Dissecting CTCF site function in a tense HoxD locus

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In this issue of *Genes & Development*, Amândio and colleagues (pp. 1490–1509) dissect the function of a cluster of several CTCF binding sites in the HoxD cluster by iterative deletions in mice. They found additive functions for some, and intriguingly found that some sites function as insulators, while others function as anchors for enhancer–promoter interactions. These functions vary depending on developmental context. The work provides new insights into the roles played by CTCF in regulating developmental patterns and 3D chromatin organization.

The function or importance of the three-dimensional organization of the genome is considered an important regulatory mechanism in gene regulation. The most compelling insights into this have been genomic rearrangements that clearly change the 3D architecture of the genome and alter human and mouse development (Spielmann et al. 2018). Many studies have shown that CTCF is important for the insulation of topologically associated domains (TADs), regions of the genome that tend to self-interact, and for enhancer–promoter interactions (Merkenschlager and Nora 2016). However a systematic dissection of CTCF motifs and their impact in vivo has been poorly explored until now. In this issues of *Genes & Development*, Amândio et al. (2021) delineate the potential importance of a cluster of CTCF sites in organizing 3D chromatin in the HoxD locus.

In their work, Amândio et al. (2021) explore the very well-studied HoxD cluster. This group of genes is an exemplar of a developmentally regulated genomic locus. Conserved from *Drosophila* to vertebrates, Hox genes are expressed in developmental sequences that are referred to as colinearity, where the first Hox gene is expressed most posteriorly in the embryo, and the remainder of the genes are expressed more and more anteriorly (Kmita and Duboule 2003). The regulation of the Hox clusters has been well defined, and in particular considerable knowledge exists for the HoxD cluster (Gentile and Kmita 2018). The HoxD cluster resides genomically at the interface of two TADs, which are populated by enhancers for expression of the HoxD genes in embryonic structures such as the trunk, genitals, or proximal and distal limb elements. The physical interactions of the HoxD genes and their regulatory elements have been well defined, but the basis of these 3D chromatin interactions are not well understood.

The HoxD cluster has a very particular arrangement of CTCF sites that correspond with the location of HoxD genes within adjacent telomeric or centromeric TADs. Five CTCF sites are located near one another, all with the same orientation. The sites are conserved across vertebrate evolution, and have enrichment of the cohesion complex subunit Rad21.

The investigators deploy extensive mouse genomic manipulation to create additive mutations of one to five deletions of CTCF sites. The effort and time underlying this experimental design should not be underestimated! Furthermore, their readout is in vivo gene expression analysis, phenotypic output, and 3D chromatin organization using spatial and quantitative measurements. The microdissection of the embryonic trunk and distal and proximal limb buds was key to determining specific functions of the CTCF binding sites in distinct regions of the embryo, greatly enriching the conclusions. The impact of studying this in the context of developing mice brings considerable importance to the interpretation and significance of the results.

One clue that not all CTCF sites are the same is that most deleted sites also lost Rad21 occupancy, but one retained a significant Rad21 ChIP signal, indicating a CTCF-independent mechanism for Rad21 enrichment at this particular site. Most intriguing is that despite the same orientation and close proximity, some sites function as insulators, while others function as promoters of enhancer contact. For example, the expression of some HoxD genes

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were increased in embryonic structures upon deletion of the first three sites, but this was reverted when the next two sites were additionally deleted. In addition whether the sites function as insulators or anchors depends on the developing embryonic structure. The regulation in the limbs is very different than in the facial vibrissae or trunk, for example. This complexity clearly shows that CTCF binding sites do not have a monotonic function, but it is likely that local sequence contexts as well as broader cellular states influence them deeply. It is certainly clear that the function of CTCF sites within the HoxD cluster cannot be strictly an insulator or boundary effect.

Several interesting observations from Hi-C data in the mutants hint at the function of clustered CTCF sites. One proposed by the investigators is that there exists a "tension" between local Hox cluster interactions and distal TAD loci. They reached this conclusion by the observation that deleting several CTCF sites increases local interaction frequencies while reducing distal ones. Furthermore, the investigators' data point to the telomeric and centromeric TADs flanking the HoxD locus engaging with one another more frequently, as if they were being dragged toward one another, as the investigators put it. It would have been informative to investigate this arrangement by DNA fluorescent in situ hybridization to determine the actual spatial rearrangement of the TADs.

How important are these CTCF sites and their interactions for the developing embryo? By examining quantitative gene expression in developing embryonic structures, the investigators measured very small changes in expression of some of the HoxD genes. More importantly, by in situ hybridization, they observed quantitative and spatial changes in expression. These are consistent with loss or reassignment of HoxD promoters to their various distal enhancers. Nonetheless, most of these changes are very modest and are accompanied by slight or undetectable changes in morphology of the developing structures. For example, patterning of the axial skeleton was altered as one would expect from disruption of some HoxD genes, while limb development was unaffected.

Other recent work has shown roles for CTCF sites at other loci (Paliou et al. 2019; Anania et al. 2021). Unlike the varying functions at the HoxD locus, combined deletions at the *Pax3* locus indicates an additive insulation function only (Anania et al. 2021). In contrast, deletion of CTCF sites at the *Shh* locus reduces interaction between the ZRS distal enhancer and *Shh*, and this study did not detect site-specific functions (Paliou et al. 2019). Thus, the work presented in Amândio et al. (2021) lays out a finely regulated and complex function of an array of CTCF sites. The data reveal surprising conclusions about how distinct these seemingly similar and closely spaced CTCF sites can be. Many questions related to the specific function of each site remains, and examining other such arrays of CTCF sites and their context will be revealing.

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