Dbx2 regulation in limbs suggests interTAD sharing of enhancers

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Abstract
Background: During tetrapod limb development, the HOXA13 and HOXD13 transcription factors are critical for the emergence and organization of the autopod, the most distal aspect where digits will develop. Since previous work had suggested that the Dbx2 gene is a target of these factors, we set up to analyze in detail this potential regulatory interaction.

Results: We show that HOX13 proteins bind to mammalian-specific sequences at the vicinity of the Dbx2 locus that have enhancer activity in developing digits. However, the functional inactivation of the DBX2 protein did not elicit any particular phenotype related to Hox genes inactivation in digits, suggesting either redundant or compensatory mechanisms. We report that the neighboring Nell2 and Ano6 genes are also expressed in distal limb buds and are in part controlled by the same Dbx2 enhancers despite being localized into two different topologically associating domains (TADs) flanking the Dbx2 locus.

Conclusions: We conclude that Hoxa13 and Hoxd genes cooperatively activate Dbx2 expression in developing digits through binding to mammalian specific regulatory sequences in the Dbx2 neighborhood. Furthermore, these enhancers can overcome TAD boundaries in either direction to co-regulate a set of genes located in distinct chromatin domains.

KEYWORDS
chromatin architecture, digit development, gene regulation, Hox, TAD boundary

1 | INTRODUCTION

For many decades, the vertebrate limb has been an efficient experimental paradigm to study the basic principles and concepts underlying developmental processes. The main reason is the congruence between the definition of specific signaling regions in the developing limb buds, on the one hand, and their association with specific
molecular markers, on the other hand. Classical experimental embryology indeed led to a fairly precise cellular definition of those regions in the limb bud, which have a particular activity and function, such as the limb apical ectodermal ridge and the zone of polarizing activity.\textsuperscript{1,2} John Fallon made seminal contributions in this early phase and was one of the pioneers of this field;\textsuperscript{3} see also in References 4, 5, as well as.\textsuperscript{6,7,8,9} Subsequently, transcription factors were cloned, which could be superimposed to such cellular landmarks, such as Hox genes\textsuperscript{10} (see Reference 11), followed by the key signaling molecules.\textsuperscript{12-14} Soon after, gain- and loss-of-function experiments helped ascertain the central roles of these genes in controlling limb patterning and morphogenesis. In this view, the developing limb was the first vertebrate cellular models and their molecular components.

Among these key factors are the Hox genes belonging to both the HoxA and HoxD clusters. They are transcribed into precisely delimited domains within the incipient limb buds\textsuperscript{6,13} and they specify the proximal and distal limb segments as well as some anterior to posterior features\textsuperscript{16-18} (reviewed in Reference 19). Hoxa13 and Hoxd13 are essentially required for the specification and development of the autopod, the distal-most limb domain that will give rise to the digits and part of the wrist. They control the size, shape, and number of autopod bones by regulating mesenchymal cell aggregation, chondrification, and ossification.\textsuperscript{17,20-22} In fact, the inactivation of both Hoxa13 and Hoxd13 in mice leads to an agenesis of the distal limb and the formation of a chondrogenic blastema at the distal extremity of the ulna/fibula and radius/ humerus.\textsuperscript{17,18} Different studies have addressed the identification of the HOXA13/HOXD13 downstream target genes in distal limb development. These included genes controlling cell adhesion, morphology, and proliferation/survival (e.g., Hand2, Shh, EphA7, EphA3, and Bmp2/Bmp7).\textsuperscript{23-29} The regulatory relationships and functional roles of many such target genes nevertheless remain poorly understood.

We previously reported that the transcription factor Dbx2 (Developing Brain Homeobox protein 2) is strongly down-regulated in distal forelimb cells lacking Hox13 function.\textsuperscript{23} Dbx2 belongs to the Dbx subfamily of homeobox-containing proteins and is expressed in the mouse embryonic brain and neural tube, as well as during limb development.\textsuperscript{23,30,31} However, while it is well established that Dbx2 contributes to the specification of the V0 spinal cord interneurons,\textsuperscript{32-35} its potential role in limb development has remained elusive. A heterozygote deletion spanning the genomic region comprising the human loci NELL2, DBX2, and ANO6 was nevertheless associated with intellectual retardation, skeletal and dental anomalies, reduced hand and feet size and clinodactyly of the fifth digit, suggesting that Dbx2 could be involved in digit development,\textsuperscript{36} where it may mediate part of the functions of HOX13 proteins.

In this study, we characterized the regulation of the mouse Dbx2 gene in developing digits. We show that the HOX13 factors directly regulate Dbx2 expression in digits, in part by binding to mammalian-specific regulatory elements located within 30 kb 5’ to the Dbx2 locus, as well as within its introns. Furthermore, we show that 5’ Hoxd genes also contribute to Dbx2 regulation by acting cooperatively and redundantly with HOX13 proteins. However, Dbx2 null mice do not display any of the major limb skeletal abnormalities displayed by any combinations of HOX mutations, suggesting either that Dbx2 is not a major downstream Hox effector or that its function is compensated for in this particular situation.

We also observed that the Dbx2 neighboring genes Nell2 (Neural EGFL Like 2) and Ano6 (Anoctamin 6) are also expressed in the distal limb. Analysis of chromatin interaction profiles revealed that, at the 3D level, the Nell2 and Ano6 genes are organized into distinct topologically associating domains (TADs), which are regions of the genome where gene-enhancer interactions are favored.\textsuperscript{37} Interestingly, the boundary between these two TADs maps at the proximity of the Dbx2 locus and of its limb enhancers, which seem to be able to control the transcription of the three genes, regardless of in which TAD they reside.

## 2 | RESULTS

### 2.1 | Dbx2 expression during distal limb development

We characterized Dbx2 expression at different stages of mouse forelimb development by whole-mount in situ hybridization (WISH) and compared it with that of Hoxa13 and Hoxd13 (Figure 1). Dbx2 transcripts were first scored during early limb development (E9.5-E10) throughout the limb bud mesenchyme, with the exception of mesodermal cells underlying the distal-most limb ectoderm (Figure 1A). This expression poorly correlates with that of Hox13 genes (Figure 1B) and, overall, the Dbx2 mRNA levels at this stage remained very low. By E11.5, proximal Dbx2 expression was confined to a small and posterior moon-shaped cellular domain (Figure 1A, asterisk). Dbx2 transcripts were also detected in the anterior portion of the autopod (Figure 1A, arrowhead). Thus, the distal limb expression of Dbx2 was delayed by approximately 24 hours when compared to the onset of Hoxa13 and Hoxd13 in the autopod (Figure 1A, B).\textsuperscript{10,38} At E12.5, Dbx2 mRNA spread to the entire distal-most portion of the autopod, both in digit and interdigit mesoderm and in a nested domain within the Hoxa13/Hoxd13 expressing cells (Figure 1A,B). However, Dbx2 transcripts were
rapidly downregulated in the interdigit region and, from E13 onwards, they were detected in sequentially formed domains reminiscent of the digit joints (Figure 1A,C,D).

To assess which cell type(s) express the \( \text{Dbx2} \) gene, we re-analyzed single cell-RNA sequencing (scRNA-seq) data sets from E11, E13, and E15 mouse hindlimbs (Figure 2). At E11.5 \( \text{Dbx2} \) is expressed in two small cell populations, most of which also express \( \text{Hoxa13} \) and \( \text{Hoxd13} \) (Figure 2A). At E13 and E15 stages, some \( \text{Dbx2}^+ \) cells did not express \( \text{Hoxa13}/\text{Hoxd13} \) but displayed the \( \text{Col2a1} \) marker for mature cartilage precursors (Figure 2B, C). \( \text{Dbx2} \) transcripts were also detected in a subpopulation of \( \text{Hoxa13} \) and \( \text{Hoxd13} \) positive cells, which expressed the \( \text{Gdf5} \), \( \text{Mkx} \), \( \text{Scx} \), and \( \text{Col1a1} \) gene as well (Figures 1C, D and 2B,C). Of note, although \( \text{Dbx2}/\text{Col2a1}/\text{Sox9}+ \) cartilage cells did not express either \( \text{Hox13} \) genes at E13/E15, they derive from \( \text{Hox13} \)-positive cells (Figure 2A).

These results showed that \( \text{Dbx2} \) is expressed during digit development in \( \text{Hoxa13}/\text{Hoxd13} \) expressing cells corresponding to tendon and cartilage precursors of developing digit joints, thus supporting the possibility that HOX13 proteins could act as direct regulators of \( \text{Dbx2} \) expression in these cells, in agreement with the reported function of 5′/\( \text{Hoxd} \) and \( \text{Hoxa13} \) genes in digit joint development.\(^{40,44,45}\)

### 2.2 Identification of HOX13-bound sequences regulating \( \text{Dbx2} \) expression in digits

\( \text{Dbx2} \) expression in distal limbs is strongly compromised in the absence of HOX13 proteins.\(^{23}\) To further evaluate whether HOX13 paralogs could act as direct regulators of \( \text{Dbx2} \) expression, we set up to characterize the extent of the \( \text{Dbx2} \) regulatory landscape both by analyzing available Hi-C data sets and by performing 4C-seq experiments (Figures 3 and 4). TADs are megabase-scale structures that constitute a unit of 3D genome organization.\(^{37,50}\) Thus, they often coincide with and delimit the extent of gene regulatory landscapes.\(^{51,52}\) TADs are generally somewhat independent from the transcriptional status of the gene(s) inside and can be identified across different cell types or tissues.

We analyzed high-resolution (5 kb bin size) Hi-C data from ES cells and embryonic cortex,\(^{36}\) as well as 40 kb-resolution Hi-C profiles from E12 mouse distal limbs (Figure 3). We observed that, in all cases, the \( \text{Dbx2} \) genomic region is organized in two large TADs, which span the neighboring loci \( \text{Tmem117} \) and \( \text{Nell2} \) (5′ TAD) and \( \text{Ano6} \), \( \text{Arid2} \), and \( \text{Scaf11} \) (3′ TAD), respectively. Although with some variation between tissues or cell types, and depending on the TAD-separation score calculation parameters, the border between these two TADs consistently falls in the close vicinity of the \( \text{Dbx2} \) gene. Noteworthy, a region of \( 150 \) kb spanning the \( \text{Dbx2} \) locus forms a micro-domain of higher contact frequency spanning the TAD boundary (hereafter referred to as interTAD domain) and the \( \text{Dbx2} \) interactions were mostly restricted to this interTAD domain. Accordingly, analysis of the binding profiles of CTCF (CCCTC-binding factor), a protein that plays an important role in the establishment of chromatin loops and TAD boundaries (e.g., references 53, 54) revealed that the \( \text{Dbx2} \) interTAD domain is flanked by two clusters of CTCF binding sites (BS) organized in large part in divergent orientations (Figure 3C). Besides, we observed a cluster of CTCF BSs located at the border of the \( \text{Nell2}/\text{Tmem117} \) interaction domain, in large part oriented convergently toward the CTCF sites lying at the 5′ side of the \( \text{Dbx2} \) locus. While CTCF sites also delimit the \( \text{Ano6}/\text{Arid2} \) interaction...
domain, they are organized in the same orientation than those located on the 3’ side of Dbx2, suggesting that both TADs flanking the Dbx2 domain are established through different chromatin looping modalities.

To confirm this, we performed 4C-seq experiments by using mouse proximal and distal forelimb cells at E12 and the Dbx2 promoter as a viewpoint (Figure 4A,B). As expected, the vast majority of Dbx2 interactions were observed within the 150 kb region matching the inter-TAD domain identified in the Hi-C data analysis. However, some diffuse Dbx2 interactions were also detected over the entire lengths of the 5’ and 3’ TADs flanking the Dbx2 locus, whereas Dbx2 contacts dramatically dropped down outside of these domains. Overall, the Dbx2 interaction profiles remained very similar in both proximal and distal forelimbs (PFL and DFL, respectively). Nonetheless, we scored a DFL-specific increase in contacts over a narrow region located 55 kb away from the Dbx2 transcription start site (TSS) on the 5’ side of the locus, as well as with a broad region comprised between 82 and 236 kb 3’ to the Dbx2 transcription start site encompassing part of the neighboring Ano6 gene (Figure 4C). These results suggested that Dbx2 expression in the developing limbs is mostly driven by mid and short-range regulatory interactions within its immediate 150 kb surroundings.

To identify putative regulatory sequences controlling Dbx2 expression in developing digits, we analyzed H3K27 acetylation data sets, a histone modification specifically enriched in active enhancers and promoters. In mouse E12 PFL and DFL cells, within the 800 kb spanned by Dbx2 and its flanking TADs, we identified only five non-coding regions specifically enriched in H3K27ac (Figure 4B,C). These regions were located within the Dbx2- interTAD domain, suggesting that they could correspond to Dbx2 regulatory elements. Of these, two were located in the intergenic region on the 5’ side of the Dbx2 locus, two others mapped within Dbx2 intronic sequences whereas one overlapped with the first Dbx2 exon and TSS. All these sequences were strongly contacted by the Dbx2 promoter (Figure 4). Other H3K27ac-positive regions were identified within the Ano6/Arid2/Scaf11 TAD, yet they were not specifically enriched in this epigenetic mark in DFL cells, arguing against a specific involvement of these sequences in Dbx2 regulation.

We also analyzed HOXA13 and HOXD13 ChIP-seq data sets to determine whether these proteins would directly interact with the Dbx2 locus (Figure 4B,C). We observed HOXA13/HOXD13 binding at several locations within the Dbx2 genomic region. While most of these HOX13 bound sequences were not located in H3K27ac-positive and Dbx2 interacting regions, we nevertheless observed strong binding of these proteins in three of the DFL-specific H3K27ac-positive sequences.

**FIGURE 2** Single-cell RNAseq analysis of Dbx2+ cell populations in developing hindlimbs. UMAP representations of the scRNAseq data from mouse E11, A, E13, B, and E15, C, mouse hindlimbs showing the expression of Dbx2, Hoxa13, and Hoxd13, as well as of different joint (Gdf5) and tendons/ligaments (Mkx, Scx) markers.
showing an interaction with \textit{Dbx2}. One of these HOX13-bound sequences partially overlapped with a Vista enhancer (mm1571) previously characterized to drive \textit{LacZ} reporter expression in the neural tube and developing limbs\cite{56,57} (Figure 5B, arrowhead). We quoted the other sequences as putative Distal Limb Enhancers (DLE) and numbered them based on their 5’ to 3’ position within the \textit{Dbx2} interacting domain (DLE1 to DLE3). These sequences are largely conserved across mammals (Figure 5A). In addition, the Vista mm1571 enhancer is also conserved across tetrapods. Furthermore, we could identify evolutionarily conserved HOX13 BSs within the DLE1-3 Vista enhancer mm1571 sequences (Figure 5A).
To assess the functional role of these DLEs, we cloned the DLE1 and DLE2 sequences in a LacZ reporter vector and tested them in transient transgenic experiments. The two elements displayed activity in E13 DFLs in a domain reminiscent of Dbx2 expression in the last forming joint of the phalanges (Figure 5B). Interestingly, DLE1 and DLE2 displayed mirror-imaged stainings, with DLE1 active in the posterior portion of the handplate and DLE2 anteriorly. Besides, DLE1 displayed a weak yet reproducible activity in a narrow strip of cells within the mesopod (Figure 5B, asterisk), possibly related to the initial expression of Dbx2 at E11.5 (Figure 1A, asterisk). This was maintained until E13, likely due to the stability of the beta-galactosidase protein. Neither DLE1 nor DLE2 displayed any transgene activity anywhere else than in developing digits. To corroborate the functional role of these elements on Dbx2 regulation, we used CRISPR/Cas9 genome editing to produce mice lacking the DLE1

**Figure 4** The Dbx2 regulatory landscape in mouse limb buds. A, 4C-seq analysis of Dbx2 interactions using the Dbx2 promoter as a viewpoint in E12 mouse distal (light blue) and proximal (green) forelimbs. Profile overlap is in dark blue. Each curve marks the average profile of three independent biological replicates. Asterisks mark the region(s) displaying increased contact frequencies in the DFL vs. PFL. TADs in A and B are depicted with thick green lines. Protein coding loci are represented by blue (Dbx2) or gray boxes (all other genes) pointing toward the gene direction. B and C, 4C-seq and ChIP-seq analysis of H3K27ac and H3K27me3 marks in distal (light blue) and proximal (green) forelimbs, and HOXA13/HOXD13 binding profiles over the Dbx2 genomic region (below) and their respective peak calling. Profile overlap is in dark blue. Data from References 23, 28. The HOX13 bound putative regulatory elements (DLE1 to 3) are shown in red. The region framed by a dashed line in B is displayed at a higher resolution in C. The Vista enhancer mm1571 is represented by a blue rectangle. Zoom in view of the 4C-seq and ChIP-seq profiles from B
impaired or delayed its transcription at later phases of joint development. Alternatively, DLE1 activity in formed joints may require the synergistic cooperation of other elements to elicit a transcriptional response.

2.3  |  Hoxa13 and 5’ Hoxd genes directly regulate Dbx2 expression

To assess the relative contribution of Hox13 paralogs to Dbx2 regulation, we measured Dbx2 expression in the forelimb autopods of either Hoxa13−/− and Hoxd13−/− mice, or of compound mutants carrying different combinations of Hoxa13 and Hoxd13 null alleles (Figure 6A), by using both WISH and qPCR. As previously described, Dbx2 was almost completely abrogated in double Hox13 mutant mice (Figure 6B,C). Instead, only a weak reduction in Dbx2 expression was observed in Hoxd13−/− single mutants. There, transcripts were maintained in the distal forelimb, with the exception of the posterior-most portion of the autopod, where Dbx2 expression was severely reduced (Figure 6B,C). In contrast, Dbx2 mRNA levels strongly decreased in Hoxa13−/− embryos and transcripts remained detectable at low levels, in the distal portion of the central digits only. Dbx2 expression was not scored neither in the Hoxa13−/−Hoxd13−/−, nor in the Hoxa13+/+Hoxd13−/− compound mutants (Figure 6B), indicating that a single allele of either genes was not sufficient to activate Dbx2 in the DFL, despite the fact that in these mutants, a reduced but correctly specified autopod is still observed. Noteworthy, a faint and spatially ill-defined Dbx2 signal was scored in the Hox13 double knock-out mice (Figure 6B), reminiscent of the early expression of Dbx2 in the incipient limb bud at E9.5 to E10 (Figure 1A). This expression was not observed in either Hoxa13+/−Hox13+/− or Hoxa13+/+Hoxd13−/− mutant embryos and may thus reflect the inability of Hox13 mutant limbs to properly terminate the early limb developmental program and to initiate the transcriptional network operating at later stages in the autopodal domain.

Because Hox genes exert largely overlapping functions in the development of the distal limb domain, we also assessed whether other Hoxd paralogs could contribute to Dbx2 regulation. We analyzed Dbx2 expression in series of mutant mice carrying deletions of different combinations of Hoxd genes (Figure 6A,D). HoxDDel (Hoxd9-Hoxd12) knock-out mice, hereafter referred to as Del (9-12), carry a deletion including all Hoxd genes normally expressed in the autopod but Hoxd13. They displayed normal levels of Dbx2 expression as compared either to control or to Hoxd13−/− mutant autopods. Instead, mice carrying a homozygote deletion including from Hoxd8 to
Hoxd13 (HoxD Del(Hoxd8-Hoxd13)−/−; hereafter Del (8-13) displayed a drastic downregulation of Dbx2 mRNA levels, which was significantly stronger than that observed in Hoxd13−/− mutant and comparable to that of Hoxa13−/− mice. This reduction was also observed in mice carrying a large genomic deletion removing the HoxD centromeric gene desert, which contains all the elements controlling Hoxd gene expression in the autopod.59 Altogether, these data indicate that although Hoxd13 is the main Hoxd gene regulating Dbx2 expression in digits, other Hoxd genes cooperatively contribute to this activation along with Hoxa13 (Figure 6E).

2.4 | Dbx2 does not significantly contribute to the distal limb skeleton development

To determine the extent to which Dbx2 contributes to HOX13 functions in distal limbs, and also to assess its importance in the hand/foot phenotype associated with the deletion of the human NELL2/DBX2/ANO6 genomic region,36 we used CRISPR/Cas9 genome editing to disrupt the Dbx2 homeodomain (Figure 7A). We designed specific sgRNAs targeting the flanking region of the Dbx2 third exon, which encodes two out of the three alpha-helices (H1-H2) of the homeodomain and part of the third (H3). We thus produced mice carrying a 377 bp large deletion, which removes the H1-2 coding sequence and produces a frameshift mutation disrupting also the third alpha-helix and the DBX2 C-terminal domain. This mutation is expected to prevent the binding of the protein to DNA and hence to inactivate its function (Figure 7A, B). The frequency of mouse pups carrying this Dbx2 mutant allele, either heterozygous or homozygous, was significantly reduced when compared to the expected Mendelian ratio (Figure 7C), suggesting that the Dbx2 mutation led to embryonic or perinatal lethality. However, no clear skeletal or hand/foot phenotype was observed in Dbx2−/− mice, neither in the length or number of phalanges, nor in their degree of ossification or in their phalangeal joints (Figure 7D, F). Therefore, although Dbx2 operates downstream of HOX13 genes in distal limb development, it is not the main contributor to the effects observed in these structures upon the loss of Hox13 and other Hoxd genes.17,40,45

2.5 | Nell2/Dbx2/Ano6 coregulation in developing limbs

The absence of limb alterations in Dbx2 null mice raised the question of whether the neighboring Nell2 and Ano6 genes may contribute to limb development. In fact, the
The entire Dbx2 genomic region has a syntenic interval in humans and other tetrapods (Figure 8A) and the deletion involved in hand-foot defects in humans also contains the NELL2 and ANO6 genes. WISH analysis as well as mining a scRNA-seq data set revealed that Nell2 and Ano6 are specifically expressed in the distal portion of mouse developing limbs, in a population of Hoxa13/Hoxd13 double-positive cells, part of which also express Dbx2 (Figure 8B-D). In both cases, their transcripts were distributed on both sides of the developing digits, displaying an indentation (Nell2) or a faint band (Ano6) corresponding to the joints of the forming phalanges (Figure 8B,C). However, we could not identify any DFL-specific H3K27ac positive region in the Nell2 or Ano6 TADs (Figure 4B,C), suggesting that their expression in developing limbs could be driven by the regulatory elements of the Dbx2-containing interTAD region.

To address this question, we performed 4C-seq experiment in E12 DFLs using either the Nell2 or the Ano6 promoters, as well as DLE1 and DLE2 as viewpoints (Figure 9A,B). As expected, the Nell2 and Ano6 promoters displayed strong interactions with sequences located in their own TADs, while they showed reduced contacts with the neighboring TAD. However, in both cases, we observed significant contacts between both the Nell2 and Ano6 promoters and the DLE1-3 region (Figure 9A,B). In the reverse experiment, DLE1 interacted not only with the Dbx2 promoter, but also with the close neighborhood of the Nell2 and Ano6 TSSs. Such interactions were also scored when using DLE2 as a viewpoint, although with a reduced intensity likely due to the fact that the DLE2 contacts remained overall strongly confined to the Dbx2 interTAD domain. Nonetheless, DLE2 contacts with Nell2/Ano6 were still higher than those displayed by the Dbx2 promoter, which contacted predominantly the interTAD domain. We did not observe any significant enrichment of H3K27me3, a histone modification usually associated with inactive enhancers and promoters, over the DLE1 to 3 regions (Figure 4B,C), ruling out the possibility that these interactions would represent contacts between H3K27me3 islands, as reported in other instances.

To further document that part of the transcription of both Nell2 and Ano6 could be driven by elements shared with the Dbx2, we analyzed their expression in mice lacking the DLE1 sequence by WISH and qPCR.
observed that *Nell2* and *Ano6* transcript levels were significantly decreased in the autopods of DLE1−/− embryos when compared to control littermates (Figure 9C,D). This decrease was more pronounced for *Nell2* than for *Ano6*, yet it remained proportionally lower than that observed for *Dbx2* (Figure 5C,D), in agreement with the differences observed in contact frequency between DLE1-2 and the promoters of these three genes. These results supported the hypothesis that the regulatory elements located in the genomic vicinities of *Dbx2* also control part of the *Nell2* and *Ano6* expression in developing digits.

2.6 | Structural differences at the *Nell2/Dbx2/Ano6* locus between birds and eutherian mammals

While the DLE1 to 3 regulatory elements are broadly conserved across eutherians, they could not be identified in nonmammalian tetrapods and we only observed a weak conservation for DLE1 in prototheria (opossum) (Figure 5A). Instead, a large syntenic region around the *Dbx2* locus is conserved across all tetrapods, except in monotremes where the *Ano6* gene was specifically lost (Figure 8A). Hi-C interaction profiles produced from embryonic chicken limbs revealed that the TAD organization of the chicken *Dbx2* region is similar to that of the mouse (Figures 3 and 10A), with *Dbx2* located in the close vicinity of the boundary between the *Nell2* and *Ano6* containing TADs (Figure 10A), in agreement with the syntenic correspondence. Accordingly, the distribution and orientation of CTCF sites in the chicken *Dbx2* genomic region are equivalent to those of the mouse, although with some differences (Figure 10A, bottom). The chicken *Dbx2* locus is also flanked by clusters of divergent CTCF sites, and the *Nell2*-containing TAD is delimited on its 5′ side by CTCF sites oriented convergently toward those on the 5′ side of *Dbx2*. Besides,
the chicken Ano6/Arid2 interacting domain is marked by CTCF sites organized in the same orientation but, unlike its mouse ortholog, the Ano6/Arid2 intergenic region bears a high density of CTCF sites, indicating that this regulatory landscape evolved differently in the mouse and chicken lineages. This suggests that the Dbx2 TAD architecture is maintained across tetrapods and arised before the emergence of mammals. We thus asked whether Dbx2, Nell2, and Ano6 were expressed in the developing wings of chick embryos.

We did not observe any expression of either Dbx2, Nell2, or Ano6 in the distal domain of embryonic chick limb buds (Figure 10B) by WISH. Although weak expression levels of Dbx2, Ano6, and Nell2 transcripts were detected in RNAseq data, they were not specifically increased in the chicken autopod, in agreement with the idea that digit-specific expression of these genes was acquired after the emergence of the eutherian lineage. Instead, Dbx2 was expressed in the developing chicken neural tube (Figure 10B), as expected from its expression in the mouse CNS as well as by the activity of the evolutionary conserved mm1571 regulatory element in the neural tube (Figure 5A,B). Likewise, Nell2 was expressed in the neural tube and somites of both species, in agreement with the function of this gene in sensory and motor neuron differentiation. Ano6 transcripts were also detected in the paraxial and lateral mesoderm of both species. Therefore, Dbx2, Nell2, and Ano6 expression in embryonic structures others than the developing digits is common to different tetrapod lineages, yet it is associated with different populations of neural and mesodermal precursors, suggesting that their transcription in these structures likely relies on gene-specific regulatory elements (Figures 8C and 10B). These results suggest an evolutionary scenario whereby the acquisition of distal limb enhancers within an ancestral TAD organization led to the co-option of Nell2, Dbx2, and Ano6, in the developing mouse digits (Figure 11). The functional consequences of this co-option remain to be established.
3 | DISCUSSION

3.1 | HOX13 mediated activation of Dbx2 in digits and its function in distal limb development

In this study, we show that Hoxa13 and posterior Hoxd genes directly activate Dbx2 expression by binding to different regulatory elements located either within the Dbx2 introns or in the 30 kb 5′ to Dbx2. This is supported by the expression of these former genes in the autopod anlage, which precedes that of Dbx2 by ~24 hours. Because HOX13 proteins have been proposed to act as pioneer factors\(^\text{25}\) (see also References 65), their binding at the Dbx2 locus may facilitate the access to other transcription factors, thus explaining why some Dbx2 expressing cells do not express any Hox13 genes in E13 and E15 distal limbs whereas mice lacking all Hox13 functions completely loose Dbx2 expression. Besides binding to the DLE1 to DLE3 sequences, HOXA13 and HOXD13 also bind to various locations within the Nell2/Dbx2/Ano6 genomic region. Many such sequences are only partially conserved across the eutherian lineage, in contrast with the high conservation of the DLE regions. While the functional significance of this large HOX13 coverage has not been addressed, it is clearly reminiscent...
of that described for the TAD flanking the \textit{HoxD} cluster, suggesting that \textit{HOX13} proteins may globally regulate the TAD activities at the \textit{Dbx2} locus.\textsuperscript{23}

Mice lacking \textit{Dbx2} function did not show any major skeletal anomaly, neither in the number of phalanges, their length or their ossification pattern, nor in their joints. Also, we did not observe any major limb alterations in the offspring of mice carrying a deletion of the DLE1 sequence, thus suggesting that \textit{Dbx2} is likely not a major mediator of \textit{HOX13} function during distal development. The observed embryonic/perinatal lethality of \textit{Dbx2}–/– mice could result from defects in the specification of neuronal type in the CNS. However, \textit{Dbx2}–/– mice could display as yet undetected anomalies in the development and/or function of digital tendons and/or ligaments, as suggested by the expression of this gene in precursors identified by the presence of transcripts from \textit{Sx}, a known marker of tendon and ligament progenitor differentiation.\textsuperscript{42,66} Therefore, our results do not support the possibility that the loss of function of \textit{DBX2} alone leads to the hand/foot defects observed in humans carrying a heterozygote deletion of the \textit{NELL2}/\textit{DBX2}/\textit{ANO6} genomic region.\textsuperscript{36} However, the observation that these genes are expressed during embryonic limb development indicates that their combined loss may generate these severe limb alterations. Accordingly, \textit{Ano6} inactivation in mice was reported to affect bone formation and to result in micromelia.\textsuperscript{67} Alternatively, the existence of a human-specific function of the \textit{DBX2}/\textit{NELL2}/\textit{ANO6} genes in hand/foot development cannot be completely ruled out.

\subsection{Chromatin architecture and enhancer activity during \textit{Nell2}, \textit{Dbx2}, and \textit{Ano6} coregulation}

We used a comprehensive set of scRNA-seq, ChIP-seq, and Hi-C data to identify regulatory elements controlling \textit{Dbx2} expression in digits and two such elements (DLE1 and 2) displayed enhancer activity in transgenic mice. The deletion of DLE1 leads to a strong down-regulation of \textit{Dbx2} transcripts. The DLE1 to 3 sequences are evolutionarily conserved across eutherians, while they were not identified in non-mammalian tetrapods. Together with our observation that \textit{Dbx2} is not expressed in embryonic chicken extremities, these observations suggest that limb-specific \textit{Dbx2} expression evolved in the mammalian lineage. Also, the comparison of Hi-C interaction profiles at the \textit{Dbx2} loci between mouse and chick revealed a similar TAD organization (Figure 11), which likely originated early in the tetrapod lineage, although we cannot exclude that more subtle changes in TAD architecture also contributed to the evolution of \textit{Nell2}/\textit{Dbx2} and \textit{Ano6} expression in digits. In this context, the different distributions of CTCF binding sites in the mouse and chicken \textit{Ano6} containing TADs may reflect this evolutionary divergence. However, while in chick the \textit{Dbx2}, \textit{Nell2}, and \textit{Ano6} promoters are regulated mostly by locus-specific short or mid-range regulatory interactions, the mouse DLEs can co-regulate these three genes despite their location in different TADs.

This regulatory organization is reminiscent of that observed at the \textit{HoxD} cluster, yet with an inversion of functionalities. At the \textit{HoxD} locus, two flanking TADs contain distinct enhancers, which act in an exclusive manner upon \textit{HoxD} genes located at the TAD boundary (e.g., 65–67). At the \textit{Dbx2} locus, the regulatory elements are located at the TAD boundary and can interact with
target genes located within the two adjacent TADs (Figure 11A), thus providing an original example of such a regulatory architecture. However, the functional contribution of this organization, as well as the mechanisms whereby the DLE sequences can differentially interact with the Nell2/Dbx2 and Ano6 promoters, remain to be determined. Recent reports have used chromosome engineering to analyze the insulating effect of TAD boundary regions, supporting the conclusion that TADs are domains where enhancer-promoters contacts are favored, if not constrained. Our results suggest that, in some cases, enhancers located in between TADs may be selected to interact with either TAD depending on the context. Accordingly, it was recently shown that boundary elements can play an important role in allowing the establishment of interTAD promoter-enhancer interactions in drosophila embryos, yet with a mechanism substantially different from the one proposed here. Another non-exclusive possibility is that Dbx2 would be expressed in developing digits as a bystander effect due to the activity of the neighboring limb enhancers.

4 | EXPERIMENTAL PROCEDURES

4.1 | Mouse strains and transgenesis essays

Mice were kept and handled following good laboratory practices. Mutant strains were maintained in heterozygosis. The Hoxa13, Hoxa13, Del (1–13), De (8–13), and Del (9–12) mutant mouse lines (Figure 4) were previously described.

To generate the Dbx2+/− and DLE1+/− mutant lines, pairs of specific sgRNA targeting both sides of the Dbx2 third exon (CTGCTGTTGAAATAGGACT; CCACTGTT CTGAGAGTCCGA) and the DLE1 enhancer (GAAAA GGAAGACCACCCGTG; AGGGGCTAGAGATCTCC CA) were co-electroporated together with the Cas9 protein (TruecutV2; Thermofisher), in fertilized mouse oocytes. To screen for each mutant allele, we designed specific primer pair flanking the Dbx2 third exon and enhancer (DLE1_F: ACACACAGATAATGCACG TGAAGTG; DLE1_R: GGAGGGCCACTCTTAGGGTGG). In each case, we selected F0 mouse mutant carrying a deletion of 377 bp (chr15: 95632232-95 632 608, mm10) encompassing the whole Dbx2 third exon, and a 1015 bp deletion (chr15:95600674-95 602 176, mm10) encompassing the DLE1 sequence. Mutants mice were backcrossed with wt B6CBAF1 mice. Mutant F1 and F2 mice were selected using specific genotyping primers for their respective wt and mutated alleles (Dbx2_F: GGAACTCCCAACCTT CGACTGACTG/ ACTGTGATTAGGGCTGGTCCA; wt alleles: 756/ mutant allele 388 bp; DLE1_wt: GGAGTGAGGTGTGCCAAGA/ ACCTGTAAGCCAAAC CCGTCA; DLE1_Mut: ACACACAGATAATGCACG TGA/ GAGGGCCACTCTTAGGGTGG).

For the transgenesis essays, the TgDLE1::Lacz and TgDLE2::Lacz plasmids were digested with NotI and KpnI. The fragment encoding the enhancer, b-globin minimal promoter and LacZ reporter was gel purified and injected in the masculine pronucleus of fertilized oocytes. Transgene injections were performed by the transgenesis platform of the University of Geneva. F0 embryos were dissected at E12-E13 and stained for LacZ activity.

4.2 | Probe, transgene, and sgRNA cloning

The sgRNA targeting guides were generated by annealing complementary pairs of oligonucleotides and cloned into the pX330 vector as described in. The plasmid encoding the mouse Dbx2 RNA probe was a gift from Thomas Jessell (Addgene plasmid 16288). Instead, specific primers were used to amplify a portion of the transcribed region of the mouse Nell2, Ano6, Gdf5, and Mlx genes as well as of the chicken Dbx2, Nell2, and Ano6 orthologs. In each case, the PCR products were cloned into the pGEM-Teasy plasmid and sanger sequenced. For the chicken Dbx2, Nell2 and Ano6 genes, primers were designed based on the exon/intron structure predicted from the UCSC Non-Chicken Refseq genes, spliced EST, chicken mRNA and Ensembl gene prediction.

For the transgenesis assays, the DLE1/DLE2 sequences were amplified with specific primers (DLE1 Fw: ATCCTGCTGTCTCTGGCTTTCAT/ GGGATCTGA TGAAGTGCCAGAATGGAATTTC; DLE2 Fw: TCCAGTTTCG TCTTCTAGGGCA/ GGAATGTGATTAACCGACC GA) and cloned into the pSK-bgl:LCZ reporter plasmid, generating the TgDLE1::Lacz and TgDLE2::Lacz reporter vectors.

4.3 | Probe and sgRNA preparation

For the sgRNA transcription, we PCR amplified the sgRNA sequence cloned into the px330 plasmid using a T7 promoter containing primer and a universal reverse oligonucleotide (TAATACGACTCACTATAG). PCR products were gel purified and transcribed in vitro using the HiScribe T7 High Yield RNA Synthesis Kit (NEB). The transcribed sgRNAs were purified using the RNase/TK mini kit (Qiagen). Gene-specific probes were synthesized in vitro by linearizing the respective coding
plasmids using specific restriction enzymes and by in vitro transcribed with either T7/T3 or Sp6 RNA polymerase. The probes were purified using the RNA easy mini kit.

### 4.4 Gene expression analysis

For the qPCR analysis, pairs of E10/E11/E12/E13 mouse DFLs, as well as HH30-31 chicken distal wings, were microdissected and stored in RNAlater. Toral RNA was extracted from each pair of DFLs/ distal wings using the Qiagen microRNA extraction kit and retro-transcribed using the Promega GOscript reaction mix with random primers. Gene expression levels were measured by real-time qPCR using the SYBR Select Master Mix for CFX (Thermofisher), and specific primer pairs for the mouse Dbx2, Hoxa13, Hoxd13 genes as well as for the chicken orthologs Hoxd13 and Dbx2 (Table 1). The mouse Hmbs and chicken Gapdh housekeeping genes were used as internal controls for the normalization of gene expression levels (2^ΔCt). WISH experiments were performed as described in Reference 79.

### 4.5 Skeletal preparation

Alcian blue and alizarin staining was performed as described in 56. Briefly, P7 mouse pups cadavers were eviscerated and skin and fat tissues were removed as much as possible. After 48 hours fixation in ethanol, cadavers were stained alcian blue solution (150 mg/L alcian blue 8 GX in 80% ethanol and 20% acetic acid) for 2 days and washed in 100% ethanol overnight. Subsequently, they were cleared for at least 3% KOH solution and stained for 2 hours in 50 mg/L alizarin red / 2% KOH solution. Finally, they were washed in 2% and 1% KOH solution and progressively dehydrated to 100% glycerol solution.

### 4.6 Hi-C/ChIP seq/scRNA-seq data analysis, 4C-seq interaction profiling

All scripts used to analyze data and generate figures are available at https://github.com/lldelisle/scriptsForBeccariEtAl2021. The calculations were performed using the facilities of the Scientific IT and Application Support Center of EPFL. For the chicken Hi-C analysis, the raw forelimb and hindlimb data were extracted from GEO (see Table 2) and processed independently using HiCUP v0.8.0 on galGal6. Valid pairs were obtained using a custom python script. Both valid pair files were merged before analysis. Valid pairs from Hi-C carried out on mouse material were downloaded from GEO (see Table 2). Valid pairs of each study were loaded in a cool file using cooler version 0.8.10 using a resolution of 5, 20, or 40 kb. The TAD-separation score and the domains were obtained using hicFindTADs version 3.5.1 with—minBoundaryDistance 100 000 and either default parameters for the choice of window size or a fixed window size of 120 kb. Plots were obtained using pyGenomeTracks version 3.6.

ChIP-seq paired-end (PE) fastq of HOXA13 and HOXD13 data, as well as single-read (SR) fastq from H3K27me3 and H3K27ac and corresponding inputs were downloaded from GEO (see Table 2). Adapter sequences and bad quality bases were removed with Cutadapt version 1.16 with options -a GATCGGAAGAGCACACGTCTGAACTCCAGTCAC -AG A T C G G A A G A G C G T C G T GTTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTA-TCATT -q 30 -m 15 (-A being used only in PE data sets).

### Table 1 Primers used for the cloning of RNA probes for in situ hybridization and for qPCR experiments

<table>
<thead>
<tr>
<th>ISH probe</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Nell2</td>
<td>GCGAAAACCCCACAGTTGAC</td>
<td>TTCTTATCCAGGGGAGGAGGAT</td>
</tr>
<tr>
<td>Mouse Ano6</td>
<td>TCCCAGGCTCTCGACGAGGC</td>
<td>ACACGTCGCAACGGAGAGGC</td>
</tr>
<tr>
<td>Mouse Gdf5</td>
<td>GGCTCCCCGTGCTTCTCACAGGA</td>
<td>GGTGTTTCCTCGCAAGCCAGAG</td>
</tr>
<tr>
<td>Mouse Mx</td>
<td>CCAGCAGTGCTGTGGGAAAC</td>
<td>CAGTGAAAGACGTGTGCTCACAACC</td>
</tr>
<tr>
<td>Chick Dbx2</td>
<td>GTCTGCCATGAATTTTGCCTC</td>
<td>GGTGTTTACGTTACCCACAGC</td>
</tr>
<tr>
<td>Chick Nell2</td>
<td>GCATCTGGTCAAGTGTGGACTC</td>
<td>GTGGATCCACAGTGGCTCAG</td>
</tr>
<tr>
<td>Chick Ano6</td>
<td>CACGTCTATACATTTTGAGA</td>
<td>CGAATTACTTGTTAGAAGGC</td>
</tr>
<tr>
<td>qPCR analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Dbx2</td>
<td>AAGTGGCCCTCCAAGAGAGGCTTT</td>
<td>GGTGTTTACGTTACCCACAGGA</td>
</tr>
<tr>
<td>Mouse Nell2</td>
<td>AATGGAACCACATGTCGAAGAC</td>
<td>ACACATGGCAGCAATGCAG</td>
</tr>
<tr>
<td>Mouse Ano6</td>
<td>TGGCTCTGCGCTGAAAAAG</td>
<td>TGCCAGACAGAGAAGTCAAGTCAG</td>
</tr>
<tr>
<td>Mouse Hmbs</td>
<td>CGCGTCTGAGACACAGAG</td>
<td>CCCTCATCTTGGAGGCTTT</td>
</tr>
</tbody>
</table>

...
Reads were mapped with bowtie 2.3.5 with default parameters on mm10. Alignments with a mapping quality below 30 were discarded with samtools view version 1.9. For HOXA13 and HOXD13, coverage and peak calling were computed by macs2 version 2.1.1.20160309 with options --call-summits -f BAMPE -B. Coverage was then normalized by the number of million fragments used in macs2 coverage. For histone marks, coverage and peak calling were computed by macs2 with options -f BAM—nomodel—extsize 200—broad using the BAM of input in -c. The coverage was then normalized by the number of million tags used in macs2 coverage. Plots were obtained using pyGenomeTracks version 3.6. For DFL_E12_H3K27ac, the two replicates were averaged.

For the scRNA-seq, matrices with counts were downloaded from GEO (see Table 2). UMAP and expression plots were obtained using Seurat package version 3.2.2 on each data set individually.

We performed our 4C-seq experiments according to. Briefly, 12 pairs of wild-type DFLs or PFLs were dissected, dissociated with collagenase (Sigma Aldrich/Fluka) and filtered through a 35 μm mesh to isolate single cells. Cells were fixed with 2% formaldehyde (in PBS/10%FBS) for 10 minutes at room temperature and the reaction was quenched on ice with glycine. Cells were further lysed with 10 mM Tris pH 7.5, 10 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 1x Protease inhibitor cocktail to isolate nuclei and stored at −80°C. Nuclei from pools of at least 10 distal or proximal limbs were digested with DpnII (New England Biolabs) and ligated with T4

### Table 2: GEO data sets used in this study

<table>
<thead>
<tr>
<th>GEO accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiC mouse E14 Cortex, ES cells</td>
<td>GSE96107, GSE161259</td>
</tr>
<tr>
<td>HiC mouse DFLs</td>
<td>GSE101715</td>
</tr>
<tr>
<td>HiC chicken HH20 FLs and HLs</td>
<td>GSM3182470, GSM3182471</td>
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<tr>
<td>HOXA13 and HOXD13 binding in DFLs</td>
<td>GSE81358</td>
</tr>
<tr>
<td>H3K27ac and H3K27me3 coverage in DFLs and PFLs</td>
<td>GSE77900</td>
</tr>
<tr>
<td>CTCF binding in mouse E12 DFLs</td>
<td>GSM2535578</td>
</tr>
<tr>
<td>CTCF binding in CHICKEN HH20 FLs</td>
<td>GSM3182452</td>
</tr>
</tbody>
</table>

### Table 3: 4C-seq primers

<table>
<thead>
<tr>
<th>Viewpoint</th>
<th>Primer (Fw and Rv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dbx2 promoter</td>
<td><strong>A34_Fw</strong>: AATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
</tr>
<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
<td></td>
</tr>
<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
<td></td>
</tr>
<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
<td></td>
</tr>
<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
<td></td>
</tr>
<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
<td></td>
</tr>
<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
<td></td>
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<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
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</tr>
<tr>
<td><strong>Rv</strong>: AAATGATACGGCGACACGAGAATCCCTGGCTCTGCGATCTTGCAGTACGCTGGGTAGTTGCAGATC</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The sequences corresponding to the Illumina adaptors and barcodes are marked in blue and in red, respectively.
DNA ligase HC (Promega) in diluted conditions to promote intramolecular ligation. Samples were digested again with NalIII (New England Biolabs) and ligated with T4 DNA ligase HC (Promega) in diluted conditions. These templates were amplified using Expand long template (Roche) and inversed PCR primers flanked with adapters allowing multiplexing (Table 3). Barcodes (4 bp) were added between the Illumina adaptor and the specific DpnII primers. Libraries were prepared by means of 8-10 independent PCR reactions using 70 to 100 ng of DNA per reaction. PCR products were pooled and purified using the PCR purification kit (Qiagen). Multiplexed libraries were sequenced on Illumina HiSeq 2500 at the Sequencug platform of the University of Geneva to obtain 100 bp single-end reads. Demultiplexing, mapping and 4C-seq analysis were performed using a local version of the pipeline described in,92 on the mouse assembly GRCm38 (mm10). The profiles were smoothened using a window size of 11 fragments and normalized to the mean score in + − 5 Mb around the viewpoint. When multiple independent biological replicates were available, average 4C-seq profiles were calculated.

4.7 Data availability

Data are available in GEO (accession number: GSE161386).

4.8 Phylogenetic footprinting and HOX13/CTCF binding site analysis

The genomic sequence of the mouse DLE1 to 3 and Vista enhancer 1571 were used to identify their orthologous sequences in other mammalian and tetrapods species using the NCBI BLAST alignment tool93 with sensitive parameters. Orthologous sequences were then aligned using the Vista alignment tools94,95 (Shuffle-LAGAN algorithm). Evolutionary conserved binding sites for HOX13 proteins were identified with the ConTra v3 tool.96 Using the Transfac97 HOXA13 position weight matrixes and stringent parameters (core = 0.95, similarity matrix = 0.85). A predicted HOX13 BS was considered evolutionary conserved when it could be identified in at least four of the six mammalian species used in the multispecies alignment (human, cat, cow, elephant, rabbit, and opossum). For the identification of the CTCF binding sites in the mouse and chicken Dbx2 genomic regions, as well as the analysis of their orientation, we used the CTCFBS Prediction Tool (http://insulatordb.uthsc.edu/).99

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AUTHOR CONTRIBUTIONS

Leonardo Beccari: Conceptualization; formal analysis; investigation; methodology; project administration; supervision; writing-original draft; writing-review & editing. Gabriel Jaquier: Investigation; methodology. Lucille Lopez-Delisle: Data curation; formal analysis; methodology; software; validation; writing-review & editing. Eddie Rodriguez-Carballo: Investigation; methodology; resources. Benedicte Mascrez: Investigation; resources. Sandra Gatto: Formal analysis; resources.

CONFLICT OF INTEREST

The authors declare no competing interests.

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