

OUTLOOK

HOX13 proteins: the molecular switcher in *Hoxd* bimodal regulation

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The striking correlation between the genomic arrangement of *Hox* genes and their temporal and spatial pattern of expression during embryonic development has been a source of fascination since its discovery. This correspondence has been used as a privileged example in the investigation of the connection between genomic architecture and function. In this issue of *Genes & Development*, Beccari and colleagues (pp. 1172–1186) make a big step forward in understanding *Hox* gene regulation during limb development by showing the pivotal role of HOXA13 and HOXD13 proteins in the transition from a proximal to a distal type of *Hoxd* transcriptional regulation.

Hox genes encode transcription factors critical for the establishment of the basic body plan of bilaterian animals (Lewis 1978). Through the acquisition of new regulatory strategies, *Hox* genes were subsequently co-opted to pattern novel structures such as the appendages. In particular, genes of the *HoxA* and *HoxD* clusters are instrumental in the generation of the tetrapod limb morphology (Kmita et al. 2005).

During the past few years, a combination of transcriptional and epigenetic profiling, chromosomal architecture characterization, and transgenic reporter assays has revealed the presence of multiple long-range enhancers located within the two gene deserts flanking the *HoxD* cluster that control the transcription of *Hoxd* genes specifically in the limbs and other secondary structures (for review, see Noordermeer and Duboule 2013). Interestingly, these regulatory landscapes overlap with topologically associating domains (TADs), regions of the chromatin with a discrete three-dimensional architecture in which internal interactions are favored (Dixon et al. 2012; Andrey et al. 2013). During limb development, *Hoxd* genes show complex patterns of expression that evolve in two successive phases (Tarchini and Duboule 2006). The early phase occurs in the emerging limb bud and drives the expression of *Hoxd8* to *Hoxd11* under the tran-

scriptional regulation of enhancers in the telomeric TAD (T-DOM) (Fig. 1A; Andrey et al. 2013). The later phase occurs in the hand plate and drives the expression of *Hoxd11* to *Hoxd13* under the control of enhancers (including an archipelago of I–V islands) in the centromeric TAD (C-DOM) (Fig. 1B; Montavon et al. 2011). The early phase specifies the morphology of the arm and forearm, while the late phase specifies the morphology of the digits. Thus, normal limb development involves a switch from a T-DOM (proximal) to a C-DOM (distal) type of *Hoxd* regulation. It has been proposed that this switch generates a zone of low *Hox* expression, the presumptive wrist/ankle, that separates the two phases of *Hox* expression and reflects the modular pattern (proximal/distal) of the tetrapod limb (Andrey et al. 2013).

Here, Beccari et al. (2016) have investigated the molecular mechanisms controlling this important regulatory switch. Previous evidence showed that the segregation between the early and late *Hoxd* phases of expression was virtually absent in *Hoxa13;Hoxd13* mutants (for simplicity, referred to here as *Hox13* mutants) together with an abnormal expression of early-phase *Hoxd* genes in the distal digit domain (Sheth et al. 2014; Woltering et al. 2014). These observations suggested a role for HOX13 paralog proteins in the control of the bimodal *Hox* gene regulation that Beccari et al. (2016) embarked to determine.

Because HOX13 proteins are considered necessary to achieve a distal identity, the investigators started by evaluating the expected proximal transformation of the *Hox13* distal limb. Besides the approximation of the expression profile of distal mutant cells to that of proximal normal cells, the RNA sequencing (RNA-seq) analysis also revealed that the changes in the expression of *Hoxa* and *Hoxd* genes were stronger than in the total set of transcripts, further supporting the involvement of HOX13 proteins in *Hox* regulation.

To resolve which regulatory phase was altered in the mutants, the investigators took advantage of their

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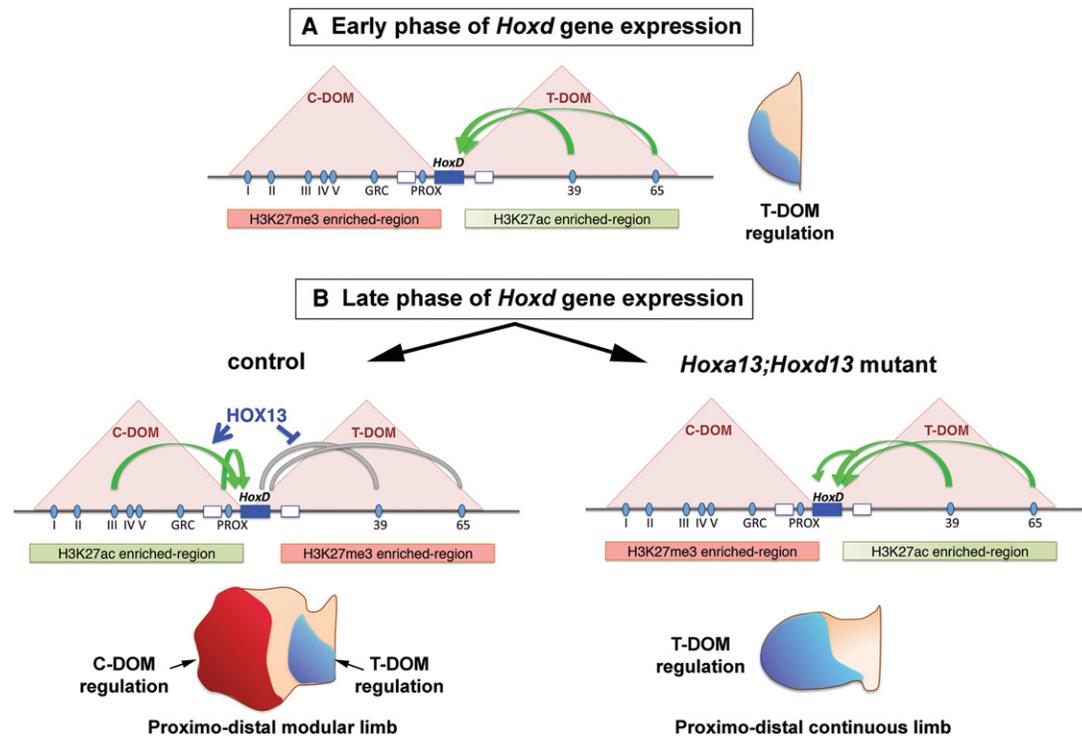


Figure 1. *Hoxd* gene regulation during limb development. (A) During the early phase, the T-DOM regulates the transcription of *Hoxd8* to *Hoxd11* in arm and forearm progenitor cells. (B, left) Subsequently, the action of HOX13 factors concomitantly terminates the T-DOM while activating the C-DOM, thereby permitting the segregation of the proximal and distal domains. (Right) In *Hoxa13;Hoxd13* mutants, the T-DOM regulation persists and abnormally affects late *Hoxd* expression. The blue ovals represent the main regulatory sequences in the C-DOM (Cs39 and Cs65) and T-DOM (islands I-V, CRG, and PROX).

previously generated modified alleles in which the C-DOM is either absent or not operative, and therefore the second phase of *Hoxd* expression is abrogated (Andrey et al. 2013). They showed that the additional removal of *Hoxa13* in the absence of C-DOM regulation rescued the expression of *Hoxd10* and *Hoxd13* in the digital plate. These results beautifully exposed the ability of the T-DOM to drive the transcription of second-phase genes such as *Hoxd13* into the digit domain as long as the HOXA13 proteins are not present (Fig. 1B). Furthermore, ChIP-seq (chromatin immunoprecipitation [ChIP] combined with high-throughput sequencing) analyses showed the abundant binding of HOXA13 within both the T-DOM and C-DOM in normal distal cells and the requirement of HOX13 binding for the H3K27 trimethylation of the T-DOM and the acetylation of the C-DOM. In *Hoxa13* distal limb cells, the T-DOM remained functionally active, while the C-DOM was never implemented, reflecting the persistence of a proximal-type regulation (Fig. 1B). Circularized chromosome conformation capture (4C) coupled with next-generation sequencing (4C-seq) additionally confirmed that the inactivity of the C-DOM as the interaction profile of *Hoxd13* and *Hoxd11* in distal mutant cells remained over the T-DOM. Most importantly, the analysis of intermediate allelic combinations showed that the impact of HOX13 proteins was dose-dependent.

Beccari et al. (2016) also noted that HOXA13 binding over the T-DOM did not localize on the main regulatory sequences (Cs39 and Cs65), raising the possibility that they could interfere with the global function of the T-DOM rather than directly repressing the enhancers. In support of this interpretation, the T-DOM enhancers randomly integrated in classical transgenic reporter assays elude the normal silencing that HOX13 factors would cause in distal cells.

Based on all of this evidence, Beccari et al. (2016) propose a model in which HOXA13 and HOXD13 act in a synergistic and dose-dependent manner to switch the regulatory landscapes by concomitantly repressing the T-DOM and activating the C-DOM (Fig. 1). The investigators also consider the potential evolutionary implications of their work. The repressive function of HOX13 proteins over the ancestral T-DOM (Andrey et al. 2013; Acemel et al. 2016) might be absent in fish, and its gaining may have been instrumental for the implementation of the bimodal *Hoxd* regulation.

The work by Beccari et al. (2016) lays the ground for further studies on the mechanisms underlying HOX13 function in *Hoxd* regulation. An intriguing major question is how HOX13 proteins can differentially regulate the activity in the two TADs, acting as repressors at the T-DOM and activators at the C-DOM. The actual mechanisms may involve interactions with other cofactors, the

identification of which seems a major challenge. Another point that remains to be clarified is how the switch between the telomeric and centromeric regulation leads to the down-regulation of *Hoxd* expression in the wrist/ankle precursors and whether the C-DOM regulation is ever activated in these progenitors.

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