

Response to Peron *et al.*

To the Editor: We thank Peron *et al.*¹ for their interest and appreciation of our work² and for their contribution to expanding the current limb copy-number-variation morbidity map. The authors describe a fetus with shortening and bowing of radius and ulna (mesomelic dysplasia). Using array comparative genomic hybridization the authors identified two de novo deletions flanking the 5' and 3' regions of the *HOXD* cluster (**Supplementary Figure S1** online). The 5' centromeric 998-kb deletion spans the region upstream of the 5' *HOXD* genes including the genes *ATF*, *ATP5G3*, and *KIAA1715* as well as the regulatory *HOXD* archipelago, a cluster of well-characterized limb enhancer elements (I-V, GCR, and Prox).³ The telomeric deletion is located downstream 3' of the *HOXD* cluster (153 kb including *MTX2*). The authors conclude that the latter telomeric deletion is likely the cause of the phenotype via the loss of *cis*-regulatory elements controlling early 3' *HOXD* genes in the proximal limb resulting in forelimb-specific mesomelic dysplasia. While the larger deletion removes all known 5' *HOXD* enhancers, we point out that the telomeric deletion does not include any of the previously characterized *HOXD* limb enhancers. Therefore, alternative explanations other than regulatory loss of function need to be considered.

Extensive mouse studies have revealed that during limb development, a subset of genes belonging to the *HoxD* cluster (*Hoxd8*–*Hoxd13*) are transcribed in two waves that determine the proximal–distal axis of the limb. The early wave is regulated by the forelimb enhancers CNS39 and CNS65 positioned in the telomeric topologically associating domain (TAD) activating *Hoxd8*–*Hoxd11* transcription that patterns radius/tibia and ulna/fibula (**Supplementary Figure S1**).³ The succeeding wave is regulated by enhancers located in the centromeric TAD that activate *Hoxd9*–*Hoxd13* expression to form the digits.⁴ Due to its strong chromatin interaction with the centromeric TAD, *Hoxd13* is only very weakly contacted by the telomeric enhancers, leading to its absence of transcription in the developing radius/ulna region.

Several structural variants (SVs) at the locus in both mice and humans have been shown to disrupt this regulatory topography of the *HOXD* cluster causing limb malformation. The mouse mutant *Ulnaless* is caused by a 770-kb inversion of the *HoxD* cluster bringing *Hoxd13* into the neighborhood of the telomeric enhancer elements and hijacking their regulatory potential. This results in the premature and ectopic onset of *Hoxd13* transcription in proximal limb, where *Hoxd13* is normally not expressed. This misexpression negatively interferes with the proliferation of mesenchymal cells in the zeugopod, which results in truncation of radius/tibia and

ulna/fibula, with the forelimb being more severely affected. Tandem duplications of the *HOXD* cluster in humans cause Kantaputra mesomelic dysplasia, a severe mesomelic shortening of the upper limbs. These duplications place *HOXD13* into the telomeric TAD 3' regulatory landscape, thereby inducing misexpression of *HOXD13* in the proximal limb, similar to the *Ulnaless* inversion.

Based on the specific upper-limb phenotype and the location of the deletions, we strongly suspect that the limb malformation reported by Peron *et al.* is not a result of a loss of function of anterior 3' *HOXD* genes, because the 153-kb deletion in the telomeric TAD does not include any known enhancer elements. Also, chromatin analyses revealed that the critical region for proximal limb enhancers in the telomeric TAD is not included into the reported deletion.³ Finally, the function of *HOXD* genes in developing limbs is largely redundant with that of *HOXA* genes and even a homozygous deletion of the entire murine *HoxD* cluster did not elicit any substantial mesomelic phenotype. Instead, we consider it more likely that the phenotype results from a gain of function of *HOXD13* in proximal cells, as already reported in various cases in the mouse. Several scenarios are possible to account for this gain of function (**Supplementary Figure S1**).

First, the deletion of the centromeric regulatory landscape might allow ectopic interactions to be established between *HOXD13* and the CNS39 and CNS65 enhancers located in the telomeric TAD. This particular mechanism of contact reallocation was previously observed in a mouse mutant where the centromeric regulatory landscape was displaced several Mb away from the *HoxD* cluster via a targeted inversion. Proximal limb bud cells showed ectopic expression of *Hoxd13* and chromatin interactions between *Hoxd13* and the telomeric landscape were increased significantly. These mice displayed a mild mesomelic dysplasia.³

Second, the presence of two deletions flanking both sides of the *HOXD* cluster might illustrate that the region between the two deletions containing the *HOXD* cluster itself is inverted in the affected individual, a condition that would remain unnoticed with the technology used by Peron *et al.* Such an inversion would place *HOXD13* on the side of the telomeric regulatory elements resulting in proximal enhancer adoption and misexpression of *HOXD13* with consequent development of severe mesomelic dysplasia. This situation would be similar to the *Ulnaless* allele in mouse and the Kantaputra duplication. This scenario is only possible if the deletions are located in *cis* on the same chromosome. Only quantitative reverse-transcription polymerase chain reaction in combination with Sanger sequencing of the breakpoints will reveal the exact nature of this complex SV. Third, another possibility is that the centromeric deletion removes a boundary element at its centromeric end resulting in a fusion of the neighboring TAD

with the 5' *HOXD* genes leading to *HOXD13* misexpression in the zeugopod.

In conclusion, SVs at the *HOXD* locus, including the case reported by Peron et al., demonstrate how complex rearrangements can reshuffle noncoding regulatory landscapes of developmental genes causing disease. A major shortcoming of our study and the report by Peron et al. is that array comparative genomic hybridization does not provide positional information nor does it detect inversions, smaller deletions, and duplications (< 20 kb). However, as highlighted above, mapping of the exact breakpoints is essential to determine the true pathogenic potential of complex SVs. Whole-genome sequencing promises to be a more appropriate method to detect the full spectrum of SVs at single-nucleotide resolution, which will help to predict their effect on gene regulation. Moreover, in vivo mouse models using CRISPR-Cas9 system provide an excellent tool to understand the effect of chromosomal rearrangements in the genomic and chromatin regulatory context.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

DISCLOSURE

The authors declare no conflict of interest.

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