Transgene- and locus-dependent imprinting reveals allele-specific chromosome conformations

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When positioned into the *integrin α-6* gene, an *Hoxd9lacZ* reporter transgene displayed parental imprinting in mouse embryos. While the expression from the paternal allele was comparable with patterns seen for the same transgene when present at the neighboring *HoxD* locus, almost no signal was scored at this integration site when the transgene was inherited from the mother, although the *Itga6* locus itself is not imprinted. The transgene exhibited maternal allele-specific DNA hypermethylation acquired during oogenesis, and its expression silencing was reversible on passage through the male germ line. Histone modifications also corresponded to profiles described at known imprinted loci. Chromosome conformation analyses revealed distinct chromatin microarchitectures, with a more compact structure characterizing the maternally inherited repressed allele. Such genetic analyses of well-characterized transgene insertions associated with a de novo-induced parental imprint may help us understand the molecular determinants of imprinting.

epigenetic | architecture | enhancer | Hox genes

Genomic imprinting is a phenomenon observed in mammals and marsupials, where some genes are expressed in a parent-of-origin–specific manner, with one of two alleles being stably silenced during its passage through the germ line (1). As such, imprinting can be considered as a regulatory process to control gene expression, and the dysfunction of this mechanism in humans leads to severe conditions, such as Angelman and Prader–Willi syndromes. Although this allelic silencing is, thus, admittedly of great benefit for the organism, both the evolutionary and ontogenetic origins of this parental-specific gene dosage remain unclear. Imprinted genes are usually associated with differentially methylated regions, a DNA mark that acts as the imprint to distinguish the parental alleles. In the mouse, many imprinted genes seem to cluster together at particular genomic loci under the regulation of a single major differentially methylated region called the imprinting control region (ICR). The ICR may, thus, control the imprinting for the full gene cluster, which can contain both maternally and paternally expressed genes as well as genes transcribed irrespective of parental origin (2, 3).

Several mechanisms have been proposed to account for imprint-associated gene silencing. One possibility is to directly trigger monoallelic DNA methylation followed by a repressive chromatin environment over one allele, whereas the other allele remains active. Gene transcription may be another mechanism used when the ICR contains the promoter for a noncoding RNA, which may target methylation and repressive histone modifications to imprinted genes (4–7). Imprinting may also rely on the presence of an insulator within the ICR, which restricts the activity of shared enhancers to either one of the target genes (8). The specificity of DNA methyltransferases (9) for ICRs and the maintenance of these marks throughout development at a time when the embryo undergoes genome-wide demethylation (10, 11) is poorly understood. Although some chromatin modifications can prevent de novo DNA methylation, others facilitate the recruitment of DNA methyltransferases (12–16). DNA or chromatin marks at ICRs are acquired in the germ line and persist

after fertilization in the developing embryo; eventually, they are erased during gametogenesis. This epigenetic reprogramming ensures a proper transmission of imprinted loci to the following generation, with appropriate parent-of-origin–specific gene expression. Defects in these processes have been reported to lead to severe congenital syndromes as well as cancer and obesity (17).

The *HoxD* locus is a cluster of genes with important functions during embryonic patterning. These genes are regulated through both local and long-range mechanisms, including regulatory sequences located within large flanking centromeric gene deserts. Although no imprinted region has been identified on this large genomic landscape on mouse chromosome 2 (18–20), some reports mention that the human syntenic region 2q31 may contain imprinted genes involved in bipolar affective disorder (21) and obesity (22). By studying long-range gene regulation at this locus, we noticed that a *lacZ* reporter transgene inserted into the *Itga6* gene displayed a clear parent-of-origin transcription. Transgene-induced imprinting was previously reported in several cases but generally in a position-independent manner (23–27). Here, we show that *lacZ* expression is silenced when the transgene is inherited from the mother and that this silencing is position-dependent, because the same transgene relocated nearby was not imprinted. We also show that the two alleles adopt different 3D conformations, and we describe how this local imprint impacts on the enhancer activity and expression of neighbor genes.

Results

Allele-Specific and Position-Dependent Expression of a *lacZ* Reporter Transgene. The mouse *HoxD* gene cluster is flanked by a centromeric gene desert (28) containing remote enhancer sequences necessary to control *Hoxd* gene transcription in developing digits (29). To investigate this complex regulation, we undertook a targeted transgenic approach, where an *Hoxd9lacZ* reporter transgene was inserted into several sites upstream of *HoxD* to capture regulatory influences. Whenever this transgene was inserted within 1 Mb of centromeric DNA, *lacZ* expression in developing digits was observed, showing its capacity to respond to the influence of the strong enhancers located nearby (30). We induced a large inversion by using a *loxP* site contained in the *Hoxd9lacZ* transgene targeted to the rel5 position (Fig. 1A) and another *loxP* site located in the *Itga6* locus (31). This 2.7-Mb large inversion [$HoxD^{Inv(rel5-Itga6)}$] repositioned both the *lacZ* reporter transgene
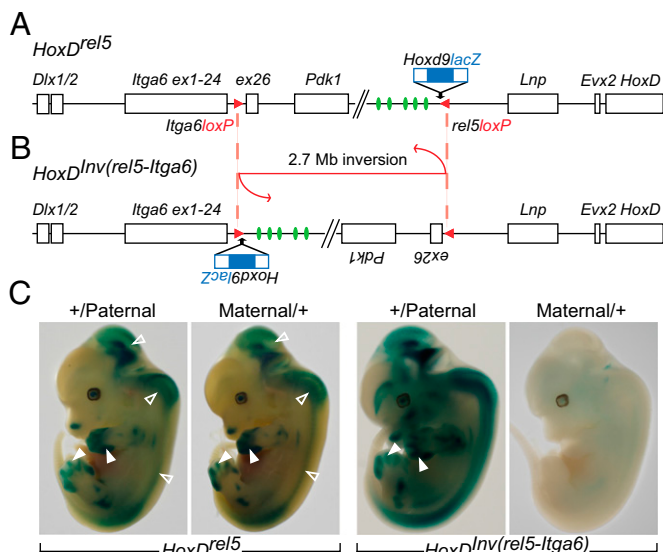
Fig. 1. Allele-specific expression of a *lacZ* transgene positioned inside the *Itga6* locus. (*A*) Scheme of the *HoxD*rel5 allele. An *Hoxd9lacZ* transgene (blue) was inserted into the *rel5* site along with a *loxP* site (red). On the same chromosome, another *loxP* site with an opposite orientation is present within the *Itga6* gene (red) substituting for a deletion of exon 25 (31). (*B*) Scheme of the same locus after the *HoxD*Inv(rel5-Itga6) inversion is induced. As a result, the *Hoxd9lacZ* transgene is repositioned within *Itga6* 2.7 Mb away from its original insertion site. Digit enhancer sequences (green) active on *HoxD* cluster genes are inverted along with the reporter transgene. (*C*) Whole-mount staining of β-gal activities of embryos heterozygous for the transgene (*Left*) before and (*Right*) after the inversion. Embryos carrying either a paternally or a maternally inherited transgene are marked +/Paternal or Maternal/+, respectively. In *Left*, β-gal reporter activities show strong and comparable patterns independent of parental inheritance. Staining is observed in the brain and the spinal cord (open arrowheads) as well as in the developing digits in response to the nearby-located enhancers. *Right* shows the imprinting of the *Hoxd9lacZ* transgene after inversion when inherited from the mother with an almost complete absence of staining. In *HoxD*Inv(rel5-Itga6) +/Paternal fetuses, staining persists in both the CNS and the digits (arrowheads), with some expression sites likely imposed by the unique transgene environment.

and the whole centromeric gene desert into the *Itga6* locus (Fig. 1*B*). Because enhancer sequences were inverted, *lacZ* activity was still scored in presumptive digits (Fig. 1*C*), whereas the endogenous, noninverted *Hoxd13* gene was severely down-regulated, because it was disconnected from the inverted enhancers (29).

When males were used to propagate the transgene, a strong *lacZ* staining was scored. In contrast, when the transgene was transmitted through the mother, F1 progenies surprisingly revealed either a very weak or no *lacZ* staining at all (Fig. 1*C*, *Right*). Females and males carrying the *HoxD*Inv(rel5-Itga6) allele were, thus, crossed to WT mice, and 309 embryos were stained between embryonic day 10.5 (E10.5) and E13.5. Of 87 embryos with the transgene inherited from the father (+/Paternal), 76 (87%) embryos showed the expected signal, whereas only 8 of 92 (9%) embryos carrying a transgene transmitted by the female (Maternal/+) displayed the expected *lacZ* activity (Fig. S1), thus suggesting that the *Hoxd9lacZ* transgene was maternally imprinted at this locus. This silencing on the maternal chromosome was not always complete, and some embryos escaped to different extents; 33% of Maternal/+ transgenic animals showed intermediate expression of the *lacZ* gene from little staining to almost no repression at all with a β-gal pattern comparable with +/Paternal transgenic animals (Fig. S1). These escapers, which were often littermates, usually showed distinct reduced patterns of expression, with clonal patches of cells and streaks showing *lacZ* activity. Likewise, some +/Paternal

transgenic animals showed little or no expression but with a much lower frequency (12%).

When located at its *rel5* position, before the inversion, the transgene showed no expression bias (Fig. 1*C*, *Left*), indicating that the imprinting was site-specific. We next assayed whether imprinting was transgene-specific by introducing another transgene into *Itga6*. We used an *Hoxd11lacZ* transgene inserted upstream of *Hoxd13* (32) to induce an inversion repositioning the transgene within the exact same breakpoint in the *Itga6* locus [*HoxD*Inv(TgHd11lacNsi-Itga6)] (33) (Fig. S2). As for *Hoxd9lacZ*, the inverted *Hoxd11lacZ* was strongly repressed when inherited from the mother, while active when inherited from the father. Before inversion, imprinting was again not observed (Fig. S2). We assessed whether the parent-of-origin trait of these transgenes was dependent on the integration site by analyzing other rearrangements where a *lacZ* transgene was inserted at various positions within the *HoxD* centromeric landscape. None of them revealed any parental-specific expression (Fig. S2) (29, 32, 34–36). Altogether, these results indicated that maternal repression was position-dependent and only occurred at the *Itga6* locus.

lacZ-Specific and Itga6 Locus-Specific Imprinting. We next investigated whether the *lacZ* sequence was required to initiate maternal imprinting and analyzed the *HoxD*Inv(HoxDRVIII-Itga6) inversion, which carries no transgene but breaks the *HoxD* cluster between *Hoxd11* and *Hoxd10* and thus, relocates the native *Hoxd11* gene into the *Itga6* locus (Fig. S3) (37). As a consequence, *Hoxd11* was placed at the same position as the imprinted *Hoxd11lacZ* transgene but without any *lacZ* sequence. We crossed mice carrying this inversion with mice lacking *Hoxd11* (38) to compare the expression of *Hoxd11* after either paternal or maternal inheritance. In situ hybridization and quantitative RT-PCR (RT-qPCR) quantifications revealed no significant variation in *Hoxd11* expression levels when derived from either parental allele (Fig. S3). Therefore, the presence of *lacZ* sequence was required to elicit maternal repression.

Because exogenous sequences may acquire the imprinted status of their insertion sites or potential imprinted genes located nearby (39), we assessed the allelic expression of the *Itga6* gene by RT-qPCR. E12.5 digits mRNA was collected from either +/Paternal or Maternal/+ *HoxD*Inv(rel5-Itga6) embryos. For each embryo, remaining body parts were separately stained for β-gal activity to ascertain that limbs from escaper embryos were not included. Because this modified allele carries an *in cis* deletion of exon 25 (31), we distinguished the parental origin of the *Itga6* alleles by using this polymorphism (Fig. S4). We used specific primers for this exon and did not detect any difference in expression levels between the paternal and maternal copies of *Itga6* (Fig. S4). This result suggested that the *Itga6* gene is not imprinted (at least for transcripts including exon 25), although the repression of both the *Hoxd9lacZ* and *Hoxd11lacZ* transgenes, when coming from the mother, was specific to this locus.

Methylation Status of the Transgene and Chromatin Marks. Both DNA methylation and the methylation of specific histone residues are associated with imprinting. Transgenes can drive parent-of-origin–dependent methylation (25), and repressed genes are generally methylated at their promoters. We compared DNA methylation profiles of Maternal/+ vs. +/Paternal *HoxD*Inv(rel5-Itga6) heterozygous embryos by performing bisulfite sequencing on DNA isolated from developing digits using a set of primers specific for the transgene. DNA methylation over this region corresponded to the activity of the transgene; the maternally inherited transgene showed almost 100% of methylation, whereas the paternal copy showed no methylation at all (Fig. 2*A*). Maternal escaper embryos showed a variable but lower rate of methylation (Fig. 2*B*). We also looked at the DNA methylation in germ cells of animals heterozygous for the transgene, and methylation was scored in oocytes but not sperm cells, illustrating a genuine maternal imprinting (Fig. 2*C*). The germ cells samples displayed the expected control patterns of methylation
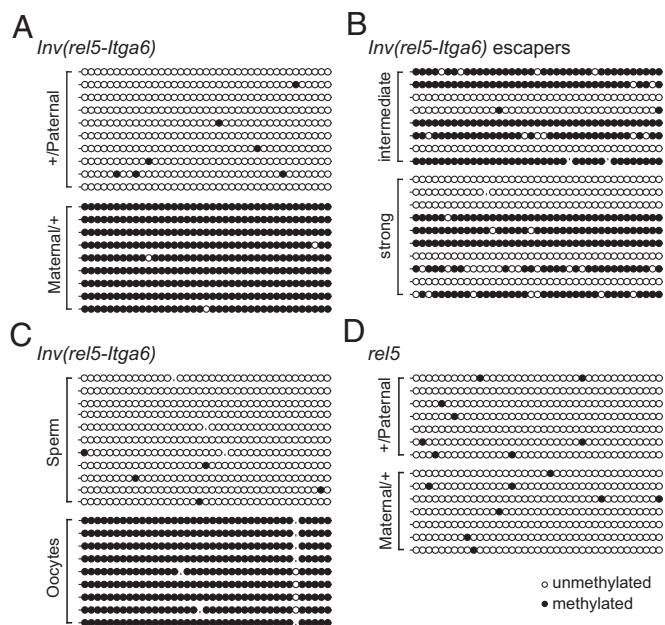
Fig. 2. Transgene imprinting is associated with differential allelic DNA methylation acquired in the germ line. (*A*) Methylation profiles on DNA from E12.5 presumptive digits of nonescaper *HoxD^Inv(rel5-Itga6)^* Maternal/+ or +/Paternal embryos using transgene-specific primers (Table S1). (*B*) Same analysis using escaper embryos. The methylation rate is strongly reduced, with variations between samples reflecting the variable expression of the transgene. (*C*) Analysis of oocytes and sperm from the inverted allele shows germ line-acquired methylation. (*D*) Differential methylation over the transgene was not found in noninverted animals.

for *H19* and *Snprn* (Fig. S5) (40). In addition, differential methylation was (as expected) not detected on the transgene in noninverted *HoxD^rel5^* animals (Fig. 2*D*), and the analysis of the *Hoxd11* gene without the *lacZ* component when positioned within the *Itga6* locus [*HoxD^Inv(HoxDRVIII-Itga6)^*] (Fig. S5) also showed no difference in DNA methylation patterns.

We investigated the trimethylation of H3K9, which usually covers repressive heterochromatin. This mark was described at most known ICRs (41) along with other specific inactive marks, such as H4K20me3 (42). We performed ChIP on *HoxD^Inv(rel5-Itga6)^* heterozygous mutant brains and digits using antibodies directed against these repressive marks as well as H3K27me3. A comparison between maternally and paternally inherited transgenes showed an enrichment of both H3K9me3 and H4K20me3 over the *Hoxd9* promoter and *lacZ* on the repressed allele compared with the active allele in both tissues (Fig. S6). The transgene was not active in the brain and covered by repressive H3K27me3 marks in +/Paternal heterozygous embryos, whereas H3K9me3 decorated the transgene in Maternal/+ embryos (Fig. S6). These results emphasized that maternal imprinting likely occurred in all embryonic cells. Studies of the offspring from a second generation of *Hoxd9lacZ* heterozygous mice showed that the imprint was reversible after passage through the germ line, such that males who had received a silent allele from their mother gave rise to *lacZ*-positive progeny (12 of 12), whereas female mice transmitted a silenced transgene (3 of 3). These epigenetic marks were, thus, correctly reset at each generation, such as for endogenous imprinted genes.

Allele-Specific 3D Conformations. We could assess potential allelic differences in 3D architectures by using the *lacZ* as a bait in chromosome conformation capture experiments. We previously showed that the enhancer elements controlling *Hoxd13* transcription in digits physically interact with the *HoxD* cluster (29) and that some of these contacts exist in tissues where *Hox* genes

are inactive, suggesting the presence of a preformed regulatory structure independent from the state of activation (29, 43) and matching a reported topological domain (44, 45). We could, thus, analyze the 3D conformation of this locus in either transcriptionally active or silenced states in the same cellular population. Allele-specific chromosome conformation capture was first performed on digits dissected from *HoxD^rel5^* embryos (i.e., specimen carrying the transgene before inversion). In these animals, the *lacZ* pattern correlates with the WT *Hoxd13* expression domain, regardless of parental origin. Primers within the *lacZ* reporter gene were used to identify interacting DNA sequences. As expected, most of these interactions involved sequences located within the gene desert centromeric to *HoxD*, and strong contacts were scored with the various enhancers previously described to control *Hoxd13* transcription in this embryonic tissue (Fig. 3*A*). The interaction profiles were indistinguishable when established from either a paternally or a maternally inherited transgene.

We next used the same bait to assess the spatial conformation of the locus in *HoxD^Inv(rel5-Itga6)^* inverted animals. In +/Paternal heterozygous embryos, the *Hoxd9lacZ* exogenous DNA contacted again all of the regulatory islands, which had been inverted along with the transgene (Fig. 3 *B* and *C*). In contrast, the interactions with either *HoxD* cluster genes or other digits enhancers located between the *HoxD* cluster and the *rel5* inversion breakpoint were absent as expected. However, new contacts were gained in the other side of the integration site, reflecting spontaneous interactions between the transgene and its novel genomic environment. We then looked at chromosome conformation when the inverted allele was inherited from the mother (i.e., when *lacZ* staining was not detected in digits). The silenced allele showed a more compact domain of interactions (*ca.* 70-kb large and equally distributed on both sides of the breakpoint), suggesting a tight folding of the transgene with closely neighboring DNA unlike the paternally inherited allele, where the bulk of contacts was mostly biased to the inverted gene desert and its enhancers (Fig. 3*B*). Noteworthy, the contacts established between the paternally inherited transgene with the digit enhancers were no longer observed in the maternally inherited copy (Fig. 3*C*). In fact, this allelic difference in chromosome conformations was more marked than the difference observed at the same *HoxD* locus between active and inactive tissues (29), suggesting that imprinted loci may generally display distinct allelic conformations associated with the two alleles (45).

Regulatory Side Effects of Imprinting. The comparison between interaction profiles on both paternal and maternal *HoxD^Inv(rel5-Itga6)^* inverted alleles revealed an unexpected effect. In digit cells, the active paternal *Hoxd9lacZ* transgene established significant contacts with the *Dlx1/Dlx2* locus, which is located *ca.* 300 kb centromeric to the *Itga6* breakpoint (Fig. 4*A*). In the maternal allele, the imprinted configuration prevented these contacts from occurring, which was seen by the absence of any 4C signal over the *Dlx* genes. In this case, the compaction of the locus isolated the transgene from both the digit enhancers (Fig. 3*C*) and the *Dlx* locus. *Dlx* genes are expressed during limb development mostly in the apical ectodermal ridge (i.e., where *Hoxd* genes are not transcribed). Thus, we checked if the contacts between the *lacZ* transgene and the *Dlx* locus initiated in the +/Paternal inversion would lead to a transcriptional enhancement of *Dlx* genes in digits after the inversion had positioned the digit enhancers closer to the *Dlx* locus than in the WT situation (Fig. 1 *A* and *B*).

We assessed the expression of both *Dlx1* and *Dlx2* by RT-qPCR in digits dissected from E12.5 embryos. In the paternally transmitted inversion, an increase in both *Dlx1* and *Dlx2* mRNAs levels was observed; these genes became regulated in digits along with the *lacZ* transgene, which was anticipated from the 4C profiles. This twofold increase was significant but not dramatic (Fig. 4*B*), indicating that the digit enhancers may preferentially target the *Hoxd9lacZ* transgene. Unexpectedly, however, the transcriptional enhancement of *Dlx* genes was reenforced when
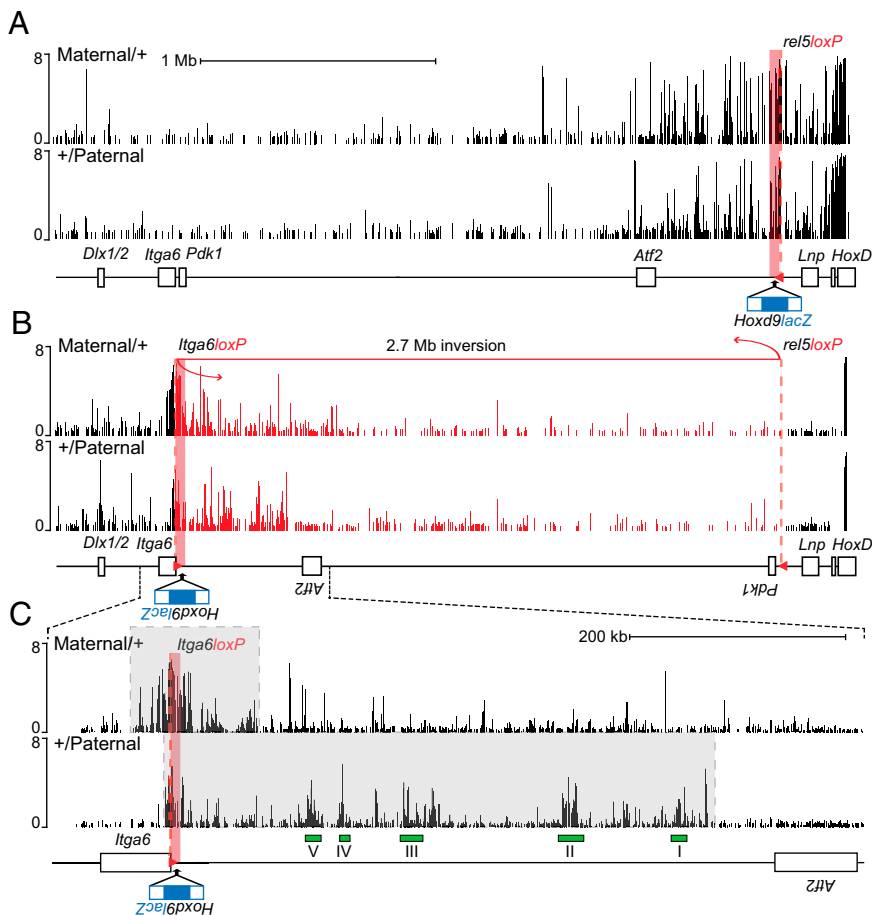
Fig. 3. Allele-specific conformations induced by the *Hoxd9lacZ* transgene when positioned into the *Itga6* locus. (*A*) The *lacZ* sequence is used as a bait for the 4C analysis (red square). Allele-specific chromosome conformation captures on chip from E12.5 *HoxD*[rel5]-dissected digits show similar interaction profiles for the maternal and paternal alleles. Contacts are mostly located in the centromeric gene desert flanking the *HoxD* cluster (from *Atf2* to *HoxD*). (*B*) Same experiment using *HoxD*[Inv (rel5-Itga6)] embryos showing allele-specific profiles of *lacZ* interactions. Inversion breakpoints are indicated with a dashed red line. For comparison, the segment corresponding to the inverted 2.7-Mb fragment (red) was manually inverted to represent the WT order in the DNA sequence. (*C*) Enlargement of the region shown under *B* with interactions on both side of the *lacZ* (gray boxes). Only the paternal allele interacts with previously identified digits enhancers (I–V, green rectangles). The *y* axis represents log2 ratio of the 4C normalized to input DNA.

the inverted allele was inherited from the mother, despite the absence of 4C contacts between the *lacZ* reporter transgene and the *Dlx* locus. In this case, the increase in transcripts levels was between four- and sixfold (Fig. 4*B*). This result suggested that, because the imprinting prevented contacts from occurring between the transgene and the enhancers, these enhancers were free to be partially reallocated to the *Dlx* locus.

Discussion

Parental imprinting leads to the nonexpression of either the maternal or the paternal allele, generally in all cells of the organism, and thus, it is an efficient regulatory mechanism to properly dose gene products. Among the various approaches used to understand the molecular bases of this epigenetic control, the analysis of imprinted transgenes can be instrumental (23–27). The imprinting that we describe here displays the hallmarks associated with this process. The silent version of the transgene was highly methylated at both the DNA and the particular residues of histone, two features ensuring correct allelic expression. Allele-specific silencing was reversible, because the passage through the opposite germ line could reset the imprint, and the epigenetic nature of the repression was confirmed by the analysis of oocytes and sperm, showing maternal germ line-specific DNA methylation.

Sequence-Specific and Position-Dependent Imprinting. However, this imprinting was locus-dependent, because the same transgene, which was maternally repressed when inserted at the *Itga6* locus, was readily expressed by both alleles when located at neighbor sites within a *ca.* 2-Mb-long DNA interval. Another *lacZ*-containing transgene also showed imprinting when moved into *Itga6*, showing that imprinting at this site was promoter-

independent. Also, when the same DNA sequence without the *lacZ* gene was positioned at this site, imprinting was not observed. From this information, we conclude that, in this instance, (*i*) the imprinting is triggered by the *lacZ* sequence, and (*ii*) this sequence must be positioned into the *Itga6* locus, which is itself not imprinted. The fact that the *lacZ* sequence may cause DNA methylation is not surprising (46), and a generic mechanism at work in the female germ line may recognize this sequence and deposit an imprint. However, this process does not explain the locus specificity reported in this work, a feature suggesting that particular DNA regions may be more prone than others to be imprinted if the right DNA sequences are there. Because some genes expressed from both alleles have been described inside imprinted clusters (47), another locus located nearby *Itga6* could be imprinted and impact on a transgene inserted in the vicinity. Also, the biallelic expression of *Hoxd11* when relocated into *Itga6* may be sustained by the neighboring of *Hoxd13*, which was inverted along with all its digit enhancers. A robust transcription of this latter gene remains in this inversion (48), which may somehow counteract the imprinting process.

Allele-Specific Chromosome Conformation and Side Effects. By using the *lacZ* reporter transgene as a viewpoint, we assessed the interaction profiles of this exogenous DNA in an allele-specific manner and showed the formation of clearly distinct, parent-of-origin interaction domains, which are similar to what was reported on the X chromosome (45, 49). In the imprinted maternal allele, contacts were mostly observed around the insertion site, suggesting a compaction of the chromatin over a *ca.* 140-kb large domain centered on the *lacZ* reporter transgene. The size of this domain indicates the extent of endogenous DNA that can be directly affected by the presence of the imprinted piece of DNA. Whether these increased contacts were caused by the
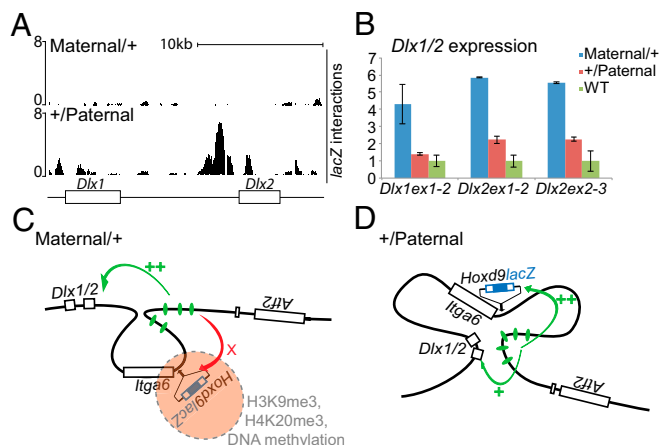
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Fig. 4. Impact of chromosome conformation on neighbor genes regulation. (*A*) *Dlx1* and *Dlx2* located 300 kb upstream of the *loxP* site within *Itga6* (Fig. 1*B*). Allele-specific 4C shows strong interactions between *Dlx* genes and the transgene when active (+/Paternal) in contrast to the silenced state (Maternal/+). (*B*) RT-qPCR on mRNA from E12.5 *HoxD^Inv(rel5-Itga6)* digits (here *n* = 2). *Dlx* genes are up-regulated in both the paternal and maternal inverted alleles but with a more robust increase in Maternal/+ embryos. (*C* and *D*) Hypothetical conformations of the inverted *Hoxd9lacZ* allele when (*C*) maternally or (*D*) paternally inherited. (*C*) On maternal repression, chromatin compaction prevents interactions from occurring between the digits enhancers present in the inversion and the transgene. As a consequence, these regulatory elements are hijacked by the *Dlx* locus. (*D*) In contrast, in the active allele, the transgene interacts with the digits enhancers, thus reducing the interaction with the *Dlx* locus.

spreading of epigenetic marks (H3K9me3, H4K20me3, and DNA methylation) from the *lacZ* reporter gene to flanking sequences remains to be assessed. On the repressed allele, although some contacts were still observed between the inverted gene desert and the transgene, interactions with the digits enhancers were lost, although their topological relationship with the target *Hoxd9lacZ* promoter remained unchanged after inversion. This observation was expected, because the imprinted transgene was no longer transcribed in these cells. Interestingly, however, this result showed that even such a robust regulatory mechanism (29), which normally sustains one of the strongest transcriptional outcomes during development (50), is unable to elicit any response from the reporter transgene when imprinted, thus illustrating the remarkable efficiency of the inherited repressive configuration.

The loss of contacts between digits enhancers and the transgene in the imprinted allele likely favored a reallocation of these regulatory elements to the *Dlx1/2* locus (Fig. 4*C*). Some contacts between this latter locus and the *lacZ* transgene were observed in the paternal, nonimprinted allele in association with a slight gain of expression of both genes in digits. We hypothesize that, after inversion, the *Dlx1/2* locus was included into a novel regulatory landscape under the control of digit enhancers. In the paternal allele, however, most of the contacts established by the *lacZ* transgene were biased to the inverted gene desert (Figs. 3 and 4*D*). In the imprinted allele, the transgene and surrounding sequences were excluded from this landscape because of the repressive configuration, and hence, the digit enhancers increased their interactions with the *Dlx* locus (Fig. 4).

Whether such enhancer reallocations routinely occur around imprinted loci remains an open question. Although the induced side effects may be functionally neutral in many instances, the potential of imprinting as a way to globally regulate enhancer–promoter interactions over large distances should not be overlooked. High-throughput studies have revealed that chromosomes are organized into domains of preferential regulatory interactions (44, 45), and our results suggest that the presence of an imprinted locus within such a domain may lead to a significant

reorganization, which may not be visible until allele-specific approaches are considered in the definition of such domains (45, 49). In any case, the potential role of imprinting in organizing enhancer–promoter interactions may be difficult to evaluate, because its benefit may occur in restricted cell populations that are not yet amenable to genome-wide studies.

Variability in Transgene Silencing. The imprinting reported here shows an unusual frequency of escapers with both the maternal and paternal alleles. Maternal escapers (escaping repression) came as a significant number and displayed patterns of variegation different from one another. For example, some embryos displayed staining in a given tissue but not in another tissue rather than having a general increase of the *β-gal* staining everywhere. Such a tissue-specific variability can hardly be explained by the sole defect in the initial imprinting process and must involve cellular-specific component. In this view, it is possible that enhancers can sometimes win over repressive configuration and still elicit a transcriptional response from an imprinted locus. This mechanism may be helped by a somehow weaker repression, which would derive from the initial imprinting process, and such escapers may, thus, reflect the combination of both factors. The study of such escapers over several generations will be of interest in this respect.

Paternal escapers were less frequent, but 1 of 10 embryos did not express (or weakly express) *lacZ*. The low frequency and variability of these escapers made their study difficult. One possibility is that this repression was caused by other mechanisms used to silence exogenous DNA. In fact, should the same frequency of nonimprinted but silenced embryos be present within the pool of maternally imprinted embryos, they would remain unnoticed. Alternatively, the presence of the transgene may induce either maternal or paternal imprinting but with different frequencies. Finally, it is also conceivable that, after inversion, the enhancer–promoter interactions were weakened because of a new genomic topology leading to the weak or nonexpression of the transgene in some cases. In this context, one should remember that the 4C approach shows an average of contacts taken at a given time for a pool of cells, and hence, it merely reflects a trend, thereby diluting potential distinct configurations formed at low frequency.

Material and Methods

Mouse Strains, in Situ Hybridization, and *lacZ* Staining. All strains were described before: *HoxD^rel5* (29), *HoxD^Inv(rel5-Itga6)* (29), *HoxD^TgHd11lacNsi* (32), *HoxD^Inv(TgHd11lacNsi-Itga6)* (33), *HoxD^Inv(HoxDRVIII-Itga6)* (37), *HoxD^DelRXIIDel(13-8)* (38), *HoxD^TpSB1* (36), *HoxD^Del(TpSB1-Atf2)* (29), and *HoxD^Inv(TpSB1-Itga6)* (51). Genotyping conditions are found in the references mentioned above. Mice heterozygous for the inversion *HoxD^Inv(HoxDRVIII-Itga6)* were crossed with animals carrying a deletion from *Hoxd8* to *Hoxd13* [*HoxD^DelRXIIDel(13-8)* or Δ]. Whole-mount in situ hybridization and *lacZ* staining were performed using standard protocols. The *Hoxd11* in situ hybridization mouse probe has been described previously (52).

Bisulfite Sequencing. Genomic DNA was isolated from E12.5 digits, and *β-gal* activity was assessed on the remaining parts of the embryos to discard escapers. Germ cells were collected directly in 40 μL DNA lysis buffer of 33 mM Tris·HCl (pH 8), 1 mM EDTA, and 10 mg/mL SDS; 2.5 μL sperm were squeezed from vas deferens, and oocytes (160 and 212 per tube) were collected from superovulated females (3–6 wk postpartum). Sodium bisulfite conversion and cleanup for methylation analysis of either 1 μg DNA (not digested) from digits or total volume of germ cells in lysis buffer were performed using the QIAGEN EpiTect Bisulfite Kit. For the analysis of the transgene, single-round PCR amplification was performed using specific unbiased primers (Table S1) for the converted DNA. *H19* and *Snrpn* were used as controls (40). Analysis of clones was performed using the BISMA web interface (53) or the BiqAnalyzer software (54). Clones were accepted at a conversion rate of 95%, and detection of clonal molecules based on nonconverted cytosine or base error was used to avoid clones originating from same template DNA. For oocytes, the conversion rate was around 80%. Lollipop methylation plots were generated with BiqAnalyzer.

RNA Extraction, RT-qPCR, and ChIP. E12.5 digits were stored at −80 °C in RNA*later* RNA Stabilization Reagent (Ambion) before genotyping. RNA was extracted using the QIAGEN RNeasy Plus Micro Kit after tissue disruption and homogenization. Reverse-transcription qPCR analyses were performed using the primers listed in Table S1. ChIP–RT-qPCR (55) was carried out on E12.5 digits and brains of nonescaper embryos. One-half of a forebrain or pools of six pairs of distal limbs fixed for 15 min in 1% formaldehyde in PBS were used for each ChIP using 4 μL anti-H3K4me3 (pAb-056-050; Diagenode), H3K27me3 (17–622; Millipore), or H4K20me3 (ab9053; abcam) antibodies and immunoprecipitated with EZview Red Protein G/A Affinity Gel Beads from Sigma (primers for RT-qPCR are in Table S1).

Chromosome Conformation Capture and Tiling Array Analysis. 4C analysis was as described (29) using E12.5 digits; 2% formaldehyde-fixed nuclei from collagenase-dissociated tissues were stored at −80 °C before genotyping. Pools of 10 pairs of mutant digits were used for each 4C analysis using *DpnII* restriction enzyme to identify interacting partners of the *lacZ* sequence from the transgene (primers are in Table S1), allowing for an allele-specific conformation capture. 4C libraries were fragmented, and then, they were labeled and hybridized on chromosome 2 Affymetrix tiling arrays. For *HoxD*$^{Inv(rel5-Itga6)}$ heterozygous embryos, two independent experiments were conducted for both parental transmissions. Tiling Analysis Software from Affymetrix was used to extract and analyze raw hybridization using gDNA input to normalize (29). Log2 data were plotted on University of California Santa Cruz genome browser after genome conversion (mm5 to mm9).

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