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Characterization of mouse *Dactylaplasia* mutations: a model for human ectrodactyly SHFM3

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Abstract SHFM3 is a limb malformation characterized by the absence of central digits. It has been shown that this condition is associated with tandem duplications of about 500 kb at 10q24. The *Dactylaplasia* mice display equivalent limb defects and the two corresponding alleles (Dac^{1j} and Dac^{2j}) map in the region syntenic with the duplications in SHFM3. Dac^{1j} was shown to be associated with an insertion of an unspecified ETn-like mouse endogenous transposon upstream of the *Fbxw4* gene. Dac^{2j} was also thought to be an insertion or a small inversion in intron 5 of *Fbxw4*, but the breakpoints and the exact molecular lesion have not yet been characterized. Here we report precise mapping and characterization of these alleles. We failed to identify any copy number differences within the SHFM3 orthologous genomic locus between *Dac* mutant and wild-type littermates, showing that the *Dactylaplasia* alleles are not associated with duplications of the region, in contrast with the described human SHFM3 cases. We further show that both Dac^{1j} and Dac^{2j} are caused by insertions of MusD retroelements that share 98% sequence identity. The differences between the nature of the human and mouse genomic abnormalities argue against models proposed so far that either envisioned SHFM3 as a local trisomy or *Dac* as a mutant allele of *Fbxw4*. Instead, both genetic conditions might lead to complex alterations of gene regulation mechanisms that would impair limb morphogenesis. Interestingly, the Dac^{2j} mutation occurs within a highly conserved element that may represent a regulatory sequence for a neighboring gene.

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Introduction

Dactylaplasia is an inherited mouse limb malformation characterized by missing phalanges and reductions or fusions of metacarpals and metatarsals in each foot (Chai 1981). The phenotype follows a semidominant pattern of inheritance, with the expression of the abnormality dependent on homozygosity for the unlinked modifier allele *mdac* in addition to the genotype at the *Dac* locus

(Johnson et al. 1995). The recessive *mdac* allele exists only in certain laboratory mouse strains, including SM/Ckc and BALB/cJ, but not in C57BL/6J. Two *Dac* alleles, Dac^{1j} and Dac^{2j}, arose spontaneously in The Jackson Laboratory and were described by Chai (1981) and Sidow et al. (1999), respectively. Both mutations map either within or close to the F-box gene *Fbxw4* on chromosome 19qC3. Dac^{1j} is an insertion of a transposon upstream of *Fbxw4* and the insertion site was previously determined (Sidow et al. 1999), while the exact molecular lesion in Dac^{2j} has not yet been characterized but is thought to be an insertion or a small inversion in intron 5 of *Fbxw4*.

Dactylaplasia is a model for human split-hand/split-foot malformation 3 (SHFM3) that maps to the syntenic locus. Both the human and the mouse phenotypes seem to be caused by disruption in normal gene expression patterning in limb bud development, but the associated mechanisms and genes are not known. In several independent SHFM3 patients, ~500 kb duplications have been described. The largest duplication contained at least five genes, *TLX1*, *LBX1*, *BTRC*, *POLL*, and *FBXW4* (de Mollerat et al. 2003), but subsequent work narrowed the minimal duplication to a 325-kb segment containing two genes (*BTRC* and *POLL*) (Lyle et al. 2006). The phenotype in SHFM3 patients could be a consequence of dosage imbalance for the genes within the minimal duplication, i.e., like a minitrisomy. Alternatively, the mutation could disrupt a long-range control mechanism either by direct alteration of an important regulatory element or as a consequence of the reshuffled organization of the region leading to so called "position-effects" (Kleinjan and van Heyningen 2005).

Here we report fine mapping of the mouse Dac^{2j} mutation and show that it is caused by the insertion of a retroelement similar to that of Dac^{1j}. We fully sequenced both inserted elements and built a phylogenetic tree, including all related sequences. Moreover, we excluded the presence of a duplication in the *Dac* region in both alleles, confirming that the mutation mechanism is different than that of human patients with SHFM3.

Material and methods

SYBR Green real-time PCR

SYBR Green assays were designed using the program Primer Express (Applied Biosystems, Foster City, CA) with default parameters in every case. Repetitive sequences were masked using RepeatMasker (www.repeatmasker.org) (Smit et al. 2004), and amplicon sequences were checked by BLAT (Kent 2002) against the mouse genome to ensure that they were specific for the region under study. Primer sequences of SYBR Green assays are provided in Supplementary Table 1. Each assay's efficiency was tested using serial dilutions of a control DNA. Only assays that accurately measured copy number were included. All reactions used 300 nM of each primer, 10 ng of genomic DNA, and Power SYBR Green PCR master mix (No. 4368702) from Applied Biosystems. PCR reactions were set up using a Biomek 2000 robot (Beckman, Fullerton, CA), in a 10-µl volume in 384-well plates with three replicates per sample. Reactions were run in an ABI 7900 Sequence Detection System (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 sec/60°C for 1 min. For data analysis, Ct values were obtained using SDS 2.0 (Applied Biosystems), input DNA quantities were normalized to five assays from Mmu16, and relative DNA copy number was obtained by pairwise comparisons of test and control DNAs. Calculations were carried out in Excel (Microsoft Corp., Redmond, WA).

Inverse PCR

Inverse PCR primers were designed in intron 5 of *Fbxw4* using Primer3 (Rozen and Skaletsky 2000). Sequences are provided in Supplementary Table 2. Eight micrograms of genomic DNA from Dac^{2j} or wild-type mice were digested overnight with *Bcl*1. DNA was then religated with T4 DNA ligase to generate circular molecules and PCR-amplified in a nested reaction with the inverse PCR primers. An approximately 700-bp fragment was recovered from the reaction on Dac^{2j} DNA but not on wild-type DNA. Sequencing from both ends identified a piece of transposon LTR and the Dac^{2j} breakpoint.

Sequencing strategy

The MusD elements at Dac^{1j} and Dac^{2j} were PCR-amplified in several overlapping PCR reactions using one primer in the MusD element and one primer mapping in the flanking genomic sequence. PCR products were sequenced form both ends and a contig was built for each insertion. Sequences were submitted to NCBI (Dac^{1j}: EF490384; Dac^{2j}: EF490385).

Phylogenetic analysis

All MusD and early transposon (ETn) elements were found by BLASTing previously described elements (Baust et al. 2002) against the mouse genome. Coordinates are given for the February 2006 mouse genome assembly. All phylogenetic analyses were performed using the maximum likelihood (ML) method implemented in the phyml program (Guindon and Gascuel 2003). Calculations were done with the GTR+G+I evolutionary model. Robustness of reconstruction was tested with the bootstrap method.

Mice

The two *Dac* alleles, Dac^{1j} (SM;NZB-$Fbxw4^{Dac}$/J) and Dac^{2j} (CBy.MRL-$Fbxw4^{Dac-2J}$/J), were obtained from The Jackson Laboratory (http://www.jax.org/). Dac^{1j} was maintained on an SM/NZB mixed background and Dac^{2j} was maintained on a BALB/cByJ background.

Results

No duplication in Dac^{1j} or Dac^{2j}

Human ectrodactyly SHFM3 is caused by a duplication at 10q24 with a minimal size of 325 kb. To test whether *Dac* mice carried a duplication in addition to the transposon insertion, we designed 25 SYBR Green assays spanning about 2 Mb of the sequence at the *Dac* locus. Quantitative PCR on genomic DNA with assays covering the genomic locus failed to identify any copy number differences between any *Dac* mutant and wild-type littermates (Fig. 1). This result confirms that the *Dactylaplasia* phenotype is caused by insertion of a transposon and that there is no duplication consequent to the insertion.

Dac^{2j} is caused by insertion of a MusD retroelement

The Dac^{2j} mutation was mapped by Sidow et al. (1999) to an interval in intron 5 of *Fbxw4*. To identify the mutation and fine-map it, we designed inverse PCR primers close to the putative mutated region. We used the Southern blot data from Sidow et al. to deduce where the mutation might lie and placed primers accordingly. An inverse PCR fragment of approximately 700 bp was recovered and sequenced. The sequence contained the expected chromosome 19 mouse genome fragment with a part of a transposon LTR. Dac^{2j} lies ~50 kb centromeric to Dac^{1j} (Fig. 2a). After identifying the breakpoint of this insertion (Chr19: 45,669,788 Mouse February 2006 assembly), we fully sequenced the element (Dac^{2j}: EF490385), which turned out to be a retrotransposon of the MusD family. The Dac^{2j} insertion is 7471 nucleotides long (Fig. 2c). Interestingly, the Dac^{2j} insertion falls within a DNA element of considerable evolutionary conservation (Fig. 2b). This segment of ~1.5 kb could represent a regulatory element dictating the correct spatial and temporal expression of a neighboring gene. If so, disruption of such an element could be the explanation for the Dac^{2j} phenotype.

Phylogenetic analysis

After sequencing the Dac^{2j} insertion, we also further characterized the Dac^{1j} mutation (mapped Chr19: 45,723,779 Mouse February 2006 assembly) by fully sequencing the inserted transposon. As for Dac^{2j}, we PCR-amplified and sequenced the element. The Dac^{1j} insertion was 7486 nucleotides, slightly longer than that of Dac^{2j}. We noted that both Dac^{1j} and Dac^{2j} belong to the MusD family of

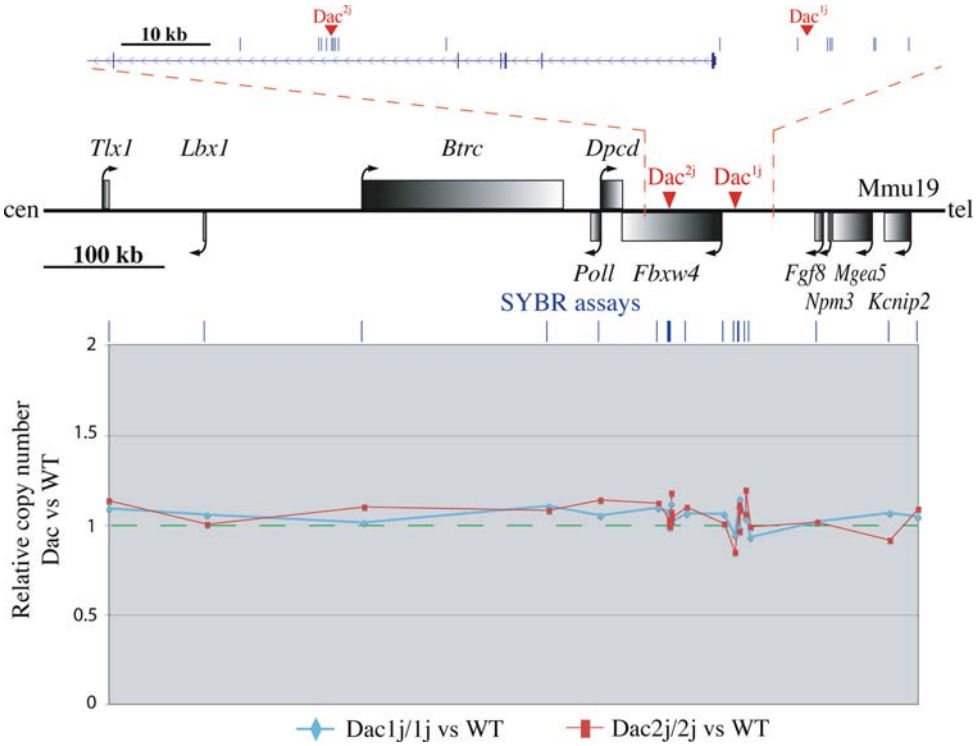

Fig. 1 *Top*: The *Dac* locus with positions of the *Dac* mutations. *Bottom*: Relative copy number of *Dac* mutants versus wild mice assessed by SYBR Green qPCR

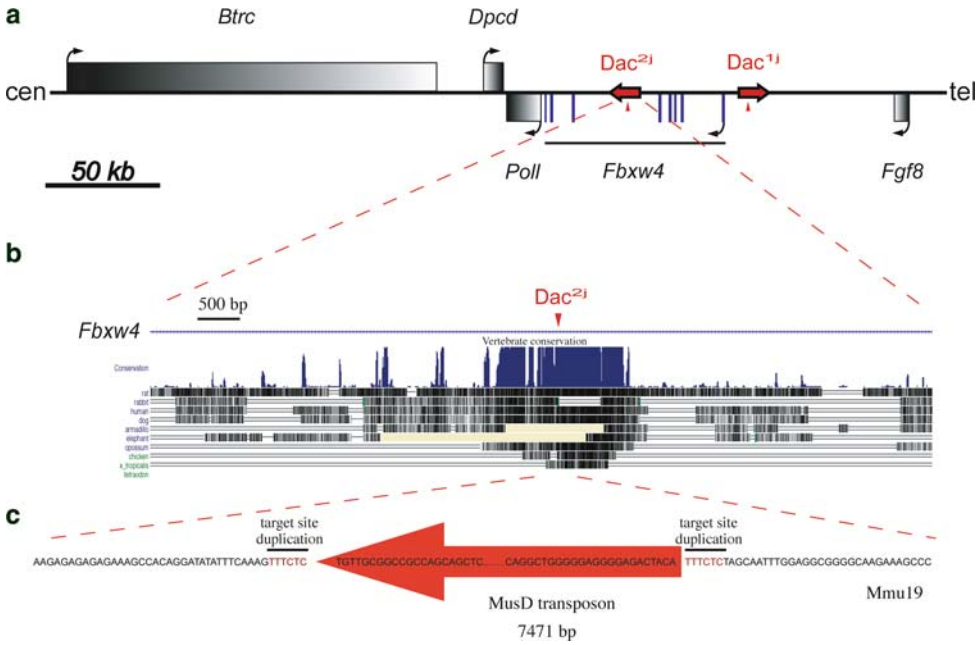

Fig. 2 (**a**) *Dac* mutations are represented by red arrows in the *Dac* locus. Dac^{1j} and Dac^{2j} are in opposite orientations. (**b**) The Dac^{2j} mutation falls within a block evolutionary conservation (from UCSC genome browser). (**c**) Sequence of the breakpoints flanking the Dac^{2j} insertion

transposable elements and have about 98% sequence identity. To our knowledge this is the first transposition event of a MusD element detected, but multiple ETn transpositions were described (reviewed in Maksakova et al. 2006). We created the alignment of retrotransposed mouse elements that includes previously annotated (Baust et al. 2003; Ribet et al. 2004) sequences as well as those recovered using genomic BLAT in UCSC genome browser. No exact match representing the parental element was found for either Dac^{1j} or Dac^{2j}; this is probably because the mouse genome reference sequence is from a different genetic background than Dac^{1j} and Dac^{2j}. The total alignment includes 8246 nucleotide positions from 47 MusD and 19 ETn elements. To clarify the structure of mouse genome mobile elements, we performed phylogenetic analysis. To escape the bias caused by possible recombination between LTRs and other positions of mobile elements, we split the total transposon alignment into two categories—5′ LTR + 3′ LTR (LTR) and the region between LTRs (non-LTR)—and built a phylogenetic tree for each category (Fig. 3, Supplementary Fig. 1). We rooted the trees between MusD elements that have the highest number of differences between 5′ and 3′ LTRs because accumulation of substitutions between 5′ and 3′ LTRs that are normally identical is a mark of inactivity and ancient transposition. Our phylogenetic analysis of the non-LTR data set revealed that Dac^{1j} and Dac^{2j} transposons group with early MusDs (100% BP), three of which have shown to be active *in vitro* (Ribet et al. 2004). Although the signal of the LTR tree is weaker because of the short sequence length (321 bp) and the phylogenetic trees are not completely congruent, we did not observe striking differences between topologies of LTR and non-LTR trees. Interestingly, open reading frames in MusD elements do not seem to be crucial for transposition activity since active ETns do not have any genes needed for transposition (Ribet et al. 2004). This suggests that these elements can utilize the proteins needed for transposition produced by other MusD elements in trans. Besides Dac^{1j} and Dac^{2j}, we have identified three other MusD elements that fit the criteria of active transposons (i.e., identical ETn-like LTRs, in boldface in Supplementary Fig. 1). Phylogenetic analyses of MusD and ETn LTRs (Supplementary Fig. 1) have shown that Dac^{1j} and Dac^{2j} transposons group within active ETnII elements. This suggests that Dac^{1j} belongs to the group of MusDs that gave rise to ETnI and ETnII elements. The fact that active transposons group together and share some particular features might indicate that the activity of a transposable element is dependent on the type of LTR as well as on identity of 5′ and 3′ LTRs. Moreover, we show that ETn elements are not monophyletic; they have lost MusD-specific genes at least twice (Supplementary Fig. 1). Interestingly, based on LTR sequences, Dac^{1j} and Dac^{2j} fall in the MusD group (95% BP) from which ETnI, ETnII-α, and ETnII β originated. The ETnII-γ branch is in a different part of the tree. ETnII-γ elements were not shown to be active and have a number of differences between 5′ and 3′ LTRs.

Discussion

Despite very similar phenotypes and association with the orthologous genomic region, the genomic basis of the *Dac* and SHFM3 ectrodactylies in mice and human seem to be very different. The comparison of the two distinct

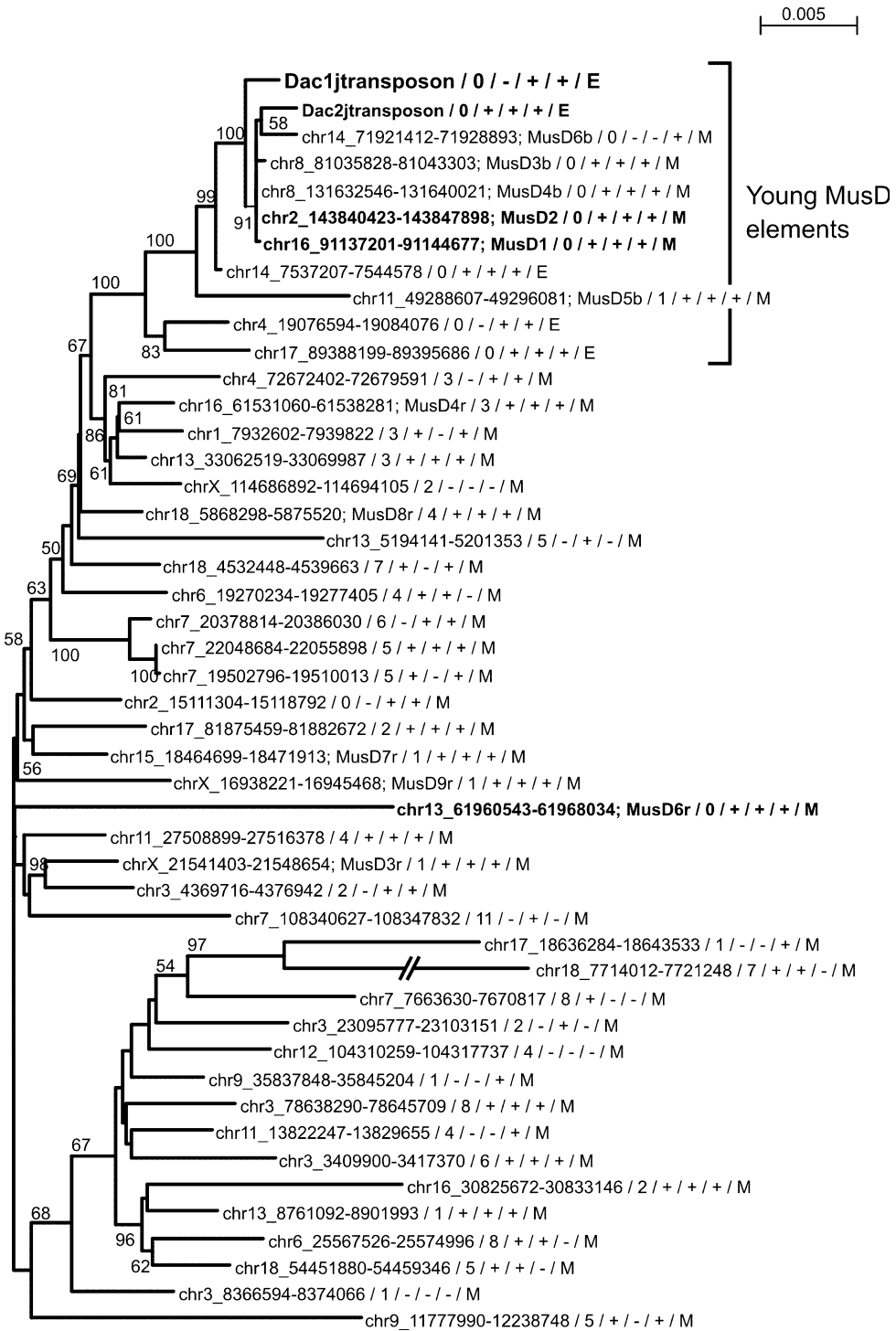

Fig. 3 Phylogenetic tree including the *Dac* mutations and related transposons. The tree was built using non-LTR sequence

situations suggests some possible mechanisms while ruling out others. Because SHFM3 has been associated with duplications of the region, a simple interpretation of the human SHFM3 mutation would be that dosage increase of genes within the minimal duplication is responsible for the phenotype. We demonstrate here that neither Dac^{1j} nor Dac^{2j} mice carry similar duplications, suggesting that gene dosage increase is not primarily responsible for the limb malformations. Accordingly, in a recent report Kano et al. (2007) failed to detect any change in expression of the genes from the SHFM3 interval in *Dac* mutant embryos. The positions of both the MusD insertions and most of the distal duplication breakpoints as well as reported expression changes have highlighted *Fbxw4* as a potential candidate (Basel et al. 2003; Sidow et al. 1999). Because it is oriented in the same direction as *Fbxw4*, the MusD

element at Dac^{2j} likely contributes to knock down this gene by induced premature termination of transcription, like ETnII with similar LTRs do for other instances (Maksakova et al. 2006). However, *Fbxw4* transcript size or dosage is not affected in Dac^{1j} (Sidow et al. 1999), and, more importantly, in a *Mdac* background where Dac^{2j} animals have normal limbs, the Dac^{2j} MusD insertion still leads to downregulation of *Fbxw4*, suggesting that *Fbxw4* may not be directly involved in the ectrodactyly.

Kano et al. (2007) showed that the inserted MusD elements harbor different epigenetic modifications in *Dac* animals depending on their *Mdac* genotype. These observations provide important insights into the possible function of the *Mdac* gene and how it might control the consequences of the MusD insertions, but the nature of these consequences and their contribution to the *Dactylaplasia* phenotype are yet unknown. The reported ectopic expression of MusD in the distal limbs of mutant animals is unlikely to occur in human patients, and the impact on the expression of the neighboring *Fgf8* gene cannot fully explain the observed phenotype. Mice heterozygous for a loss of function of *Fgf8* +/− have normal limbs (Moon and Capecchi 2000). Furthermore, the complete loss of expression of *Fgf8* in the central part of *Dac* heterozygous animals cannot be explained by a *cis* effect as proposed by Kano et al. (2007) since the *Fgf8* copy on the wild-type chromosome should be expressed normally. Instead it indicates a progressive loss of the apical ectodermal ridge (AER), even though we cannot rule out that a reduction of *Fgf8* could contribute to the phenotype. Moreover, no limb phenotypes were observed in mice haploinsufficient for an interval comprising the genes duplicated in SHFM3 (Keller et al. 1994; Sidow et al. 1999), and thus we favor the hypothesis that *Dac* is a gain-of-function allele. Several genes mapping around the SHFM3 interval (e.g., *Fgf8*, *Ikkα*, *Wnt8b*, *Sufu*, *and Nfκb2*) are involved in limb development, sometimes with antagonist activities (Capdevila and Izpisua Belmonte 2001). We propose that the *Dac* insertions or the SHFM3 duplications may alter the delicate regulatory interactions between these genes and their remote *cis*-regulatory elements, leading to a potential rerouting of limb regulatory elements to different genes. Several recent examples have pointed out that structural changes in the organization of a genomic locus can deeply alter the regulation of genes even localized at a distance (Kleinjan and van Heyningen 2005; Kokubu et al. 2003; Reymond et al. 2007). This includes deletions or duplications of several hundreds of kilobases, but also smaller changes like insertion of novel genes or creation of novel ectopic promoter regions (De Gobbi et al. 2006). However, further work will be required to identify the genes and regulatory elements involved, as well as the nature of the underlying molecular mechanism(s).

Interestingly, we noted that Dac^{2j} falls within a region of considerable evolutionary conservation (Dermitzakis et al. 2005), which could represent a regulatory element. This hypothesis is supported by a recent study that showed that 45% of a set of 167 extremely conserved sequences could function reproducibly as tissue-specific enhancers of gene expression (Pennacchio et al. 2006). It would therefore be interesting to test whether the block of conservation disrupted by the Dac^{2j} insertion can function as a regulatory element for a neighboring gene. However, to trigger the gain-of-function Dac^{2j} phenotype, this element would need to behave not only as an enhancer but also as a silencer or tethering element, functions that are less straightforward to assess *in vivo*. However, it is also possible that this element does not contribute to ectrodactyly (it is not disrupted in Dac^{1j}) but may lead to a yet unknown phenotype specific to Dac^{2j} mice. For example, the insertion at Dac^{2j} at the same time may knock down *Fbxw4*, disrupt a regulatory element for another gene, and induce limb malformations through a different gene/mechanism. The similar limb malformations in the *Dac* strains and SHFM3 patients may represent only a shared and dominant trait among genetic conditions with additional and specific recessive defects.

Dac^{1j} and Dac^{2j} are the only cases of disease-causing insertions of MusD elements reported to date (Maksakova et al. 2006).There are about a hundred MusD elements in the mouse genome, with only a subset of them active. MusD elements carry three genes that are absolutely required for retrotransposition: *gag*, *pro*, and *pol* (Ribet et al. 2004). By building a phylogenetic tree with the elements inserted at the *Dac* loci and other related MusD and ETn sequences, we found that *Dac* insertions cluster together and in the same branch as the active ETnII elements of the family. We therefore describe here a new subgroup of the MusD family, formed by autonomous active elements which, similarly to ETnII, can cause phenotypes by insertional mutagenesis, as exemplified by the knockdown of *Fbxw4* in Dac^{2j}. These MusDs have the same LTRs as ETnII elements, explaining why they are highly active. Other MusDs (1, 2, 6), selected for having an open reading frame (ORF) in *gag*, *pro*, and *pol*, were also shown to be active although to a lesser extent (Ribet et al. 2004). We found three other MusDs in the mouse genome (C57BL/6J) that have ETnII-like LTRs, two with full ORFs and one with a stop codon in POL. It would therefore be interesting to test their activity.

Characterizing the *Dac* mutation is an important step toward the comprehension of the complex molecular mechanisms that lead to the phenotype and illustrate how mouse genetics can highlight the underlying causes of human genetic diseases. The complementary approaches taking advantage of both human SHFM3 and mouse *Dac* will be important in identifying the causative genes that are

likely shared between the two conditions and uncover the regulatory mechanisms associated with each case.

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