MOLECULAR BIOLOGY

*HOX*s and lincRNAs: Two sides of the same coin

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The clustered *Hox* genes play fundamental roles in regulation of axial patterning and elaboration of the basic body plan in animal development. There are common features in the organization and regulatory landscape of *Hox* clusters associated with their highly conserved functional roles. The presence of transcribed noncoding sequences embedded within the vertebrate *Hox* clusters is providing insight into a new layer of regulatory information associated with *Hox* genes.

Hox gene clusters are one of the most ancient and highly conserved multigene loci in the animal kingdom (*1*, *2*). Tandem duplication and unequal crossing over in an ancestral organism created a cluster of *Hox* genes, which then underwent further duplication and divergence from the common ancestral cluster (*3*). Functional studies in a wide range of invertebrate and vertebrate species have underscored the conserved roles of the HOX family of transcription factors as central players in the regulation of axial patterning during elaboration of the basic body plan in the evolution of animals (*2*, *4*–*7*). The series of genome duplications associated with vertebrate evolution have generated multiple *Hox* complexes and sets of paralogous genes within a species. This creates a situation whereby a subset of *Hox* genes or clusters may fulfill ancestral functions in axial patterning, whereas the others are available to evolve new roles or activities and become coupled to regulation of different developmental processes (*1*, *3*, *8*).

Many fundamental properties of the organization, regulation, and function of *Hox* gene clusters, such as colinearity, posterior prevalence, response to major signaling pathways, auto-, para-, and cross-regulation, and long-range or global regulation, appear to be common features of the regulatory landscape of *Hox* clusters among widely diverse species (*2*, *9*–*11*). Studies on the expression, regulation, and evolutionary origins of *Hox* gene clusters have primarily focused on the protein-coding regions (*12*). However, recent advances in genomics have unearthed a treasure trove of transcribed sense and antisense noncoding sequences embedded within the vertebrate *Hox* clusters and their flanking regions (for example, *Hotair*, *Hottip*, *Hobbit*, *Halr1*, *Hotdog*, *mir10*, and *mir196*), providing insight into a new layer of regulatory inputs for temporally and spatially restricted patterns of *Hox* expression. Ironically, Lewis, in his original analysis, postulated that many cis-regulatory regions in the *Drosophila bithorax* complex were regulatory RNAs (*7*). This has proven to be correct, but for a long time, this concept received little attention from the community (*13*–*15*). This raises the intriguing question of whether noncoding transcripts are also common features of the ancestral *Hox* clusters or newly evolved properties of complex vertebrate genomes. Here, we will focus on the current state of knowledge of noncoding transcripts associated with mammalian *Hox* clusters, their regulation, and putative functions as a basis for thinking about their implications in development, disease, and evolution.

Extensive transcription of long intergenic noncoding transcripts (lincRNAs) is a key characteristic of many multigene loci, including globin, immunoglobin, and *Hox* gene clusters (*16*–*19*). These intergenic transcripts are implicated in activation and repression through opening large chromatin domains, maintenance of active chromatin state, or RNA interference–mediated silencing processes, as shown for the globin gene cluster (*16*, *18*, *20*). These lincRNAs can affect gene regulation through both cis and trans mechanisms on *Hox* and non-*Hox* genes (*21*–*25*). Mammalian and invertebrate *Hox* clusters show extensive transcriptional activity from both strands of coding and noncoding regions during development (*19*, *26*–*31*). The functional significance of such noncoding transcription is beginning to emerge as evidence from several groups suggests the important roles of noncoding transcription in the regulation of *Hox* clusters (*19*, *26*, *32*–*34*) (Fig. 1). Intergenic transcripts are often associated with active *Hox* genes. Analysis of human *HOX* clusters identified 15 antisense transcribed regions that represent 38% of spliced transcripts from these clusters (38.46% for *HOXA*, 33.11% for *HOXB*, 13.16% for *HOXC*, and 34.84% for *HOXD*) (*31*). Figure 2 illustrates extensive syntenic or positional conservation of many noncoding transcripts, including *Mirs* between human and mouse *Hox* clusters. This suggests that there may be common functional roles for these transcripts.

There appear to be more noncoding transcripts both within and flanking the *HoxA* cluster relative to other clusters (Fig. 2). Positioned 50 kb 3′ of the *HoxA* cluster, in the intergenic region between *Hoxa1* and *Skap2*, is a ~16-kb region (*Heater*) that gives rise to a large number of spliced and unspliced polyadenylated transcripts originating from both strands (*Halr1* and *Halr1os1*) (*30*, *34*, *35*). These transcripts have multiple isoforms and epigenetic marks (H3K4Me3 and H3K27Me3), and occupancy of Pol II (RNA polymerase II) indicates that they arise from at least four different start sites. From a regulatory perspective, mouse *halr1*, *halr1os1*, and their isoforms are among the most rapidly induced transcripts upon retinoic acid (RA) treatment of embryonic stem (ES) cells and also respond to RA in developing embryos (*30*).

The *Heater* region may be important for potentiating the response of *Hoxa1* to retinoids (Fig. 3A) because knockdown of three *Halr1* isoforms leads to increased levels of *Hoxa1* in uninduced ES cells (*34*). *Halr1* interacts with PURB (purine-rich element binding protein B), a single-stranded DNA/RNA binding protein involved in transcriptional regulation (*36*), and knockdown of *PURB* leads to increased expression of *Hoxa1*. There appears to be a strict threshold on the number of *Halr1* molecules per cell (<10 transcripts per cell) (*34*), and RA treatment of ES cells alters this relationship by increasing both the levels and composition of *Halr1* and *Halr1os1* transcripts through addition of new isoforms (*30*). These changes to the transcriptional repertoire have the potential to decouple interactions between *Halr1* and PURB, altering regulatory input on *Hoxa1* (Fig. 3A). Retinoids appear to directly induce the *Heater* lincRNAs because two flanking regions (*H-AR1* and *H-AR2*)

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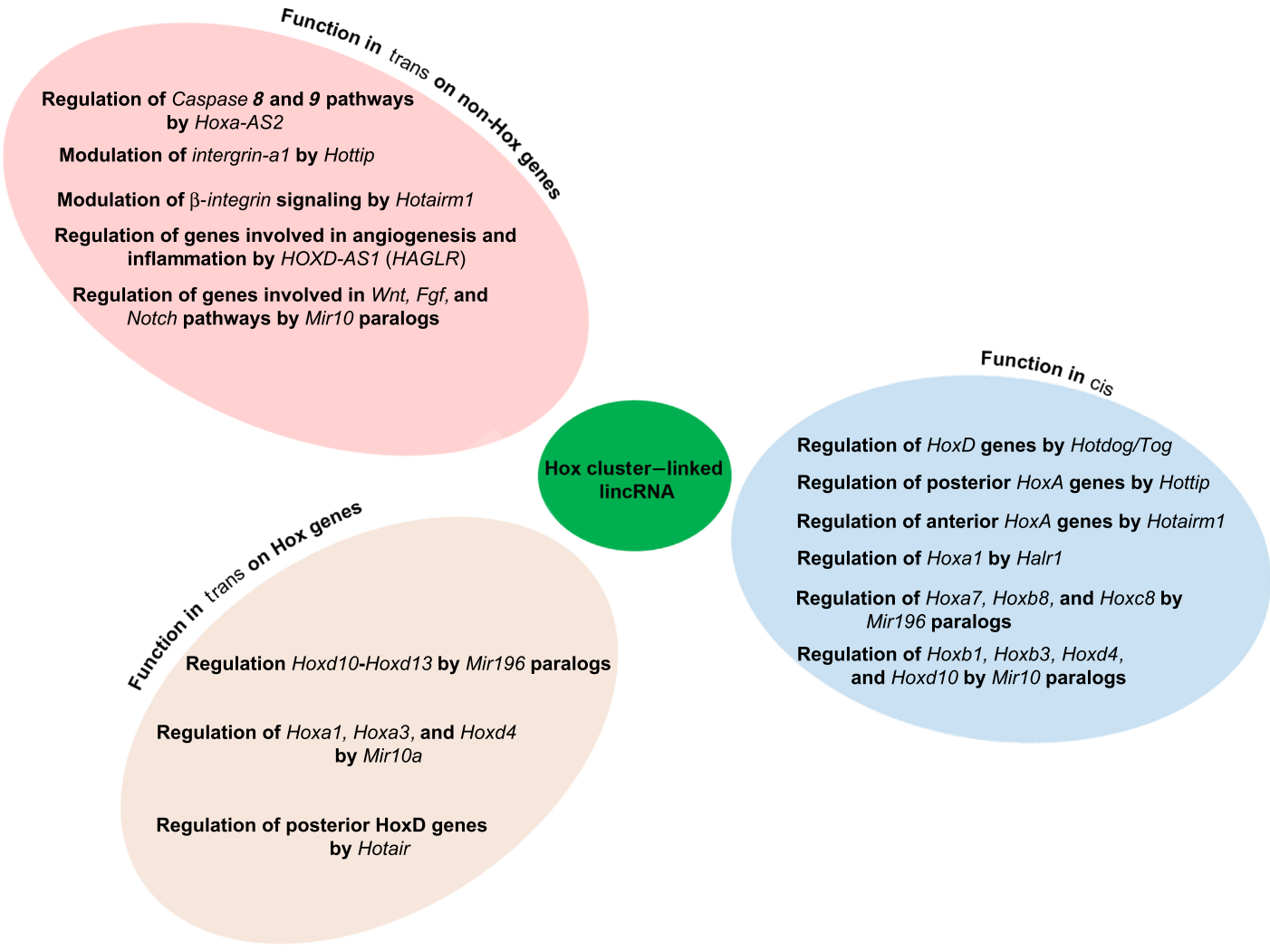

Fig. 1. Functions of *Hox* cluster lincRNAs and *Mirs* in cis and trans. Cis function is defined as the functional impact of a lincRNA and *Mirs* on *Hox* genes from the same cluster.

contain multiple retinoic acid response elements (RAREs) that display dynamic occupancy of retinoic acid receptors [RARs and RXRs (retinoid X receptors)] (*30*). This suggests a model for how a key signaling pathway in development (retinoids) may regulate noncoding transcripts that, in turn, affect the expression of the adjacent *Hoxa1* gene.

Within the human *HOXA* cluster, at the 3′ end between *HOXA1* and *HOXA2*, is *HOTAIRM1*, a lincRNA from the noncoding strand initially identified in association with myelopoiesis in humans (*28*). In mice, along with *Hotairm1*, a new isoform and a novel transcript, *Hotairm2*, has been mapped to this region, and they display dynamic expression patterns during development (*30*). Intriguingly, these lincRNAs are also rapidly induced by RA in human myeloid lineages and during mouse ES cell differentiation and embryonic development (*28*, *30*). This reveals conservation in both their syntenic position and regulatory response to retinoids. *HOTAIRM1* feedbacks into RA induced changes in gene expression because its knockdown in NB4 cells results in alterations related to RA-induced growth arrest at G_1 and granulocytic maturation (*37*). This further highlights regulatory interactions between retinoids and multiple *Hox* lincRNAs.

Functionally, *HOTAIRM1* modulates gene expression in both cis and trans (Fig. 1). Reducing the levels of *HOTAIRM1* results in a loss of gene expression of 3′ *HOXA* cluster genes (cis) and alterations in β_2-integrin signaling through CD11b and CD18 and in integrin switch mechanism involving CD11c and CD49d (trans) (*28*, *37*). This implicates *HOTAIRM1* in cell cycle regulation through moderation of G_1/S transition.

Further 5′ in the *HoxA* cluster, *HoxA-AS2* is a lincRNA with several isoforms expressed from the noncoding strand between *Hoxa3* and *Hoxa4* (*38*). *HOXA-AS2* is induced by RA, IFN-γ (interferon-γ), and TNF-α (tumor necrosis factor–α) and is functionally linked with the repression of apoptosis through modulation of the *caspase 8* and *9* pathways (*38*). Overlapping with the promoter region of *Hoxa11* is a conserved antisense RNA, *HOX11AS* (human) and *Hoxa11os* (mouse), which shows mutually exclusive expression throughout development with *Hoxa11* (*39*, *40*). This is illustrated by the expression of human *HOXA11AS* during the menstrual cycle, which peaks at midproliferative stage in an inverse relationship to *HOXA11* expression (*41*). Mechanistically, ectopic expression of *Hoxa11os* does not down-regulate endogenous

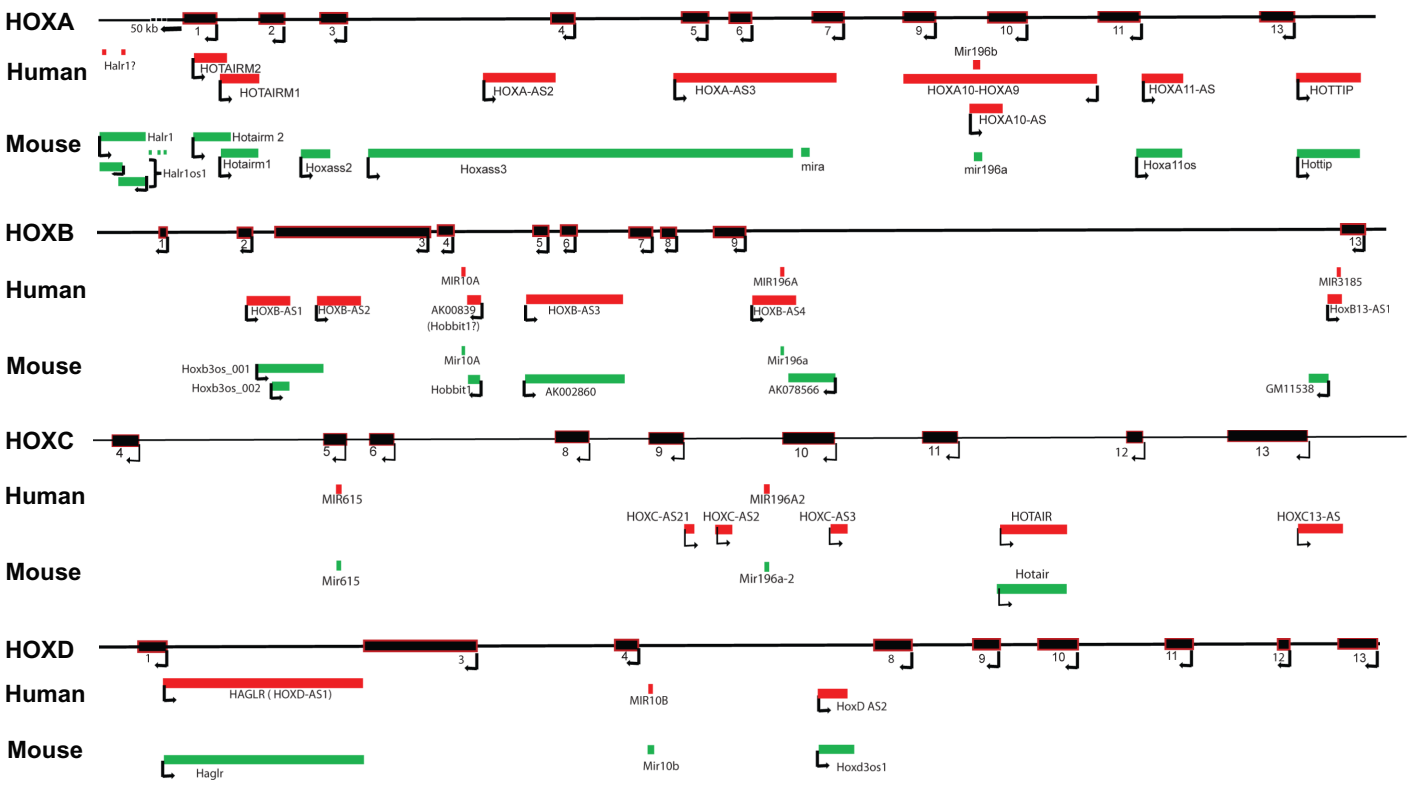

Fig. 2. Comparative alignment of human and mouse coding and noncoding transcripts originating from the four *Hox* clusters. Each *Hox* cluster is scaled on the basis of human coordinates with the gene name listed below. Relative positions of mouse (green) and human (red) noncoding transcripts are shown on the basis of human coding genes as landmark. Arrows indicate the direction of transcription.

Hoxa11 expression in murine uterus, which appears to rule out potential degradation of *Hoxa11* by sense-antisense pairing and raises the possibility of modulation via promoter interference (*41*). In addition to the examples above, there are an extensive series of lincRNAs embedded in and spread throughout the *HoxA* cluster in human and mouse that display varying degrees of syntenic conservation, but their functional significance is yet to be explored (Fig. 2).

The 5′ end of the *HoxA* cluster is also marked by two lincRNAs, *HIT18844* and *HOTTIP*. *HIT18844* contains a highly conserved 265-bp block in vertebrates that maps 1.8 kb upstream of *Hoxa13* gene (*29*). *HOTTIP* is expressed from the noncoding strand 330 bp upstream of *HOXA13*, and the *HOTTIP* region displays both H3K4me3 and H3K27me3 epigenetic marks (bivalent) that change upon activation and expression (*22*). Depletion of *Hottip* leads to shortening and bending of distal bony elements in the limb similar to the loss-of-function phenotype of *Hoxa11* and *Hoxa13*. Regulatory analyses suggest that *HOTTIP* is implicated in the regulation of 5′ *HOXA* genes in a directional manner, in that it only alters expression of adjacent *Hox* genes, not *Evx2* (*22*). Furthermore, the strongest effects are seen on the immediately adjacent *Hoxa11* and *Hoxa13* genes, and progressively less severe reductions are observed on *Hoxa10-Hoxa7*, consistent with the limb phenotypes. This may be related to the ability of *Hottip* to bind WDR5 (WD repeat–containing protein 5)–MLL (mixed lineage leukemia protein 1) complexes, which could provide a means for targeting the MLL trithorax group of histone methyl transferases to the adjacent posterior *HoxA* genes to modulate their activity. Knockdown of *Hottip* leads to a loss of H3K4Me2 and Me3 from whole *HoxA* complex including *Hottip*, whereas HeK27me3 is increased only over *Hottip*. However, ectopic expression of *Hottip* in lung fibroblasts does not lead to activation of posterior *Hox* genes or changes in the nature of bivalent marks over the *HoxA* cluster. Thus, the precise biochemical mechanism through which *Hottip* modulates posterior *HoxA* genes is yet to be established.

Hottip has other functional roles outside of input into *Hox* regulation. In combination with microRNA *mir-101*, it regulates cartilage development through modulation of *integrin-a1* by means of *DNMT-3B*–mediated epigenetic regulation (*42*). Together, all these studies on the *HoxA* cluster clearly indicate the large extent and emerging importance of noncoding transcription, which needs to be integrated in thinking about the general roles and regulation of *Hox* clusters. For example, many of the observed changes in epigenetic marks over the *Hox* clusters may be related to expression of noncoding transcripts.

Many of these features on expression and regulation of noncoding RNAs are also observed to a lesser degree in other clusters. *HoxD-As1* (*Haglr*), a noncoding RNA from the intergenic region of *Hoxd1* and *Hoxd3*, is implicated in the regulation of RA-induced differentiation and activation by the PI3K (phosphatidylinositol 3-kinase)/AKT pathway. This RNA is implicated in metastasis through regulation of genes associated with angiogenesis and inflammation, on the basis of knockout analyses in SH-SY5Y cells (*43*). An interesting feature of a lincRNA (*Hobbit1*) from the *HoxB* cluster transcribed from the sense strand between *Hoxb4* and *Hoxb5* is that it shares cis-regulatory elements with the adjacent coding genes (Figs. 2 and 3B). The unspliced and polyadenylated *Hobbit1* transcript is expressed in developing embryos and rapidly induced during RA-mediated differentiation of murine ES cells (*30*). Consistent

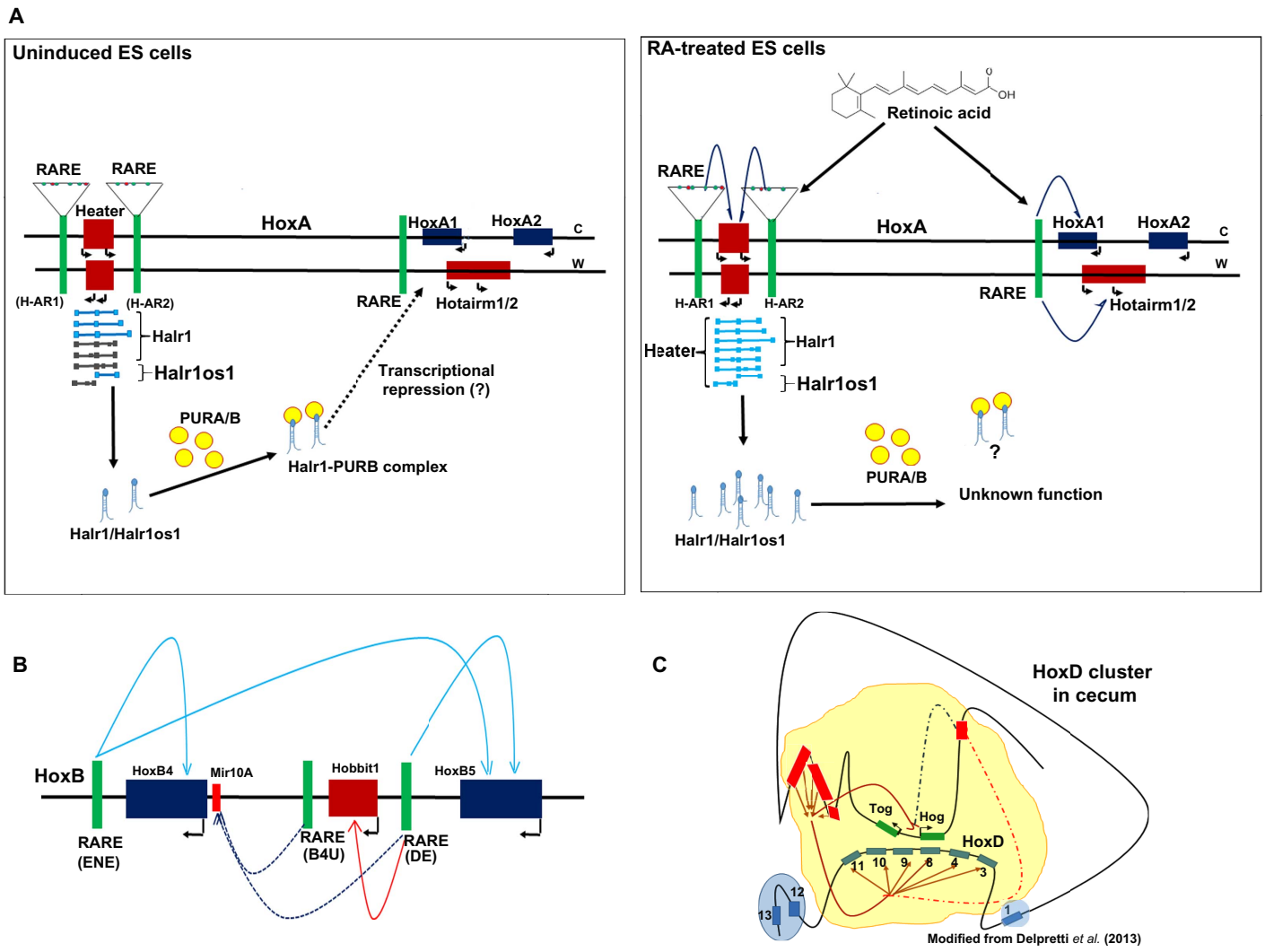

Fig. 3. Models for activities of lincRNAs from *Hox* clusters. (**A**) Regulation of *Hoxa1* by *Halr1* in mouse ES cells. Isoforms of *Halr1* and *Halr1os1* from the *Heater* region expressed in ES cells are indicated below the locus in blue. RA treatment changes the repertoire of transcripts, depicted as a shift in color from black to blue. The positions of various RAREs are also indicated. (**B**) A distal element–RARE (DE-RARE) enhancer is shared between *Hoxb4*, *Hoxb5*, *Hobbit1*, and *Mir10a*. Arrows indicate the regulatory influences of the ENE (early neural enhancer), *B4U*, and *DE*-RAREs on *Hoxb4*, *Hoxb5*, *Mir10*, and *Hobbit1*. (**C**) Long-range interaction between enhancers, the *Hog* and *Tog* noncoding RNAs, and *Hoxd3-d11* genes. The shaded yellow area depicts topologically active domains (TADs). Red solid boxes indicate enhancers. Long-range interactions between *Hog*, *Tog*, and *HoxD3-11* are noted by brown arrows. In the cecum, *Hoxd1*, *Hoxd12*, and *Hoxd13* (blue-shaded area) are outside the TAD region.

with the kinetics of induction, there is a rapid gain of the H3K4Me3 mark, associated with gene activation. In mouse embryos, *Hobbit1* expression is dependent on an RARE that plays a role in the regulation of multiple *Hox* genes (Fig. 3B) (*30*, *44*). This opens the possibility that many noncoding RNAs embedded in the *Hox* clusters may share common regulatory components with the protein-coding genes.

LincRNAs may also be associated with long-range regulation and sharing through physical interactions. For example, a pair of noncoding transcripts, *Hotdog* (*HoxD telomeric desert lncRNAs*) and *Tog* (*twin Hotdog*), arise from the gene dessert downstream of the *HoxD* cluster. *Hotdog* and *tog* are transcribed from the noncoding strand and display restricted expression in developing cecum (*45*). Furthermore, their transcription start sites show enrichment of H3K4me3 and Pol II and display strong physical interactions with active *HoxD* genes (*Hoxd4* or *Hoxd11*) in the cecum. *Hotdog* and *Tog* expression levels are completely abolished by deletion of the region from *Hoxd9* to *Hoxd11*. Disruption of contact between *Hotdog*/*Tog* and *Hoxd* genes by chromosomal inversion leads to complete loss of *HoxD* expression. This suggests a model of long-range enhancer sharing between lincRNAs (*Hotdog* and *tog*) and *HoxD* genes (Fig. 3C) (*45*).

One of the most studied lincRNAs is *HOTAIR* (HOX transcript antisense intergenic RNA), which is a spliced, antisense, and polyadenylated transcript generated from the intergenic region between *HOXC11* and *HOXC12* (*19*). *HOTAIR* serves as a scaffold for interaction with PRC2 (Polycomb repressive complex 2) and LSD1 (lysine-specific demethylase 1A) complex. The interaction between *HOTAIR* and PRC2 modulates enzymatic activity, which is mediated by interplay between EZH2 (enhancer of zeste homolog 2), EED (embryonic ectoderm

development), and JARID2 (jumonji, AT-rich interactive domain 2). Thus, *HOTAIR* works in trans to play roles in development and disease by localizing the PRC2 complex on its genome-wide targets, which include posterior *HoxD* genes and *WIF-1* (*Wnt inhibitory factor-1*) (*46*–*49*). Quantitative proteomic analysis following knockdown of *HOTAIR* in HeLa cells reveals differential expression of a large number of proteins (~170) involved in diverse cellular processes, including the dynamics of the cytoskeleton and mitochondrial structure and function (*50*). Overexpression of *HOTAIR* is a hallmark of many human cancers and is linked to aspects of carcinogenesis, including metastasis, epithelial-to-mesenchymal transition, invasion, aggression, and apoptosis (*46*). In mouse development, *Hotair* is expressed in limb buds and the posterior trunk, in an area corresponding to the future lumbosacral vertebra (*47*, *48*). Deletion of *Hotair* in mice leads to vertebral transformations and abnormal development of metacarpal and carpels, including deletion and/or fusion of digit elements. These phenotypes are attributed to anterior expansion of *Hoxd10* and *Hoxd11* in the trunk and ectopic expression of the imprinted gene *Dlk1*. In addition, there is a large-scale derepression of target genes, further confirming its role as part of a repressor complex (*48*).

It is challenging to establish orthologous relationships between vertebrate lincRNAs because, in light of their noncoding nature, they display varying degrees of sequence conservation. *HOTAIRM1*, *HoxA11as*, and *HOTAIR* display some level of sequence similarity in mammals (*51*). For example, exon 1 of *HOTAIRM1* is conserved and displays similar expression profiles across all mammals. *HOXA11AS* is highly conserved in eutherian mammals but shows less conservation in marsupials, suggesting that it arose after the eutherian-marsupial divide. In the case of *HOTAIR*, there are larger transcripts and different exon/intron organizations in human compared to other species. For example, the two exons of mouse *Hotair* match with exon 4 and exon 6 of the human transcript (*47*, *51*). Exon 4 is highly conserved in all mammals, whereas exon 6 is conserved in eutherian mammals, but in marsupials there is a reduced degree of conservation.

In addition to the lincRNAs, the human and mouse *Hox* clusters are embedded with multiple microRNAs, including *Mir196a/b*, *Mir10a/b*, *Mir615*, and *MIR3185*. With the exception of *MIR3185*, these microRNAs are conserved between mouse and human and seen in syntenic regions (Fig. 2). *Mir10* family members are positioned between the group 4 and 5 paralogous genes in the *HoxB* and *HoxD* clusters (Fig. 2). Their expression correlates with the adjacent *Hox* genes, including direction of transcription and response to RA and ethanol (*52*–*56*). As illustrated with *Mir10a* (Fig. 3B), this may reflect the role of shared regulatory elements in potentiating expression of nearby *Hox* genes, lincRNAs (*Hobbit1*), and *Mirs*. The *Mir10* family of microRNAs regulate *Hox* genes in both cis and trans (Fig. 1) (*56*–*59*), and major signaling pathways (Wnt, Fgfs, and Notch) are key non-*Hox* targets of the *Mir10* family (*60*). Because these signaling pathways are also targets of *Hox* genes, *Mir10* RNAs have feedforward regulatory inputs into *Hox* gene regulatory networks.

Three *Mir196* family members, *Mir196a*, *Mir196b*, and *Mir196a-2*, are present in the mouse and human *HoxA*, *HoxB*, and *HoxC* clusters (Fig. 2). These *Mir196* paralogs also regulate the expression of *Hox* genes in cis and trans and play important roles in the patterning of mid-thoracic skeletal element through modulating *Hox* genes and *Wnt* signaling (*61*). *Mir196* paralogs directly regulate the expression of *Rar*β, which, in turn, affects axial patterning (*62*). Transcriptome analyses suggest that *MIRs* in *Hox* clusters are functionally involved in the progression, metastasis, and prognosis of various diseases, including cancers. *MIR10b*, *MIR196a*, *MIR196b*, and *MIR615* are up-regulated in Huntington's disease (*63*), whereas *MIR196a*/b is up-regulated and *MIR10* is down-regulated in head and neck cancer (*64*). Current evidence implies that integration of *MIRs* in *Hox* clusters provides another layer of regulation for *Hox* genes and their targets, in fine-tuning developmental processes under *Hox* control.

From an evolutionary perspective, microRNAs provide insight into exploration conservation of noncoding transcripts in *Hox* clusters. The *Mir10* family is located near *Hox* paralogous group 4 in vertebrates and adjacent to *Antennapedia* in *Drosophila* (*65*, *66*). *Mir10*s appear to be the most ancient microRNAs because they are present in the common ancestor of eumetazoa (*67*, *68*). *Mir10* in *Nematostella vectensis* indicates origins predating the cnidarian-bilaterian split (*69*–*71*). *Mir10*s are not only seen in conserved syntenic regions but also display sequence conservation among bilaterians. The loss of *Mir10* family is linked to disintegration of anterior *Hox* genes as in the case of nematodes and tunicates (*72*). The presence of *Mir196* paralogs in jawless lamprey but not in nonvertebrate chordates suggests their origin at the base of vertebrate evolution before initial cluster duplication.

With respect to evolutionary conservation of lincRNAs, in a manner analogous to the vertebrate *Hox* clusters, large numbers of intergenic noncoding transcripts (*iabs*) arise from *Drosophila HOM-C* and exhibit spatial colinearity in expression and function (*7*, *13*–*15*). Their domains of expression are normally delimited by insulator elements, and if transcription proceeds through these elements, there is a loss of insulator function and associated segmental transformations (*73*–*76*). Sustained expression of these *iabs* also alters Polycomb-mediated repression and serves to maintain active chromatin states in the *bithorax* complex. Hence, they are linked with modulation of segment-specific expression of genes in the *bithorax* complex (*74*). In red flour beetle, *Tribolium castaneum*, the *Hox* cluster has three noncoding transcripts, one located in the first intron of *Utx/Tc-Ubx* and two positioned between *ptl/Tc-Antp* and *Utx/Tc-Ubx* (*77*). In hemichordates, in addition to the syntenically conserved position of *Mir10*, their single *Hox* cluster has two sense and antisense noncoding transcripts (*78*). Because some of the lincRNAs and *Mirs* in vertebrates show syntenic conservation in insects and hemichordates, noncoding transcripts within *Hox* clusters may be an ancestral feature with functional relevance. It will be interesting to see how intermingled the functional and regulatory relationships between *Hox* coding genes and lincRNAs are as we learn more about their roles in evolution, development, and disease. They may well be two sides of the same coin in *Hox*-associated gene regulatory networks.

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