale) threshold, were identified by visual inspection for stretches of positive signals above background. The most significant intergenic transcriptional activity (red bars) mapping to the region between *HOXA1* and *HOXA2* was identified in mature neutrophils and in ATRA-induced NB4 cells. A more subtle induction of this intergenic transcriptional activity was detectable in ATRA-treated HL-60 cells. No significant transcription activity in this intergenic region was detected in RNA from placental, HeLa, or GM06990 cells.

In contrast to previously identified signals at *HOXA1*, the intergenic transcription signals were not aligned with any entries of putative genes or mRNAs in the region. The distribution of thresholded signal and p-values indicates the presence of at least two TARs located close to the boundaries with their genic neighbors. The regions correspond roughly to two common exons shared by several expressed sequence tags (ESTs) (Figure 1), but these could not be compiled into a common consensus form, particularly with

regard to the 5' and 3' ends. Some additional, inconsistent intergenic signal was present, possibly due to non-specific hybridization in a long CpG island (Figure 1B).

To determine the strand specificity of the intergenic transcripts, we used the GeneChip HG_U133 platform (Affymetrix), which includes probe sets within the region for each strand and can be hybridized with amplified single-stranded antisense RNA preparations. We examined HG_U133 profiles of NB4 cells and neutrophils, as well as two GEO profiles of CD34+ hematopoietic stem progenitor cells. Positive signals were only scored for the probe set that targets the plus strand of EST BC031342 (illustrated in light blue in Figure 1B, see also Figure 6). This sequence partially overlaps both intergenic TARs close to the boundaries with their genic neighbors as well as a TAR slightly further from *HOXA2*. Two other probe sets targeting intergenic region ESTs (AK022839, AW207863) on the minus strand were scored as absent without exception (Supplemental Figure 2).

Northern blot analysis of the transcript was performed on RNA from neutrophils, HL60 cells, NB4 cells, and ATRA-induced NB4 cells. Probes corresponding to the two TARs both hybridized to a band about 0.5 kb (Figure 1C). 5' and 3' RACE using RNA from differentiated NB4 cells revealed a 483 nt (Supplemental Figure 3) spliced RNA consisting of 2 exons, corresponding to the two TARs identified by tiling array (Figure 1B). The splicing pattern of this RNA is shared by a small set of EST and mRNA deposits in GenBank that are aligned to the region; the group has been assembled as a putative gene "rumora" (alternative variant eApr07 in the AceView program). We have termed this transcript *HOTAIRM1* (HOX Antisense Intergenic RNA Myeloid 1) after the nomenclature for HOX region intergenic transcripts developed by Rinn et al.[7] Current RNA folding algorithms, such as the minimum free-energy structure methods used by the *mfold* program, do not predict any distinctive secondary structure for the *HOTAIRM1* transcript (Supplemental Figure 3).

As shown in the conservation track of the UCSC Genome Browser (Figure 1B) and confirmed by an interspecies search in GenBank, the *HOTAIRM1* sequence has not been conserved during evolution.

However, similarly localized transcriptional activity in the *HOXA1-A2* region occurs in some other species, e.g. mouse and rat, with conservation at the level of splicing structure (apparent in the mouse), but incorporating complex gaps which break homology at the DNA sequence level (Supplemental Figures 4-5).

HOTAIRM1 is a ncRNA transcribed by RNA polymerase II

There is virtually no coding potential in the sequence of *HOTAIRM1*. The longest predicted open reading frame of HOTAIRM1would generate a 51 amino acid product with a estimated molecular weight less than 6 kD and no homology to any sequences in current protein databases. To test experimentally for translation of the transcript, we performed sucrose gradient fractionation of ATRA-induced NB4 cells. As shown in Figure 2, we used multiplex RT-PCR to screen sucrose density gradient fractions for transcripts representing *HOXA1* (423 bp amplicon), intergenic *HOTAIRM1* (246 bp) and alpha tubulin (*TUBA1B*, 101 bp). Fractions containing free RNA, ribosomal subunits, monoribosomes, and polyribosomes (indicated Figure 3A) were identified (respectively left to right in the figure) by optical absorbance at 254 nm and 280 nm and by the relative intensities of 18s and 28s rRNA bands on agarose gel electrophoresis. Intergenic *HOTAIRM1* transcripts appeared largely in non-ribosomal or mono-ribosomal fractions, in contrast to *HOXA1* and alpha tubulin transcripts, which are represented in the higher density polysomal fractions to the right. Both *HOXA1* and alpha tubulin proteins can be detected in NB4 cells (data not shown). This polysome profile, along with sequence data, indicate that *HOTAIRM1* is most likely a ncRNA.

RNA ligase-mediated RACE demonstrated 3'-polyadenlyation of the *HOTAIRM1* transcript and revealed a canonical polyadenylation signal 18 nt upstream of the 3'end (Supplemental Figure 3). To test whether RNA polymerase II (RNAP II) is responsible for its transcription, we performed chromatin immunoprecipitation of *HOTAIRM1* DNA from NB4 cells, using a mouse monoclonal antibody to RNAP II. PCR detection of the transcript from both total DNA (input) and DNA pulled down with anti-RNAP II antibody was performed using primers directed to junction of first exon and intron (primer

5'HOTAIRM1) and the 3' end of *HOTAIRM1* gene (primer 3'HOTAIRM1), as illustrated in Figure 1B. Sequences from the distal promoter of the U6A snRNA gene (*RNU6A*) [27] and the 3' end of the alpha tubulin gene (*TUBA1B*), as illustrated in Figure 3, were concurrently amplified as positive controls in multiplex PCR. The 3' end of *HOTAIRM1* was amplified from DNA immunoprecipitated by anti-RNAP II antibody from ATRA-induced NB4 cells (Figure 3), but much less signal was detected in immunoprecipitated DNA from native NB4 cells and none from HeLa cells, which do not express *HOTAIRM1*. The 5' end of *HOTAIRM1* was amplified in DNA immunoprecipitated from both untreated and ATRA-induced NB4 cells, but not detected in Hela cells. This apparent RNAP II binding does not exclusively indicate active transcription, as the signal could also reflect the frequency of RNAP II stalling within proximal promoter regions throughout the genome [28] or the expression of *HOXA1* from the opposite strand in NB4 cells.

Retinoid responsive expression

Exogenous RA, via RARs, drives terminal granulocytic differentiation of committed myeloid progenitors by targeting myeloid development regulators and interacting with relevant cytokine pathways.[29,30] To further investigate ATRA-induced expression of *HOTAIRM1* during myeloid differentiation, we examined the time course of induction by ATRA (1uM) in NB4 cells and the ATRA-resistant subclone NB4r2 by real-time RT-PCR. NB4 cells showed a latency of at least 6 hours in the rise of *HOTAIRM1* expression after ATRA treatment (Figure 4A). *HOTAIRM1* induction was abrogated in the ATRA-resistant NB4r2 subline that bears a mutation in the ligand-binding domain of the promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) fusion gene (Figure 4B).[31] To examine the RAR dependence of this induction, we examined gene expression in response to 9-cis-RA, a ligand that activates both the retinoid X receptor (which is not stimulated by ATRA) as well as all RAR isotypes.[32] 9-cis-RA proved to be as potent as ATRA for induction of *HOTAIRM1* expression in NB4 cells (Figure 4A). In ATRA-resistant NB4r2 cells, 9-cis-RA was similarly unable to induce expression of *HOTAIRM1* (Figure 4B), indicating dependence upon RAR for expression of these transcripts.

To test whether regulation of *HOTAIRM1* expression represents a direct effect of retinoic acid receptor ligation, we pre-treated NB4 cells with the translation inhibitor cycloheximide for 30 min before addition of ATRA. Induction of *HOTAIRM1* was largely abolished by cycloheximide treatment under these conditions (Figure 5A), suggesting that its up-regulation depends at least in part upon newly-synthesized intermediary protein(s), which may explain its relatively late response to ATRA. Indeed during differentiation of NB4 cells by ATRA, induction of *CEBPE* occurred before *HOTAIRM1* expression, which paralleled that of *HOXA1* (Figure 4C). *CEBPE* encodes an ATRA-responsive transcription factor critical for terminal differentiation of granulocytes and its expression is not inhibited by cycloheximide.[33]

Expression in the myeloid lineage

In the initial tiling array data *HOTAIRM1* expression appeared to be related or restricted to the myeloid lineage. To better define its lineage specificity, we examined cell line and tissue cDNA panels for *HOTAIRM1* transcripts. Multiplex RT-PCR assays measured *HOTAIRM1* and *HOXA1* expression relative to the ubiquitously expressed reference genes *TUBA1B* or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). *HOTAIRM1* expression demonstrated myeloid lineage specificity (Figure 5, panels A and B). Strong positive signals were observed only in leukocytes on the tissue panel and in myeloid NB4 and K562 cells in the cell line panel. A significant increase of expression was induced by ATRA in both NB4 and K562 cells. Low level *HOTAIRM1* expression and very subtle induction by ATRA could be detected in HL-60 cells, which does not respond to ATRA efficiently and lacks a PML-RARα fusion gene. Embryonic kidney HEK293 cells showed strong expression of *HOXA1* but only very low signal for *HOTAIRM1*, indicating discordant expression of the two neighboring genes in another context.

We further assembled a comprehensive gene expression data set from human genome array studies of hematopoietic cells at different stages of differentiation. Public GEO data series for hematopoietic progenitor cell sub-populations (GSE2666, GSE3823) and human whole blood leukocytes (GSE3284) were compiled with our transcription profiling datasets from peripheral blood progenitor subpopulations, ATRA-induced NB4 cells and circulating human neutrophils, monocytes and dendritic cells.[34-36] Only

GEO datasets available as probe level intensity "raw" data were included. To ensure the cross-laboratory comparability, we applied the same quantile approach for normalization at the probe level and other statistics described above for the cross-laboratory analysis of tiling array data. As shown in Figure 6, *HOTAIRM1* expression showed a striking association with myeloid differentiation. Relative levels of *HOTAIRM1* in cell populations enriched in hematopoietic stem and progenitor cells were strikingly lower than in myeloid leukemia cells and mature leukocytes. Quantitatively, *HOTAIRM1* expression among the progenitor populations was marginal or absent, suggesting that the transcript is not involved in earlier stages of stem or progenitor cell development and lineage commitment.

A significant increase in *HOTAIRM1* expression also occurred during differentiation of the NB4 cell line, as observed in experiments described above. Uninduced promyelocytic NB4 cells expressed a low but detectable baseline level of *HOTAIRM1* transcripts and showed dramatically increasing expression during neutrophilic differentiation induced by ATRA. A much lesser degree of induction was observed during monocytic differentiation induced by TPA, confirming the results obtained with tiling arrays (Figure 1). Among primary leukocytes, *HOTAIRM1* expression was 4 to 5 fold higher in neutrophils than in monocytes or dendritic cells. E. coli lipopolysaccharide (LPS), a strong stimulus of the neutrophil inflammatory response,[22,37] dramatically down-regulated *HOTAIRM1* expression (Figure 1, Supplemental Figure 2). Although similar expression patterns for *HOTAIRM1* and *HOXA1* were observed in NB4 cells, their expression was not coordinated in either progenitor cells or mature neutrophils.

shRNA mediated silencing of HOTAIRM1 RNA attenuates expressions of 3' HOXA genes

The intergenic ncRNAs within HoxA cluster have been proposed to function as epigenetic regulators of neighboring HOX genes or remote paralogs. To assess the potential impact of HOTAIRM1on the transcriptional regulation of local HOXA cluster members and test for functional effects on myeloid differentiation, we transduced promyelocytic NB4 cells with lentiviral vectors that express shRNAs targeting the HOTAIRM1 transcript. Figure 7A shows more than 70% reduction of HOTAIRM1 RNA, in

two representative ATRA-treated stably shRNA-transduced clones , compared to non-transduced cells or cells transduced by the viral vector expressing a “scramble” shRNA.

To test the effects of HOTAIRM1 knockdown on Hox gene expression, we measured transcript levels for other HOXA cluster genes by quantitative real-time RT-PCR during ATRA-induced myeloid differentiation in the two clones of transduced NB4 cells described above. HOXA1, A4, A5, A9, A10, and A11 were selected as the most relevant to myelopoiesis in NB4 cells, based on our genomics data (Supplemental Figure 2) and the published literature.[38-41] As shown in Figure 7B, HOXA1 and A4 demonstrated striking, significant induction of expression in native and scramble shRNA-transduced NB4 cells, but not in HOTAIRM1 knock-down cells. HOXA5 showed a subtle increase in expression during three days of ATRA induction in native and negative control cells, but transcript levels fell approximately 3–fold in HOTAIRM1 knockdown cells (Figure 7B). In contrast to the suppression of these genes from the 3’ end of the HOXA cluster, knockdown of HOTAIRM1 had no significant effect on the induced responses of HOXA9, A10, or A11 on the 5’ end of the HOXA cluster (figure 7B). Accordingly no significant impact of its knock-down was observed on the induction of the phagocyte oxidase gene CYBB (Supplemental Figure 6), a known target of HOXA9 and HOXA10 modulation.[42,43] There was no apparent effect of *HOTAIRM1* knock-down on morphological maturation detectable by Wright-Giemsa staining of ATRA-induced NB4 cells (not shown).

In addition, knock-down of HOTAIRM1 during NB4 granulocytic differentiation significantly attenuates the induction of beta2 integrin CD11b and CD18 transcripts (Supplemental Figure 6), two hallmark granulocyte maturation genes.[44-46] Adhesion molecule genes such as beta3 integrin [47] are well-documented HOX targets, but CD11b and CD18 genes have not yet been linked to HOX modulation. However, these results suggest that HOTAIRM1 also has a potential role in myeloid transcriptional regulation involving, directly or indirectly, targets outside the HOXA cluster.

Discussion

In this study, we present evidence that *HOTAIRM1*, a small intergenic transcript from the plus (opposite) strand between the *HOXA1* and *HOXA2* genes, is a ncRNA expressed specifically in the myeloid lineage. We have demonstrated its expression pattern in mature myeloid cells and during granulocytic differentiation using a comprehensive collection of transcription mapping data from tiling array analyses of hematopoietic cells and circulating leukocytes. In myeloid cell lines, expression of *HOTAIRM1* was induced by ATRA and this response was abrogated in an ATRA-resistant NB4 subclone. We further showed that high level expression of *HOTAIRM1* in circulating neutrophils could be decreased by lipopolysaccharide stimulation. These data indicate a lineage-specific pattern of regulated expression for *HOTAIRM1* that parallels functional genes associated with late granulocytic maturation and with inflammatory or apoptotic functions. The lack of an open reading frame or association with polyribosomes indicates that the transcript probably represents a ncRNA. shRNA-mediated knockdown experiments indicate a functional role for HOTAIRM1 in regulating the expression of neighboring genes at the 3' end of the HOXA cluster and impact the expression of genes encoding Beta2 integrins CD11b and CD18.

HOX genes of the A and B paralog groups have emerged as a class of key transcriptional regulators in definitive hematopoiesis.[48] Functions of homeobox pathways have been well documented in normal hematopoiesis and acute myeloid leukemia.[39,49,50] However, the general collinearity rule of Hox gene expression seen in body axis patterning during embryogenesis does not hold for hematopoiesis. Although there is a 3' to 5' orderly activation sequence, with 3' genes expressed during hematopoietic stem cell renewal and expansion and 5' genes (HoxA7-10) expressed mainly in committed progenitors and mature cells, the "HOX code" associated with adult hematopoietic lineage development remains unclear. For example, both the 3'-located HOXB4 and 5' gene HOXA9 are required for normal hematopoietic stem cell function both in vitro and in vivo as.[51,52] Moreover, the HOXA9 and HOXA10 genes remain

detectable in mature myeloid cells and regulate the transcription of genes encoding the phagocyte proteins gp91-phox, p67-phox, , and β3 integrin .[42,47,48]

The ncRNAs have emerged as an important new feature of the human transcriptome, representing a surprisingly high proportion of all transcripts identified from the 1% of the human genome analyzed in the ENCODE project.[12] The particularly extensive intergenic transcriptional activity within the HOX gene clusters has been proposed to generate ncRNAs with transcriptional regulatory functions [7,9,10]. In the HOXA region, Rinn et al.[7] have identified a class of transcripts, termed *HOTAIR* (for HOX Antisense Intergenic RNA) which act in *trans* on remote targets as recruiters of histone methyltransferases.[7] Other HOXA intergenic transcripts have been speculated to serve in *cis* control of coordinately expressed neighboring HOXA genes.[9,10] Although miRNAs appear to exert their regulatory influence through processes involving sequence homology, both the molecular mechanisms and functions of other ncRNAs in gene regulation remain largely unknown.

In addition to the burgeoning roles of miRNAs in hematopoiesis and leukemogenesis,[53] recent evidence has associated other forms of ncRNA with leukocyte development. A long ncRNA, termed *EGO*, which is transcribed from an intron of the inositol triphosphate receptor type 1 gene (*ITPR1*), transcriptionally regulates secondary granule protein expression during eosinophil development.[54] In T cell leukemia cell lines, expression of a transcript paired divergently with a homeobox gene *TLX1/HOX11* (T-cell leukemia homeobox 1) and hence termed *TDI* (*TLX1* divergent), correlates with the expression of *TLX1*, a proto-oncogene inappropriately activated in human T-cell acute lymphoblastic leukemia (T-ALL) and reported to inhibit hematopoietic differentiation in a number of murine models.[55,56] No direct targets have yet been established for those regulatory hematopoietic ncRNAs.

DNA sequence analysis of *HOTAIRM1* shows poor evolutional conservation at the sequence level even among mammals, with no homologs found in current genome assemblies. However, the similarity of localized ESTs in other mammals, including murine ESTs with similar splicing patterns suggest the more

general conservation of intergenic transcription activity. Also, a long CpG island is associated with the transcription start site, as seen in almost all mammalian intergenic RNAs. This structure, suggesting the presence of a bi-directional promoter shared by the divergent coding and non-coding RNAs, has been proposed to facilitate the *cis* action of intergenic ncRNAs on their genic partners.[9,10] However, the expression of *HOTAIRM1* and *HOXA1* is not always synchronized, suggesting that *HOTAIRM1* may also function independently from the immediately adjacent gene.

Expression of early HOX paralogs in anterior-posterior body patterning during embryogenesis is mediated by flanking retinoic acid response elements, including a site downstream of *HOXA1* that has been verified experimentally.[57] Additional putative retinoid response elements have been predicted within the 3' side of the HOXA cluster, including one within the CpG island embedded the shared promoter region of *HOXA1* and *HOTAIRM1*.[10] Our current finding that cycloheximide did not entirely abrogate the induction of *HOTAIRM1* suggests that retinoid receptors or other transcription factors may also mediate induction of *HOTAIRM1*.

Overall, the current studies indicate a close association of the HOXA intergenic transcript *HOTAIRM1* with differentiation and function in the myeloid lineage and lay the foundation for further analysis of its potential regulatory function in hematopoiesis and leukemogenesis.

Acknowledgements

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Authorship: X.Z. designed research; collected, analyzed and interpreted data; performed statistical analysis; and wrote the manuscript; Z.L. and C.P. collected data; M.B.G., J.R., M.S., T.G., and P.K.

contributed vital analytical tools, analyzed data, and performed statistical analysis; S.M.W. and P.E.N. designed research, analyzed and interpreted data, and reviewed the manuscript.

Conflict of Interest Disclosure: Thomas R. Gingeras and Philipp Kapranov are employees of Affymetric, Inc., and own stock in the company.

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FIGURE 1. **Identification of intergenic transcript *HOTAIRM1* within the HOXA cluster.** *Panel A*: Transcription mapping of the human HOXA cluster in myeloid cell lines and mature neutrophils was performed on Affymetrix ENCODE tiling arrays; each panel (labeled with cell type and conditions) presents data from at least 3 biological repeats. Pseudo-median signals are color scaled by p-value, as indicated in the right margin, to highlight the significant TARs. The median point (threshold for positive) was set to $p=10^{-5}$, based on visual inspection of well aligned *HOXA1* signals in NB4 cells. *Panel B*: Alignment of the sequence of *HOTAIRM1* (mapped by 5'- and 3'-RACE), spliced ESTs, and HG_U13 probe sets with the human genome sequence for the intergenic region between *HOXA1* and *HOXA2* in the March 2006 human reference sequence (NCBI Build 36.1). Also shown are primer sites used for ChIP-PCR amplification of *HOTAIRM1* (5'sequence: "5HOTAIRM1" and 3' sequence: "3HOTAIRM1") in experiments presented in Figure 3. *Panel C*: Northern blot analysis shows *HOTAIRM1* as a 0.5 kb RNA in NB4 and HL60 myeloid leukemia cells and in human peripheral blood neutrophils.

FIGURE 2. **Polyribosome distribution of *HOTAIRM1* and *HOXA1* transcripts in ATRA-induced NB4 cells**. *Panel A*: Polyribosome distribution among fractions from top to bottom (1-22) along the gradient of increasing sucrose density. *Panel B*: multiplex RT-PCR analysis of *HOTAIRM1* transcripts in gradient fractions, compared to the distributions of *HOXA1* and alpha tubulin (*TUBA1B*) mRNAs, as indicated.

FIGURE 3. **ChIP assay of RNAP II binding to the *HOTAIRM1* gene.** The 5' and 3' ends of the *HOTAIRM1* gene (HOTAIRM1-5 [557bp] and HOTAIRM1-3 [267bp], respectively), along with 3' end of the alpha tubulin (*TUBA1B*) gene (484bp) and the distal U6 snRNA gene promoter (118bp), were amplified from anti-RNAP II pull-downs and from input DNA of NB4, ATRA induced NB4, and Hela cells. *Upper panel*: Agarose gel electrophoresis of multiplex PCR products of the indicated DNA species and cell types. *Lower panels*: Positions of the U6 snRNA and alpha tubulin amplicons are shown schematically as shaded bars (the *HOTAIRM1* amplicons are illustrated in Figure 2, above).

FIGURE 4. **Retinoid induction of *HOTAIRM1* transcription**. *Panel A:* Induction of *HOTAIRM1* transcripts in NB4 cells incubated for up to 96 h with ATRA (1uM) or 9-cis-RA (1uM), as indicated above each graph. Induction of HOTAIRM1 by ATRA was also measured in the presence of cycloheximide (100ug/ml) in the culture media, as indicated. *Panel B:* Induction of the ATRA-resistant subline NB4r2, under the same conditions. *Panel C:* Comparison of *CEBPE*, *HOXA1* and *HOTAIRM1* induction in NB4 cells during 12 hour incubation with ATRA (1uM).
Relative expression levels of targets were measured by quantitative real time RT-PCR, with results shown as ratios to the levels of uninduced reference cells. Bars and error lines represent means and standard deviations of results, normalized to an endogenous reference gene (*TUBA1B* or *RPS6*), of at least triplicate experiments for each group.

FIGURE 5**. Tissue distribution of *HOTAIRM1* expression**. *Panel A*: Multiplex RT-PCR analysis of *HOTAIRM1* and internal reference *GAPDH* transcripts on a multiple tissue human cDNA panel, with each tissue type indicated above the image of a representative agarose gel. *Panel B*: Multiplex RT-PCR analysis of *HOTAIRM1* and internal reference *TUBA1B* (101bp) transcripts on a multiple cell line panel, with each cell type and its treatment with ATRA ("-" untreated, "+" treated with 1uM ATRA for one day) indicated above the image of a representative agarose gel. The vertical line indicates the location of redundant lanes cropped from the gel image.

FIGURE 6. **Expression pattern of *HOTAIRM1* during myelopoiesis**. HG_U133 transcription profiles of the human HOXA cluster region were assembled for human hematopoietic stem/progenitor cells (HSPC), primary leukocytes, and myeloid cell line NB4. The stem/progenitor cell subpopulations are labeled according to the presence ("+") or absence ("-") of lineage development markers (CD33, CD34, CD38 or Lin) and low or high rhodamine 123 retention (Rh^{low}, Rh^{high}). Also indicated are the sources of the stem/progenitor cells: bone marrow (BM), cord blood (CB), fetal blood (FB), and G-CSF-mobilized peripheral blood (MPB). NB4 cells were examined either without treatment or after incubation with ATRA and TPA as indicated. Primary leukocyte populations included total monocytes (MC), adherent monocytes, monocyte-derived dendritic cells (DC), resting neutrophils (PMN), and neutrophils incubated with LPS (10 ng/ml) for 30 or 120 minutes, as indicated. The bar plot presents means and standard deviations for *HOTAIRM1* and *HOXA1* transcript levels in each sample group.

FIGURE 7. **shRNA-mediated silencing of *HOTAIRM1* RNA**. *Panel A:* Stable knockdown of *HOTAIRM1* transcripts in two NB4 cell clones transduced with pLKO.1 lentiviral vector expressing shRNA targeting *HOTAIRM1* transcripts and incubated with ATRA (1uM). The effectiveness of silencing is compared to pLKO.1 expressing negative control "scramble" shRNA. Quantitative RT-PCR results are shown as the percentage of *HOTAIRM1* expression in each transduced cell type relative to the level in non-transduced NB4 cells. *Panel B:* Expression of myelopoiesis-relevant HOXA genes by *HOTAIRM1* knockdown cells during three days of ATRA (1uM)-induced granulocytic differentiation of NB4 cells. Relative expression levels of the indicated targets were measured by quantitative real time RT-PCR in two stable knock down NB4 clones. The bars and error lines represented means and standard deviations of at least triplicate experiments for each group, normalized to the geometric mean of a panel of endogenous reference genes.

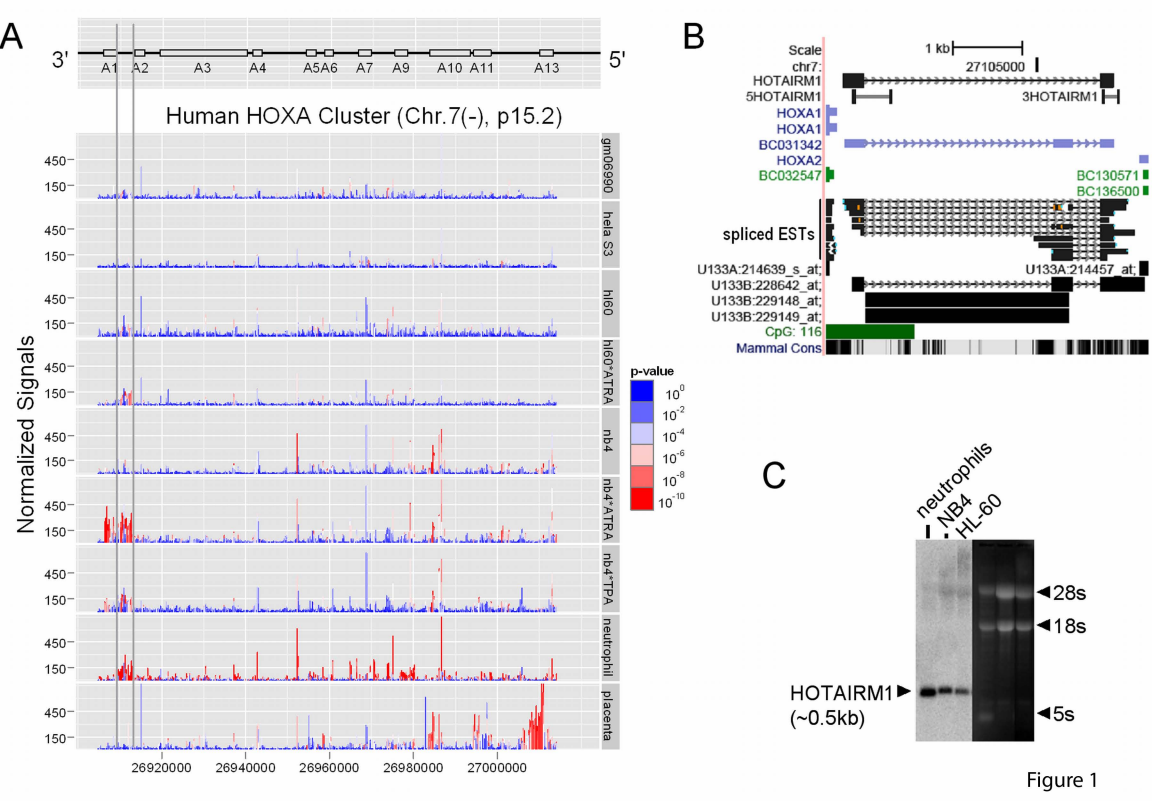
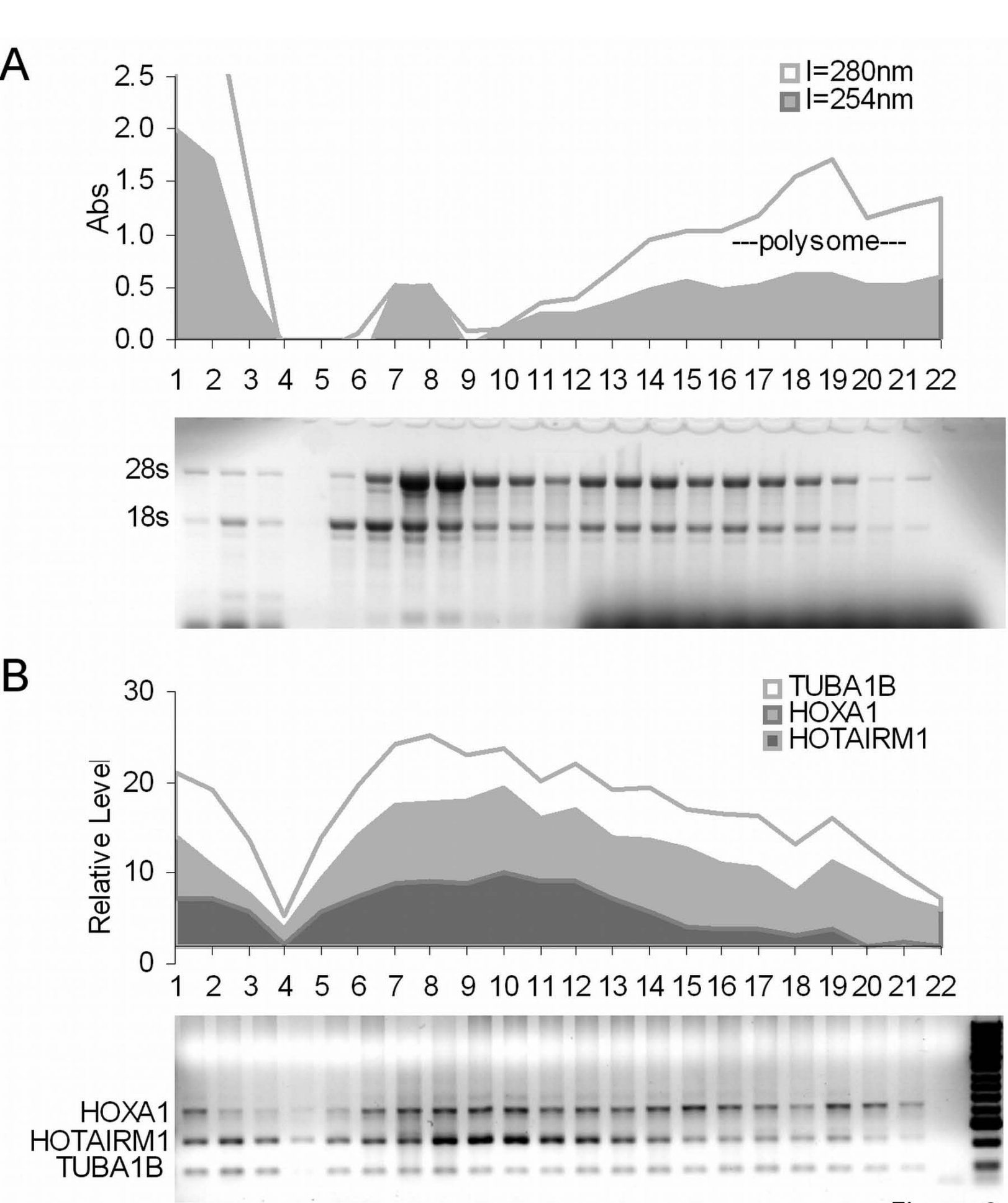

A
3'
A1
A2
A3
A4
A5A6
A7
A9
A10
A11
A13
5'
Human HOXA Cluster (Chr.7(-), p15.2)
Normalized Signals
gm06990
hela S3
hl60
hl60*ATRA
nb4
nb4*ATRA
nb4*TPA
neutrophil
placenta
450
150
p-value
10^{0}
10^{-2}
10^{-4}
10^{-6}
10^{-8}
10^{-10}
26920000
26940000
26960000
26980000
27000000
B
Scale
1 kb
chr7:
27105000
HOTAIRM1
5HOTAIRM1
3HOTAIRM1
HOXA1
HOXA1
BC031342
HOXA2
BC032547
BC130571
BC136500
spliced ESTs
U133A:214639_s_at;
U133A:214457_at;
U133B:228642_at;
U133B:229148_at;
U133B:229149_at;
CpG: 116
Mammal Cons
C
neutrophils
NB4
HL-60
28s
18s
5s
HOTAIRM1
(~0.5kb)

Figure 1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l=280nm
l=254nm
Abs
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2.0
1.5
1.0
0.5
0.0
---polysome---
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
28s
18s
B
TUBA1B
HOXA1
HOTAIRM1
Relative Level
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
HOXA1
HOTAIRM1
TUBA1B

Figure 2

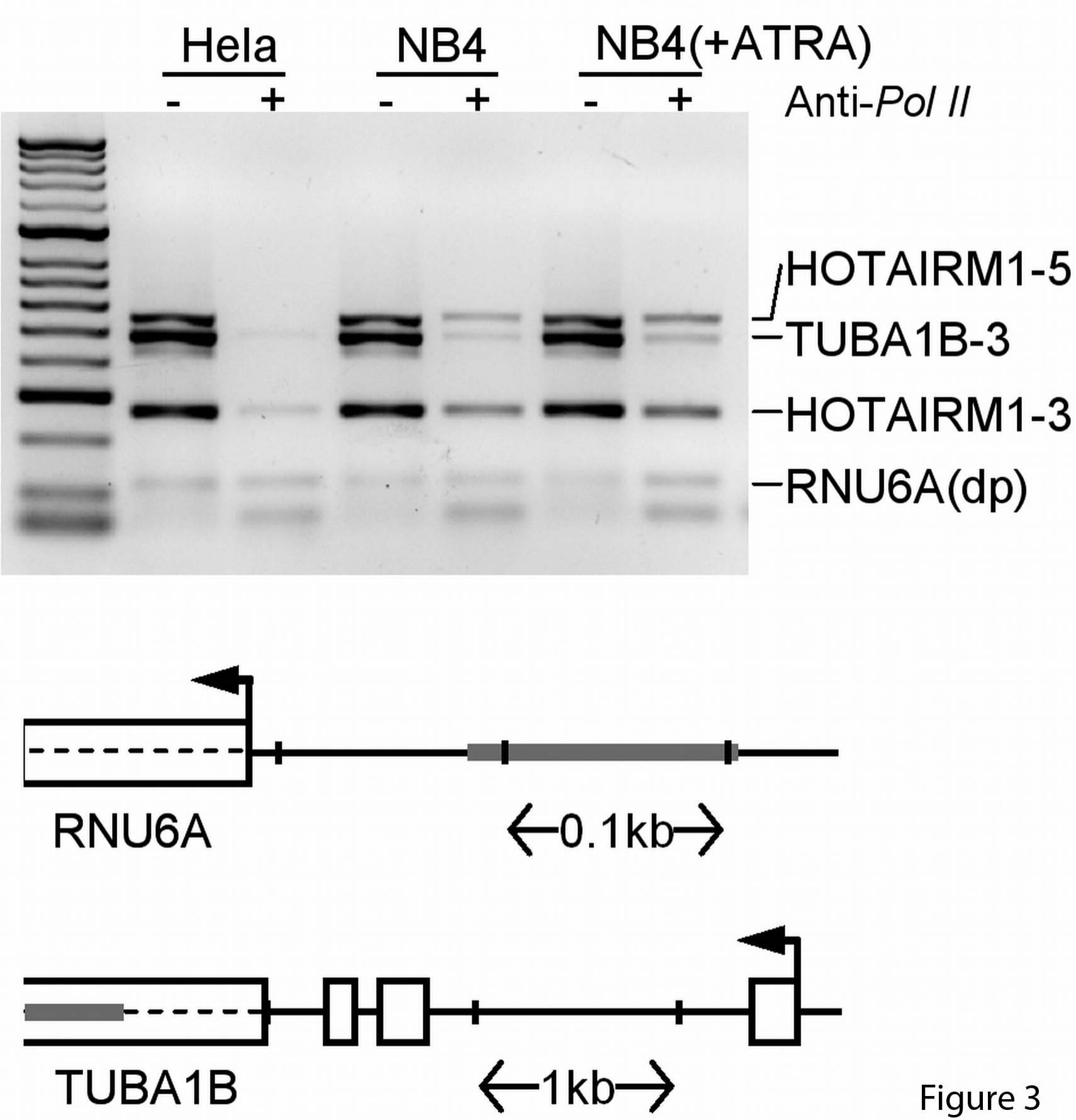

Hela
NB4
NB4(+ATRA)
- + - + - +
Anti-Pol II
HOTAIRM1-5
TUBA1B-3
HOTAIRM1-3
RNU6A(dp)
RNU6A
0.1kb
TUBA1B
1kb

Figure 3

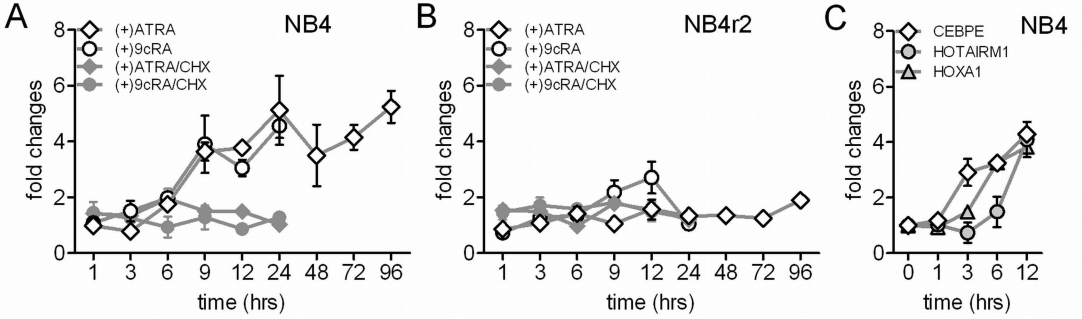
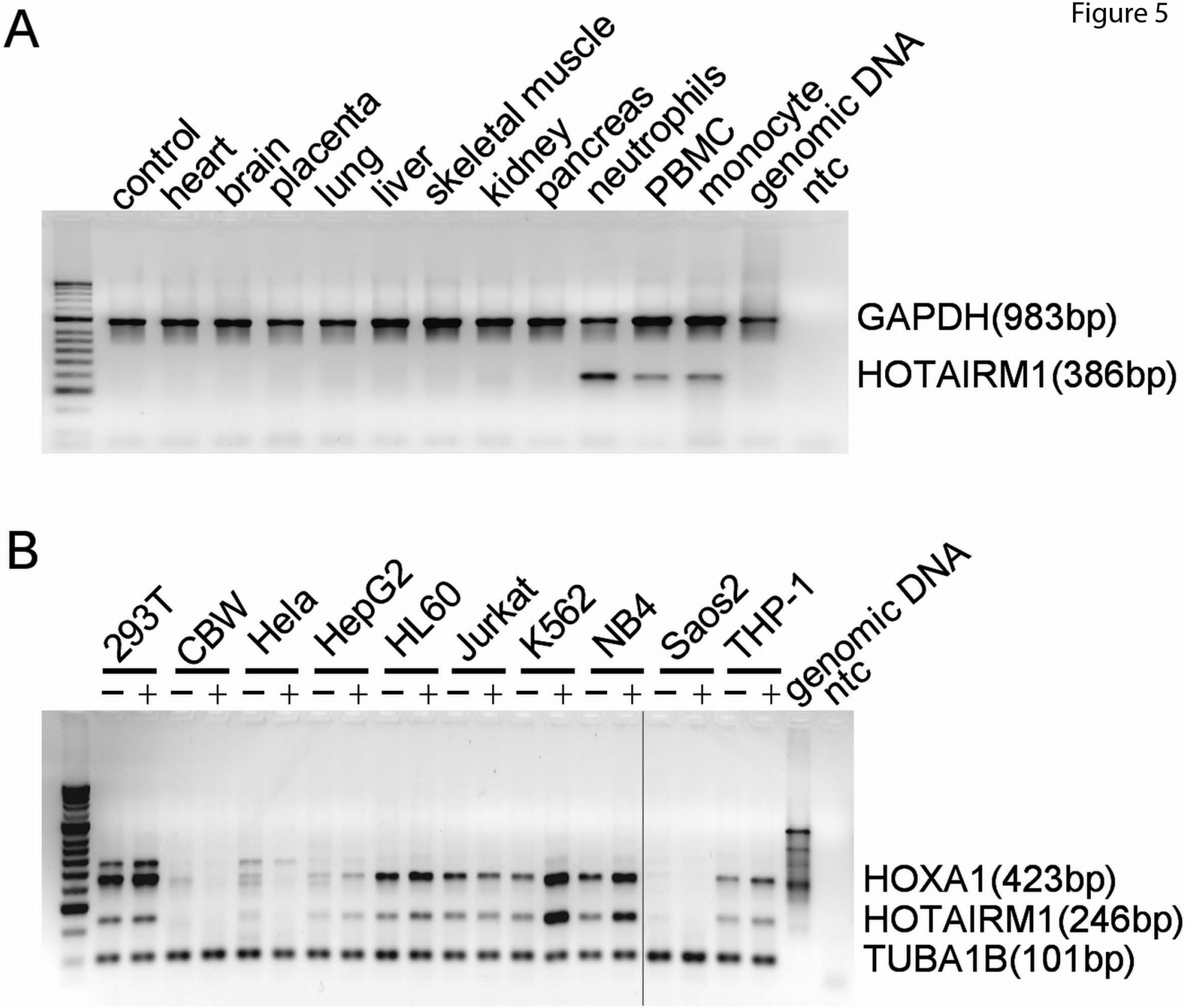

A
NB4
(+)ATRA
(+)9cRA
(+)ATRA/CHX
(+)9cRA/CHX
fold changes
time (hrs)
B
NB4r2
C
CEBPE
HOTAIRM1
HOXA1

Figure 4

Figure 5


A
control
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
neutrophils
PBMC
monocyte
genomic DNA
ntc
GAPDH(983bp)
HOTAIRM1(386bp)
B
293T
CBW
Hela
HepG2
HL60
Jurkat
K562
NB4
Saos2
THP-1
genomic DNA
ntc
− + − + − + − + − + − + − + − + − + − +
HOXA1(423bp)
HOTAIRM1(246bp)
TUBA1B(101bp)

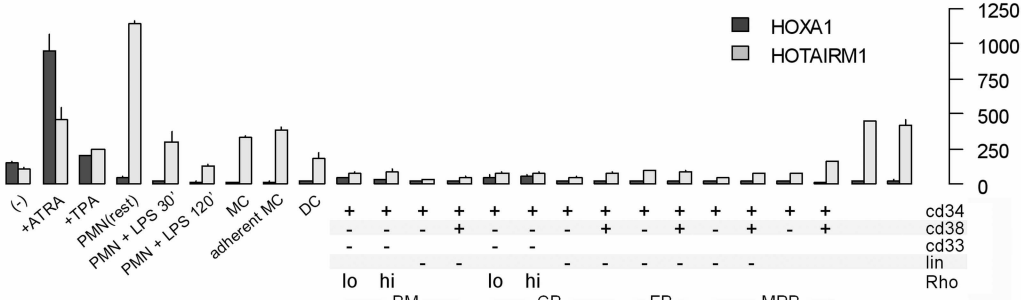

HOXA1
HOTAIRM1
1250
1000
750
500
250
0
(-)
+ATRA
+TPA
PMN(rest)
PMN + LPS 30'
PMN + LPS 120'
MC
adherent MC
DC
cd34
cd38
cd33
lin
Rho
lo
hi
lo
hi
BM
CB
FB
MPB
NB4
leukocyte fractions
HSPC
Whole leukocytes

Figure 6

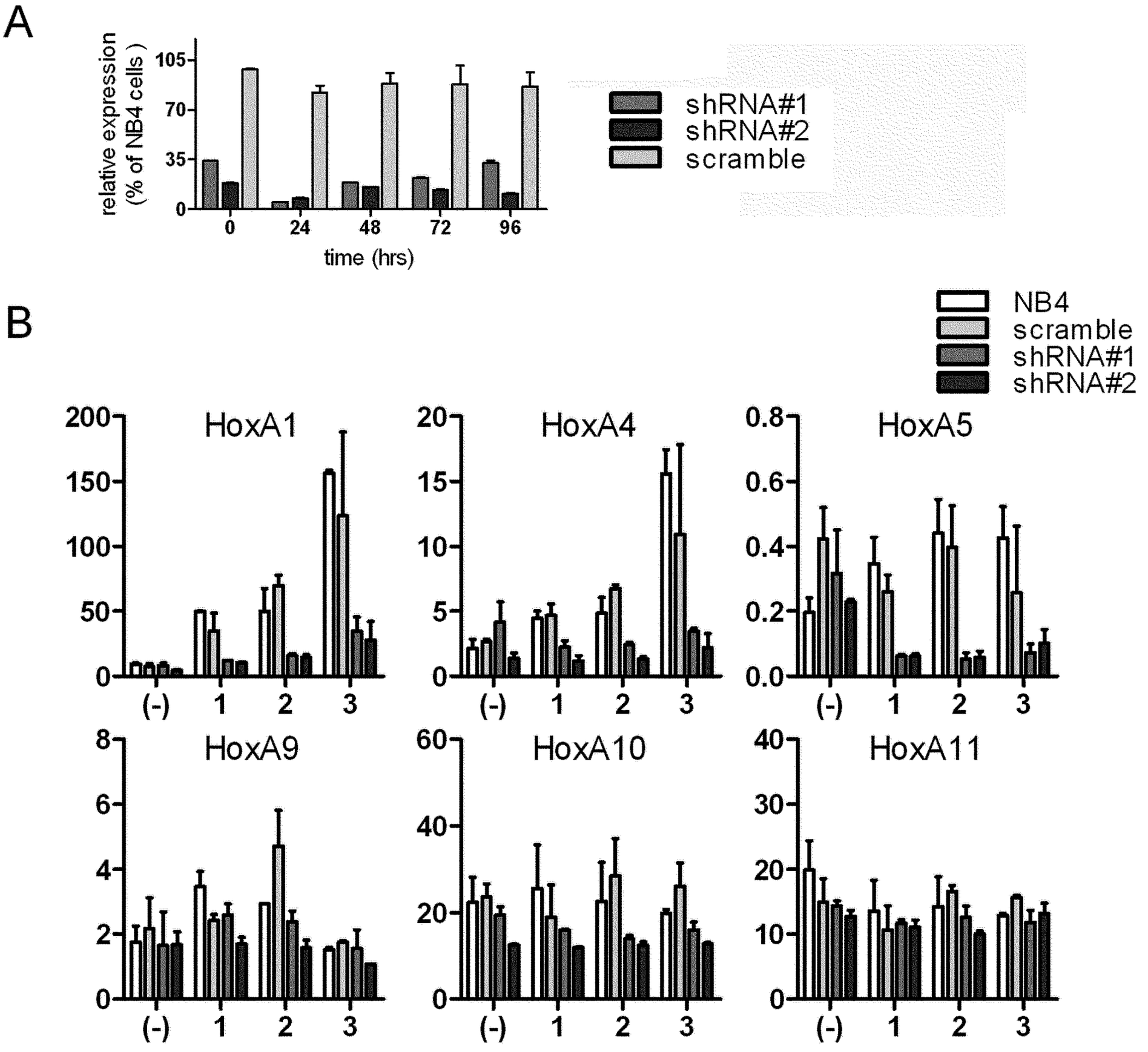

A
relative expression
(% of NB4 cells)
105
70
35
0
0
24
48
72
96
time (hrs)
shRNA#1
shRNA#2
scramble
B
NB4
scramble
shRNA#1
shRNA#2
HoxA1
HoxA4
HoxA5
HoxA9
HoxA10
HoxA11
(-)
1
2
3

Figure 7