

## ARTICLE

# Mesomelic dysplasia Kantaputra type is associated with duplications of the *HOXD* locus on chromosome 2q

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Mesomelic dysplasia Kantaputra type (MDK) is characterized by marked mesomelic shortening of the upper and lower limbs originally described in a Thai family. To identify the cause of MDK, we performed array CGH and identified two microduplications on chromosome 2 (2q31.1–q31.2) encompassing ~481 and 507 kb, separated by a segment of normal copy number. The more centromeric duplication encompasses the entire *HOXD* cluster, as well as the neighboring genes *EVX2* and *MTX2*. The breakpoints of the duplication localize to the same region as the previously identified inversion of the mouse mutant *ulnaless* (*Ul*), which has a similar phenotype as MDK. We propose that MDK is caused by duplications that modify the topography of the locus and as such result in deregulation of *HOXD* gene expression.

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**Keywords:** *HOXD*; MDK; microduplication

## INTRODUCTION

Mesomelic dysplasia Kantaputra type (MDK, MIM 156 232) is a rare dominantly inherited skeletal disease characterized by symmetric marked shortening of the upper and lower limbs.<sup>1,2</sup> The ulnae are very short, and the radii are bowed. The distal humerus has a dumbbell shape, the hands are relatively normal but show progressive flexion contractures of the proximal interphalangeal joints. Carpal and tarsal synostoses are observed in some individuals. In the lower limbs, feet are fixed in plantar flexion with sole facing backward, causing 'ballerina-like standing'. The prominent distal fibula on the ventral aspect is a consistent feature and considered as the 'signature' finding of the syndrome. Calcaneus is small or missing. Small fibula and talus, and fibulo-calcaneal synostosis are characteristic features. Tibial bony knot articulates with the proximal end of the fibula. Since the original description, several other cases have been described that show similar changes.<sup>3,4</sup> In an Italian family with a mesomelic dysplasia, a balanced translocation t(2;8)(q31;p21) had previously been observed.<sup>5,6</sup> On the basis of the phenotypic similarities with MDK, chromosomes 2 and 8 were tested as candidate regions and a region on chromosome 2 (2q24–q32) spanning about 22.7 cM was identified to harbor the *MDK* gene.<sup>7</sup> Interestingly, this interval contains the *HOXD* cluster. However, sequencing of individual genes of the *HOXD* cluster revealed no mutation associated with MDK. We here describe genomic rearrangements including the entire *HOXD* cluster identified in the Thai family originally described by Kantaputra *et al*<sup>1,2</sup> as the likely cause for MDK.

## MATERIALS AND METHODS

Genomic DNA samples were obtained from EDTA blood. DNA of the nine living affected individuals and one unaffected individual (IV.23) from pedigree of the publication of Kantaputra *et al*<sup>2</sup> were available for genomic analysis by

microarray-based comparative genomic hybridization (array CGH) and quantitative real-time PCR (qPCR). In addition, we obtained DNA samples of the MDK-affected individual II.1 from the publication of Shears *et al*<sup>3</sup> and II.1 from the publication of Kwee *et al*.<sup>4</sup>

## Microarray-based comparative genomic hybridization

DNA of the affected individual III.26,<sup>2</sup> II.1<sup>3</sup> and II.1<sup>4</sup> were available for array-CGH analysis. Array CGH was performed using a whole-genome oligonucleotide array (244K, Agilent Technologies, Santa Clara, CA, USA). 244K image data were analyzed using Feature Extraction 9.5.3.1 and CGH Analytics 3.4.40 software (Agilent Technologies) with the following analysis settings: aberration algorithm ADM-2; threshold: 6.0; window size: 0.2 Mb; filter: 5probes, log2ratio=0.29. The hybridization on a custom tiling array (AMADID no. 023120; Agilent Technologies), which covers 3 Mb of the critical region at high density allowed a more precise determination of the duplication size and breakpoint positions and was performed exclusively with the Thai family member III.26.<sup>2</sup> All genomic positions given in the text are according to human genome version hg18 (UCSC Genome browser, Mar. 2006; <http://www.genome.ucsc.edu/>).

## Quantitative real-time PCR

Confirmation of the array-CGH result of individual III.26<sup>2</sup> and analysis of further nine members (eight affected, one unaffected) of the Thai MDK family<sup>2</sup> to show the segregation was carried out by qPCR. Amplicons P1 and P3 are flanking the centromeric duplication and amplicons P4 and P6 are flanking the telomeric duplication. Amplicons P2 and P5 are located within the centromeric and telomeric duplication, respectively. Primer sequences can be obtained on request.

qPCR was performed on ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in a total volume of 12  $\mu$ l in each well containing 6  $\mu$ l of SYBR-Green PCR Master Mix (ABI SYBR Green PCR Master Mix, Applied Biosystems), 10 ng of genomic DNA (10  $\mu$ l) and 1  $\mu$ l

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primers (0.2  $\mu$ mol each). Samples were run in triplicates in separate tubes to permit the quantification of the target sequences normalized to albumin (ALB). PCR conditions were according to manufacturer's protocol: initial denaturation step at 95°C for 8 min followed by 40 cycles with denaturation at 95°C for 15 s and a combined annealing/elongation step at 60°C for 1 min. Using calibrator samples of normal control genomic DNA, the gene copy number was estimated based on the ddCt method. In addition, we performed an identification of the individuals' genders by calculating the coagulation factor VIII (F8, Xq28) relative to the two-copy-control ALB to assure its reliability.

### Fluorescence *in situ* hybridization

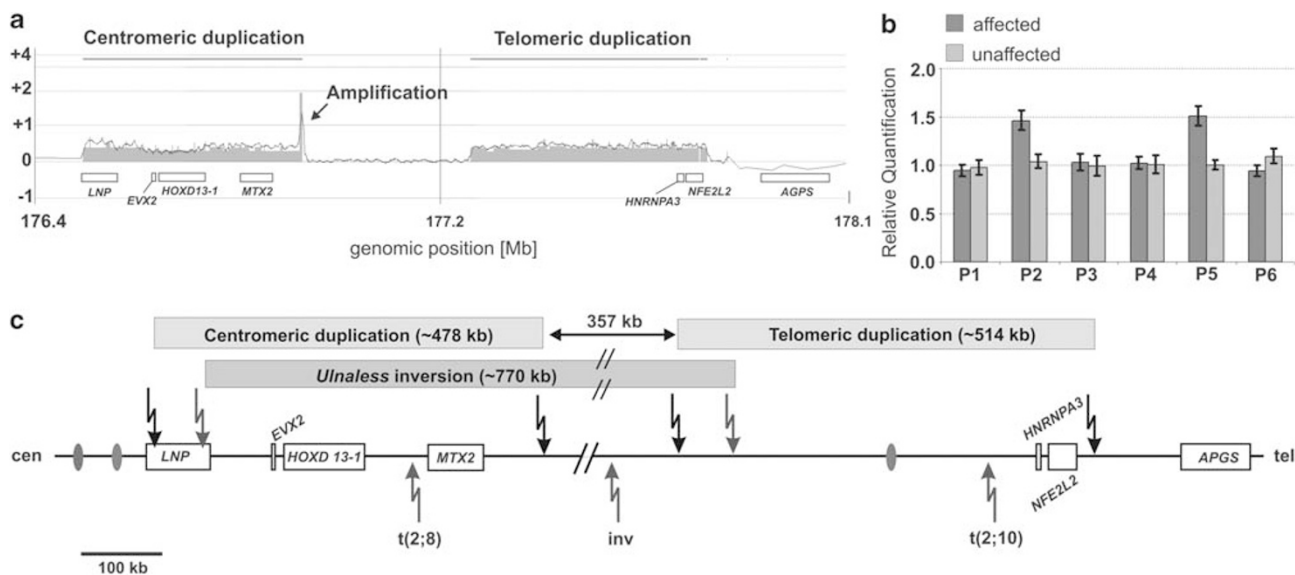
BAC clones RP4-745K6 and RP11-279N12 (obtained from the imaGenes, Berlin, Germany) were fluorescently labeled using nick translation, and hybridized to metaphase spreads of the individual III.11's lymphocytes using standard procedures. BAC clones were labeled with Spectrum Orange (RP4-745K6, located in centromeric duplication; Vysis Inc., Downers Grove, IL, USA) and Spectrum Green (RP11-279N12, located in telomeric duplication; Vysis Inc.). Chromosomes were counterstained with DAPI. Image analysis was performed using the ISIS analysis system (Metasystems, Altlußheim, Germany).

### RESULTS

As submicroscopic aberrations are a known cause for congenital limb malformations,<sup>8–10</sup> we performed array CGH using a whole-genome oligonucleotide array (244K, Agilent Technologies) of one affected individual (III. 26) from the MDK family initially described by Kantaputra.<sup>2</sup> Using this technology, we identified two microduplications on chromosome 2 (2q31.1-q31.2) encompassing

~481 and 507 kb, respectively, separated by a segment of normal copy number. The centromeric duplication contained the entire *HOXD* locus plus *EVX2* and *MTX2*, whereas the telomeric duplication contained the genes *NEF2L2* and *HNRNPA3*. To determine the duplication sizes and the breakpoints in more detail, we designed a custom tiling array of the entire region of ~3 Mb. This technology confirmed both duplications in the affected MDK family member III.26<sup>2</sup> and positioned them at 176 506 567–176 980 136 (centromeric duplication) and 177 342 561–177 854 004 (telomeric duplication), respectively (Figure 1a). A cryptic translocation or insertion of the duplicated regions into another chromosome was excluded by fluorescence *in situ* hybridization analysis (data not shown).

Using qPCR analyses, we were able to confirm the duplications and further narrow down their boundaries: (1) centromeric duplication, proximal breakpoint between 176 506 582 and 176 507 065 and distal breakpoint between 176 985 080 and 176 986 597; (2) telomeric duplication, proximal breakpoint between 177 342 108 and 177 342 602 and distal breakpoint between 177 856 721 and 177 857 706 (data not shown). The proximal breakpoint of the centromeric duplication was thus located within the gene *KIAA1715* and the distal breakpoint ~221 kb distal of *MTX2*. The telomeric duplication started ~357.5 kb away from the centromeric duplication and ended in the noncoding region between the genes *NFE2L2* and *APGS*. In addition, we identified a small amplification at the distal end of the centromeric duplication (chr2:176 973 781–176 980 910) (Figure 1a). However, this region overlaps with a benign copy-number variation



**Figure 1** (a) Profile of custom tiling array-CGH analysis, which covers 3 Mb of the critical region on chromosome 2q at high density demonstrating two duplications separated by a segment of normal copy number in individual III.26.<sup>2</sup> The centromeric duplication encompasses the *HOXD* cluster. A small amplification at the distal end of the centromeric duplication is indicated. Regions with a log<sub>2</sub> ratio >0.3 represent duplications and are shaded in gray. Genomic positions are according to human genome version hg18. (b) Microduplications and segregation confirmed by qPCR. For one affected (individual III.16;<sup>2</sup> dark gray bars) and one unaffected (individual IV.23;<sup>2</sup> light gray bars) individual, mean values and SDs (error bars) for each target amplicon relative to Albumin as a two-copy reference gene were calculated. The amplicons P2 and P4 located within the centromeric and telomeric duplicated region, respectively, show a ratio of ~1.5 in the affected individual indicating a copy-number gain, whereas the unaffected individual shows a ratio of ~1.0, which corresponds to a normal copy number (two copies). The unaffected individual is the daughter of the affected individual III.26 with whom we performed the array CGH shown in (a). (c) Schematic illustration of aberrations at the *HOXD* cluster and flanking region on chromosome 2q associated with limb malformations. The two duplications detected in the MDK family (gray bars), as well as the inversion in the mouse mutant *ulnaless* (green bar) are indicated. The centromere (cen) is on the left, and telomere (tel) on the right. The breakpoints are indicated by arrows in the corresponding colors. Red arrows below indicate published translocations and the inversion (inv).<sup>6,17</sup> The translocation t(2;8) and the inversion result in mesomelic shortening. Regulatory elements are indicated by colored ovals (blue: global control region (GCR); red: centromeric repressor (CenR); green: early limb control region (ELCR)). Genomic positions are according to human genome version hg18. The color reproduction of the figure is available on the html full text version of the paper.

(chr2:176 973 862–176 980 178) described by Kim *et al*<sup>11</sup> and it is therefore most likely not clinically relevant. Segregation of the duplications with the MDK phenotype was confirmed by qPCR analysis of further nine family members from the Thai family (eight affected, one unaffected). Amplicons P2 and P5, which localize within the duplications, detected a normal copy number in the unaffected individual IV.23 (Figure 1b). The unaffected individual is the daughter of the affected female III.26 tested by array CGH. To investigate other nonrelated individuals with MDK, we tested two previously published cases by array CGH.<sup>3,4</sup> We were not able to identify a clinically relevant copy-number change, not within the region on chromosome 2q, nor elsewhere in the genome, indicating that MDK is a genetically heterogeneous condition.

## DISCUSSION

Homeobox genes, and the proteins they encode, the homeodomain proteins, have important roles in the developmental processes of many multicellular organisms. In mammals, four such clusters exist that harbor a total of 49 genes. During development of the limbs, *Hox* genes of the A and D clusters, and in particular those of the 5' end, that is, *Hoxa10*, *-a11*, *-a13*, *Hoxd9*, *-d10*, *-d11*, *-d12*, *-d13*, are of particular importance.<sup>12</sup> Overall they appear to systematically involve loss or reduction of skeletal elements, and their function has accordingly been interpreted to specify individual segments of the limb in a proximo-distal manner.

In humans, mutations in *HOX* genes results in a number of limb malformations including synpolydactyly (*HOXD13*), and hand-foot-genital syndrome (*HOXA13*).<sup>13</sup> Larger deletions of the entire *HOXD* cluster and a smaller deletion removing just the *HOXD9–13* and *EVX2* genes were reported to result in mild limb malformations, including fifth-finger clinodactyly, variable cutaneous syndactyly of the toes, hypoplastic middle phalanges of the feet and synpolydactyly.<sup>14,15</sup> This phenotype is similar to the mild synpolydactyly phenotype commonly found in carriers of specific *HOXD13* mutations, indicating that even deletions of the entire *HOXD* cluster need not to produce major limb defects.<sup>13</sup>

Several chromosomal translocations located within the vicinity of the *HOXD* cluster have been described that result in severe phenotypes (Figure 1). Two rearrangement breakpoints located on the telomeric side of the *HOXD* cluster were described to result in proximal malformations of the limbs.<sup>16</sup> In one individual, a symmetrical limb phenotype consisting of ulnar aplasia, radial shortening, and absence of third to fifth rays was described in association with the translocation t(2;10)(q31.1;q23.33). The breakpoint was localized ~950 kb away from the 3' end of the *Hoxd* cluster. In the other case, a paracentric inversion (inv(2)(p15q31)) was associated with hypoplastic ulnae, radii and fibulae and small tibiae. This phenotype is somewhat reminiscent of the changes seen in MDK. The breakpoint in this case was closer to the cluster with a distance of ~500 kb from *Hoxd1*.<sup>16</sup>

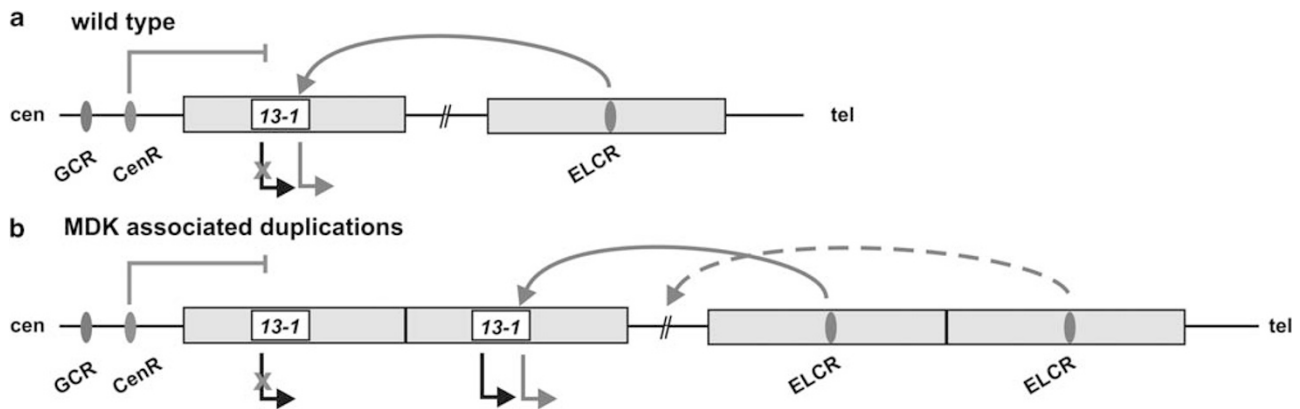
Furthermore, a refinement of the translocation t(2;8) of the patient with mesomelic shortening originally described by Ventruto *et al*<sup>5</sup> located this breakpoint 56 kb away from the 3' end of the *Hoxd* cluster<sup>6,17</sup> (for overview see Figure 1c).

The above-described breakpoints localize within or close to the duplications described here. Several other genes besides the *HOXD* cluster are present in this region that are potential candidates for MDK. The centromeric duplication contains parts of *KIAA1715*, *EVX2* and *MTX2*. *KIAA1715*, also known as *Lunapark* (*LNP*), is expressed in the developing digits but does not seem to have an important function during digit development.<sup>18</sup> *EVX2*, located next to

*Hoxd13*, is also expressed in the digits. Inactivation of *Evx2* in the mouse results in weak skeletal alterations of the digits.<sup>19</sup> An additional copy of *EVX2* may thus have an effect on limb development, but the main effect of *Evx2* appears to be in the autopod, the region of the limb that shows little changes in MDK. *MTX2* (MIM 608555) encodes for Metataxin, a protein that localizes to the outer mitochondrial membrane. It is not expressed in the developing limb<sup>17</sup> and as such unlikely to contribute to the phenotype. The telomeric duplication neighbors the *AGPS* gene, a member of the FAD-binding oxidoreductase/transferase type 4 family involved in long-chain fatty acid metabolism. Mutations in this gene have been associated with rhizomelic chondrodysplasia punctata, type 3 (MIM 600121) and Zellweger syndrome (MIM 214100). Located within the duplicated region are *HNRNPA3* and *NFE2L2* (MIM 600492). The latter encodes a transcription activator that is important for the coordinated upregulation of genes in response to oxidative stress. *HNRNPA3* has a role in cytoplasmic trafficking of RNA and may be involved in pre-mRNA splicing. In summary, it is unlikely that either of these genes is involved in the pathogenesis of MDK.

Several lines of evidence suggest, however, that *HOXD* genes are the most likely candidates for the MDK phenotype. *Hox* genes show specific proximo-distal expression patterns that specify the future skeletal elements. *Hoxd9* and *-d10*, for example, are expressed in the stylopod, the future humerus, *Hoxd11* and *Hoxa11* in the zeugopod, the future radius/ulna, and *Hoxd13*, *Hoxd12* and *Hoxa13* in distal limb (autopod). Inactivation of paralogous genes results in severe shortening of the corresponding elements. A knockout of *Hoxd11* and *Hoxa11*, for example, results in a severe reduction deficiency of radius and ulna, similar to the MDK phenotype.<sup>20,21</sup> MDK-like malformations are also present in the mouse mutant *ulnaless* (*Ul*), which is a semidominant X-ray-induced mutant with very short zeugopods with an almost complete absence of the ulna. Gene expression studies have shown that *Ul* embryos show an ectopic expression of *Hoxd13* and *Hoxd12* in the proximal limb (zeugopod) and a reduction of *Hoxd13*, *Hoxd12* and *Hoxd11* in the autopod.<sup>22,23</sup> The skeletal reductions of the *Ul* mutant were interpreted as a consequence of posterior prevalence, whereby proximal misexpression of *Hoxd13* and *Hoxd12* results in the transcriptional and/or functional inactivation of the more anterior, that is, *Hox* group 11 genes. The hypothesis of misregulated *Hoxd13* expression was supported by the finding that *Ul* was caused by a balanced paracentric inversion of ~770 kb in size, with one breakpoint centromeric to the *Hoxd* cluster disrupting the *Lnp* gene, and the other breakpoint telomeric to the *Hoxd* cluster<sup>18</sup> (Figure 1b). The inversion included *Evx2*, the *Hoxd* locus and *Mtx2*, and is thus very similar to the centromeric duplication identified in the MDK family.

The pathogenesis of *Ul* was further clarified by an elegant series of experiments that led to the identification of a *Hoxd* control region located at the 5' (centromeric) side ~200 kb away from the 5' end of the *Hoxd* cluster.<sup>18</sup> This so-called global control region (GCR, blue oval, Figures 1c and 2) positively influences the expression of *Evx2* and other 5' *Hoxd* genes expressed in the distal limb and suppresses their expression in the proximal limb. Recently, Tarchini and Duboule<sup>25</sup> specified an additional regulatory element centromeric to the *HOXD* cluster, which controls the posterior restriction of 5' *Hoxd* genes (CenR, red oval, Figures 1c and 2). These authors claimed that the GCR preferentially controls the expression in the presumptive digit domain. In the case of the *Ul* inversion, the centromeric breakpoint is located between the GCR, the additional centromeric regulator, and the cluster thus separating the cluster from the regulatory influence of



**Figure 2** (a) Regulation of *HOXD* cluster during early limb development. A complex time-dependent mechanism involving two control elements on the centromeric and telomeric side of the *HOXD* cluster controls the expression of *HOXD* genes in the early limb bud. One regulatory element centromeric of the *HOXD* cluster (CenR, red oval) represses the expression of 5' *HOXD* genes (*HOXD10–13*) indicated by the red bar.<sup>25</sup> The transcriptional activation of the 3' *HOXD* genes (*HOXD1–9*) is simultaneously induced by the early limb control region (ELCR, green oval), which is located on the telomeric side of the *HOXD* cluster indicated by the green arrow<sup>24</sup> (Tarchini and Duboule, 2006). Small green arrow marks active genes. The centromere (cen) is on the left, and telomere (tel) on the right. GCR, global control region; CenR, centromeric repressor. (b) Hypothesis on disturbance of *HOXD* cluster regulation in MDK. The two duplications detected in the MDK family (gray bars) are arranged in direct tandem orientation and result in a duplication of the *HOXD* cluster (centromeric dup) and of the ELCR (telomeric dup). As the ELCR is promoter independent, the onset of transcription depends on the distance between gene and ELCR. The duplicated *HOXD* cluster can be activated by the ELCR (small arrows); however, the 5' *HOXD* genes (*HOXD10–13*) escape the repression by the centromeric regulator CenR and thus the posterior restriction. As a consequence, *HOXD13* is expressed too early and ectopically leading to the MDK phenotype. The later *HOXD* expression in the digit domain controlled by the GCR is unaffected by the genomic duplications. The color reproduction of the figure is available on the html full text version of the paper.

the GCR and the additional centromeric regulator. The authors suggested that the disconnection of 5' *Hoxd* genes from the regulator results in a downregulation of 5' *Hoxd* genes in the distal limb (autopod) and an upregulation in the proximal limb.<sup>25</sup> The duplication described here may have a similar effect. Similar to the *U1* inversion, the duplicated cluster would be located more than 500 kb away from the GCR and the additional centromeric regulator thus escaping its control (Figure 2b).

The presence of two breakpoints at the telomeric side of the cluster that result in an MDK-like phenotype suggests an alternative explanation in which the responsible regulator is located 3' of *HOXD1*. Indeed, Zakany *et al*<sup>24</sup> propose the presence of an early limb control region (ELCR, green oval, Figures 1c and 2) localized telomeric to the cluster, which is needed to implement colinear expression of the *HOXD* cluster. The exact position of the ELCR is not known. One of the duplications detected in the Thai family is expected to disconnect the 5'-located cluster from the 3'-located regulator and vice versa. As a consequence, *HOXD13* is expressed too early and ectopically leading to the MDK phenotype (Figure 2b). Thus, it is possible that one or both regulators have to be affected to produce the MDK/*U1* phenotype. To verify our results of the Thai family, we tested the further described nonrelated MDK individuals.<sup>3,4</sup> We were not able to identify a clinically relevant copy-number change anywhere in the genome of these two additional individuals. However, as the array-CGH analysis does not exclude balanced structural rearrangements, one can speculate that the MDK phenotype in patients reported by Shears *et al* and Kwee *et al*<sup>3,4</sup> and the Thai patients reported in this paper is caused by chromosomal rearrangements affecting *HOXD* locus regulation. The identification of further rearrangements in this region will help to clarify this.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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