POINT OF VIEW

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Epigenetic regulation of vertebrate *Hox* genes

A dynamic equilibrium

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Temporal and spatial control of *Hox* gene expression is essential for correct patterning of many animals. In both Drosophila and vertebrates, *Polycomb* and *Trithorax* group complexes control the maintenance of *Hox* gene expression in appropriate domains. In vertebrates, dynamic changes in chromatin modifications are also observed during the sequential activation of *Hox* genes in the embryo, suggesting that progressive epigenetic modifications could regulate collinear gene activation.

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Ever since the effect of *Polycomb* mutations were observed on Drosophila homeotic genes,[1] the regulation of *Hox* gene expression has been a model to study the establishment and maintenance of heritable transcriptional states during development. *Hox* genes encode transcription factors that are essential for patterning along the animal anterior to posterior (AP) body axis. In flies, HOX proteins control segmental identity and modifications in their distribution result in body transformations involving either the duplication, or the loss of body structures.[2] The analyses of such mutants has provided a basis for the identification of series of genes involved in epigenetic control of gene expression, in particular genes members of the *Polycomb* (PcG) and *Trithorax* (trxG) groups.[3]

Spatial Control of *Hox* Genes by Polycomb and Trithorax Complexes

In many organisms, *Hox* genes are organized in genomic clusters[4] and there is a correspondence, within the cluster, between the respective positions of genes and their domain of expression along the AP axis;[5] genes at one extremity of the cluster are activated in anterior parts of the developing embryo, whereas genes located at the opposite end are transcribed in more posterior areas, a phenomenon refereed to as spatial collinearity. In early Drosophila embryos, activator and repressor proteins encoded by gap and pair-rule genes bind cis-regulatory elements within the homeotic clusters and thus determine the spatial expression coordinate of each *Hox* gene along the AP axis.[6] In this system, *Hox* gene promoters interpret a previously organized AP polarity. As these factors are only transiently present, PcG and TrxG gene products maintain appropriate *Hox* expression domains during subsequent developmental stages.[6] While PcG proteins keep *Hox* genes repressed,[7] TrxG gene products are required to counteract PcG silencing and maintain *Hox* genes active wherever necessary.[8]

Biochemical studies have revealed that PcG and TrxG form multi-protein complexes, containing both histone methyltransferase activity as well as proteins binding methylated histone lysine residues.[6,9,10] The components of the Polycomb Repressive Complex 2 (PRC2) tri-methylate H3K27(me3)[11-14] and bind to the same histone mark[15,16] that is essential for inheritable long-term repression of target genes. Recruitment of PRC1 members to H3K27me3 further facilitates gene silencing via ubiquitination of H2A at K119,[17-19] a modification that interferes with RNA Polymerase II activity.[20-22]

On the other hand, TrxG/MLL protein complexes catalyze the trimethylation of H3K4,[23,24] which is generally associated with active transcription. Accordingly, H3K4me3 modified nucleosomes are found enriched at the promoters of active genes.[25-29] It was also recently shown that both TrxG and PcG complexes associate with histone demethylases (KDM).[30,31]

The presence of enzymes specifically removing methyl groups from histones explained the dynamics of changes in chromatin states, which can occur during cellular differentiation. Genome-wide studies in mouse and human embryonic stem (ES) or tissue culture cells have revealed a good correlation between the positive and negative transcriptional activities of promoters and the presence of either H3K4me3 or H3K27me3 marks, respectively.[28,29,32,33] Interestingly, in both pluripotent and terminally differentiated cells, many loci are modified by both H3K4 and H3K27 tri-methylations,[34-37] an unexpected pattern called 'bivalent domains.' Treatment of undifferentiated cells with retinoids, which results in the activation of target genes, induced the resolution of bivalent domains into active marks only at target promoters.[32,35] This rapid loss of repressive marks, which can hardly reflect a passive dilution of nucleosomes during replication, was associated with the recruitment of the UTX and JMJD3 histone demethylases to promoters during the transcriptional activation of *Hox* genes in cultured cells.[38-41]

Activation of gene transcription may thus rely upon both the deposition of active marks by the TrxG/MLL complex and the removal of repressive marks by H3K27me3/2 histone demethylases.[39,41,42] Conversely, the combined activities of PcG complexes and H3K4 or H3K36 demethylases may silence target genes.[43-45] Proteolytic cleavage of histone tails may also contribute to silencing, as a fraction of histone H3 looses a N-terminal fragment containing both K4 and K9, but leaving K27 on a shorter tail, during ES cell differentiation.[46]

Epigenetic Control of Vertebrate *Hox* Gene Temporal Expression

The function of bivalent domains remains elusive and this equilibrium between positive and negative marks may keep genes encoding transcription factors in a poised state, ready for rapid activation during cell differentiation.[34,35] Bivalent marks are mostly found at GC-rich promoters[47] where they may prevent DNA methylation and silencing, at least in stem cells.[48] Consistently, these domains have not yet been clearly observed in Drosophila, and the recent observation that 6.5 percent of H3K27me3 positive regions potentially overlap with H3K4me3 marks (versus 90 percent in mammalian cells[49,36]) may reflect cellular heterogeneity rather than the co-existence of both marks. However, the analysis of chromatin modifications in Drosophila syncytial blastoderm cells, a stage where DNA methylation may occur[50,51] remains to be done.

This early developmental stage corresponds to the concomitant deployment of *Hox* gene transcripts, in response to maternal and gap gene products, a strategy that evolved along with insects that develop their anterior and posterior aspects concomitantly.[52] In contrast, in those animals implementing a progressive developmental strategy, whereby segments are progressively added from anterior to posterior,[53,54] *Hox* genes ought to be activated in a time sequence, such as to delay the transcription of posterior genes until the tail end of the embryo is formed. This is achieved via 'temporal collinearity,' the property of *Hox* genes, in some species, to be activated in a time sequence that reflects their position in the gene cluster.[55]

To better understand the mechanisms underlying temporal collinearity, the distribution of H3K4me3 and H3K27me3 chromatin marks was recently examined in vivo during the sequential activation of *Hoxd* genes in developing tail buds.[56] A highly dynamic equilibrium was uncovered, involving both the removal of H3K27me3 marks, the methylation of H3K4 and the acetylation of H3, along with progressive gene activation. The region of transition between these two states of chromatin corresponds to the window wherein *Hoxd* genes become transcriptionally active, a window that shifts from one end of the cluster to the other during gastrulation (**Fig. 1**). In this view, the tri-methylated K27 are progressively de-methylated, along with the tri-methylation of K4, in a directional manner, with a kinetics of ca. 100 kilobases over less than two days.

An alternative possibility is that individual cells at the posterior end of the embryo acquire a fixed epigenetic identity, directly from a self-renewing pool of undifferentiated stem cells. Like ES cells, these stem cells would have their *Hox* clusters fully decorated by H3K27me3. At different times, daughter cells would readily inherited their final states of repressive versus active chromatin domains, without the need for dynamic modifications to occur within the same cells.

Even though this latter alternative better fits the idea of 'epigenetic memory', we do not consider it as plausible. Should a persistent population of axial stem cells indeed exist, carrying an ESC-like epigenome, this population would expectedly be rather large to rapidly generate an entire set of cells expressing a novel combination of HOX proteins at a given AP level. Consequently, one would expect to detect background chromatin marks associated with this stem population in dissected tail buds. Yet, not only bivalent domains were not observed in these samples, but H3K27me3 marks were completely removed from the anterior part of the cluster along with gene activation. We thus suggest that these marks are erased from all tail bud cells, rather than persisting in a large stem cell reservoir to be differentially removed, subsequently.

Conclusions and Perspectives

Temporal collinearity is tightly associated both with animals developing their AP polarity in a time sequence and with a fully clustered set of *Hox* genes.[4] It was proposed that an integral gene cluster is required for the progressive replacement of a repressive state by a transcription-competent state, from one extremity of the cluster to the other, coincident with transcriptional activation.[57] Our analysis of chromatin dynamics in mouse embryos where the *HoxD* cluster was split into two independent pieces argues against a simple interpretation of this proposal. It also shows that clustering is not necessary for the initial definition of the H3K27me3 and H3K4me3 landscapes,[56] ruling out a

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mere spreading mechanism for the distribution of these marks. However, the two sub-clusters displayed a premature appearance of H3K4me3 marks, suggesting both that gene neighborhood is required for an efficient coordination of chromatin modifications in time and that some of these modifications may precede transcription rather than being consequential.

This genetic system illustrates the various ways complex regulations have evolved along with the modifications of developmental strategies. Unlike in flies where homeotic genes respond to positional information (i.e., gap genes), vertebrates *Hox* genes encode positional information. A parsimonious solution to prevent posterior determinants to be transcribed early on (in supposedly anterior structures) evolved, whereby the gene cluster, originally 'closed for business,' is progressively opened, such as to make genes accessible to rather generic transcriptional activators. Future biochemical and genetic analyses will tell to what extent modifications in the status of chromatin are instrumental in this process.

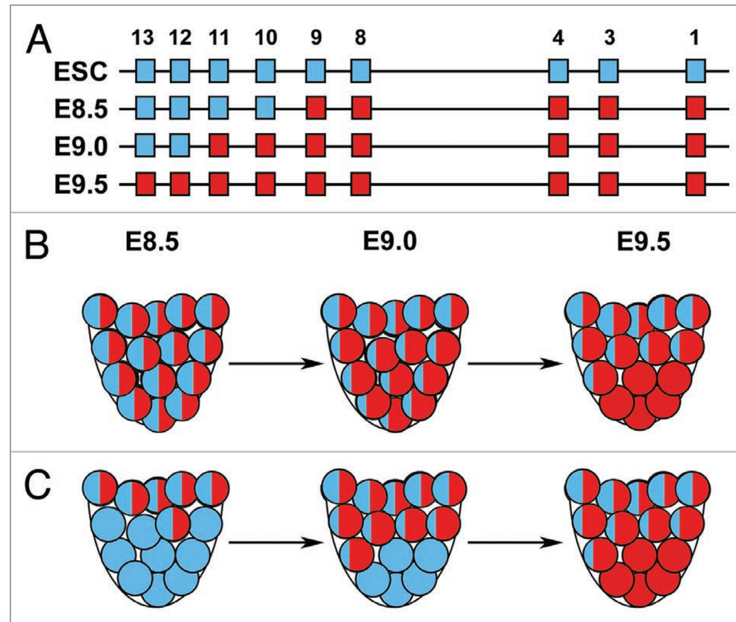

Figure 1. Two alternative possibilities can account for the observed transitions in chromatin marks over the *HoxD* gene cluster in vertebrate tail buds. (A) Schematic representation of the transcriptional activity within the mouse *HoxD* cluster at different time points. *Hoxd* genes are silent (blue boxes) in embryonic stem cells (ESC). Genes located in the telomeric part of the cluster, from *Hoxd1* to *Hoxd9*, are expressed (red boxes) in embryonic day 8.5 (E8.5). *Hoxd10* and *Hoxd11* are transcribed at E9.0. The transcriptional activity then spreads over *Hoxd12* and *Hoxd13* by E9.5. (B) Temporal progression of chromatin states in the *HoxD* cluster. Anterior is to the top. In HOX positive cells at E8.5, the telomeric half of the cluster is covered by H3K4me3 mark (red semi-circles), whereas the centromeric half of the cluster is decorated by repressive H3K27me3 mark (blue semi-circles). The most anterior cells will memorize this chromatin state. In more posterior cells H3K27me3 mark continues to retract towards the centromeric part of the cluster. Concomitantly, H3K4me3 progressively spread over the *Hoxd10* and *Hoxd11* loci, covering three quarters of the cluster at E9.0. In the most posterior cells the whole cluster contains H3K4me3 positive nucleosomes (red circles). (C) In the second model, a stem cell population, with *Hox* clusters entirely decorated by H3K27me3, constantly remains present at the posterior end of the embryo. At different times, cells are produced from this population with a different—but fixed—equilibrium between positive and negative marks.

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