A Discrete Transition Zone Organizes the Topological and Regulatory Autonomy of the Adjacent Tfap2c and Bmp7 Genes

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Abstract

Despite the well-documented role of remote enhancers in controlling developmental gene expression, the mechanisms that allocate enhancers to genes are poorly characterized. Here, we investigate the cis-regulatory organization of the locus containing the Tfap2c and Bmp7 genes in vivo, using a series of engineered chromosomal rearrangements. While these genes lie adjacent to one another, we demonstrate that they are independently regulated by distinct sets of enhancers, which in turn define non-overlapping regulatory domains. Chromosome conformation capture experiments reveal a corresponding partition of the locus in two distinct structural entities, demarcated by a discrete transition zone. The impact of engineered chromosomal rearrangements on the topology of the locus and the resultant gene expression changes indicate that this transition zone functionally organizes the structural partition of the locus, thereby defining enhancer-target gene allocation. This partition is, however, not absolute: we show that it allows competing interactions across it that may be non-productive for the competing gene, but modulate expression of the competed one. Altogether, these data highlight the prime role of the topological organization of the genome in long-distance regulation of gene expression.

Introduction

Differential regulation of gene expression transforms shared genomic information into the cell type-specific programs underlying organismal development and homeostasis. In vertebrates, it is not uncommon to find gene regulatory elements, in particular enhancers, hundreds of kilobases away from their target gene (reviewed in [1,2]). The mere scale of this genomic distance raises the question of how enhancers and promoters can find each other, and how enhancers distinguish between their specific target and other neighboring genes, which may even lie much closer. Understanding the molecular basis of such specific interactions is essential as their impairment can lead to mis-expression of the normal target gene [3,4] or to inappropriate activation of neighboring genes [5–8], with often severe phenotypic consequences [7,9–12].

Enhancers can typically activate transcription from different promoters, a property that is part of their initial definition [13] and which has been amply used to assess enhancer activity. Many enhancers act pervasively across their endogenous genomic surroundings [14,15], and enhancer sharing is not unusual between neighboring genes, particularly within multigene clusters [16–22]. Noteworthy, this can also occur between genes with no functional relationship except genomic proximity [9,23–25]. Nonetheless, in many loci, adjacent genes exhibit distinct expression patterns, implying the existence of mechanisms that limit the promiscuous potential of enhancers.

Different mechanisms and genomic elements have been invoked to explain enhancer-target gene specificity. They can be divided into two main categories, depending on whether they may promote interactions (eg. nature of the promoter, tethering elements [26,27]), or block them. Amongst the latter, insulators prevent contact of an enhancer with an adjacent promoter, when placed in between [28–30]. This capacity of insulators to organize the genome in separate regulatory compartments designate them as critical components in ensuring specificity of cis-regulatory interactions [31]. However, only a handful of insulator elements have been functionally assessed in their native genomic context, and therefore their mode(s) of action is still poorly understood. Contrary to earlier models, a growing body of evidence suggests that insulators do not function autonomously, but rather through higher-order 3D conformations [32].

The necessity to consider the genome’s three-dimensional organization is further highlighted by genome-wide high-resolution interaction maps obtained by chromosomal conformation capture techniques [33]. These studies revealed that the genome is
Author Summary

The specificity of enhancer-gene interactions is fundamental to the execution of gene regulatory programs underpinning embryonic development and cell differentiation. However, our understanding of the mechanisms conferring specificity to enhancers and target gene interactions is limited. In this study, we characterize the cis-regulatory organization of a large genomic locus consisting of two developmental genes, Tfap2c and Bmp7. We show that this locus is structurally partitioned into two distinct domains by the constitutive action of a discrete transition zone located between the two genes. This separation restricts selectively the functional action of enhancers to the genes present within the same domain. Interestingly, the effects of this region as a boundary are relative, as it allows some competing interactions to take place across domains. We show that these interactions modulate the functional output of a brain enhancer on its primary target gene resulting in the spatial restriction of its expression domain. These results support a functional link between topological chromatin domains and allocation of enhancers to genes. They further show that a precise adjustment of chromatin interaction levels fine-tunes gene regulation by long-range enhancers.

compartmentalized in topologically-associating domains (TADs) [34,35]. TADs have been proposed to contribute to gene expression by limiting enhancer action [36,37]. In support of this view, genes located within the same TAD tend to be expressed coordinately [35,38], and TADs have been found to encompass the regulatory domains defined by long-range enhancer activities [15,39]. Recent works have addressed the finer-scale structural organization of TADs, revealing a complex hierarchy of interactions, which may contribute to mediate long-distance interactions between enhancers and promoters [40,41] and to subdivide them into distinct regulatory domains [15]. In most instances, the functionality of structural contacts is difficult to evaluate precisely and the causal relationship between structural conformation and gene regulation remains unclear.

To better understand the relationships between 3D structural properties of the genome and enhance-promoter allocations, we focused on a large interval of approximately 0.5 Mb containing two different developmental genes, Bmp7 and Tfap2c. These two genes, which encode a secreted signaling molecule and a nuclear transcription factor, respectively, are active in multiple tissues and organs during embryogenesis [42–48]. Both genes have promoter architectures compatible with tissue-specific and long-distance regulatory inputs [49]. Their expression overlaps in the limbs, forebrain and branchial arches of mid-gestation mouse embryos, while in other contexts, their expression is specific of one or the other and exclusive. Therefore this locus constitutes an ideal system to study the control of long-distance enhancer specificities.

To investigate the regulatory organization of this locus, we used a transposon/recombination-based chromosomal engineering approach [14]. We show here that the genomic interval consists of two largely independent regulatory domains, corresponding to each of the two genes. Analysis of the chromatin conformation of re-engineered genomic configurations identified a central transition zone (TZ) that defines different topological sub-domains. Importantly, the allocation of enhancers to one or the other gene is determined by this partition. Altogether, our data support the view that the topological organization of the genome restricts enhancers to specific domains, determining therefore their “specific” target gene choice. Interestingly, we found that the presence of Bmp7 in cis has a mild influence on the expression level of Tfap2c in the developing forebrain, indicating that the position of the two genes to different topological domains does not lead to an absolute insulation.

Results

Mapping the regulatory landscapes of the Tfap2c-Bmp7 locus

To determine the regulatory organization of the Tfap2c-Bmp7 locus, we adapted the GROMIT (Genome Regulatory Organization Mapping with Integrated Transposons) strategy [14]. Firstly, at the 3’ end of the endogenous Bmp7 gene, we inserted a transgene consisting of a Sleeping Beauty transposon comprising 1) a regulatory sensor gene (a LacZ reporter under the control of a short naive synthetic promoter region derived from the human β-globin gene [14,50]) and 2) a loxP site. After establishment of a mouse line carrying the correct insertion, we removed the selection marker used to identify candidate targeted ES clones, a step which left behind an additional loxP site, next to the Sleeping Beauty transposon. We designated this allele as SB-B(3end) (Fig. 1). By serial remobilisation of the transposon in vivo [14], we obtained several insertions located in this region of mouse chromosome 2 (S1 Table). Of these, seven insertions were distributed along the Tfap2c-Bmp7 locus (Fig. 1A): three very close to SB-B(3end) (within 23 kb), one (SB-B(up)) 20 kb upstream of Bmp7, and another one in the first intron of Bmp7 (SB-B(in)). The remaining two (SB-A1 and SB-A2) lie within the large intergenic region separating Tfap2c and Bmp7. In parallel, we established a mouse line (BA0758) from an ES clone carrying a βgeo gene-trap insertion in Tfap2c [51].

The Tfap2c-Bmp7 locus consists of two distinct regulatory domains

We analyzed the expression pattern of the regulatory sensor at different insertion sites in E10.5 to E12.5 mouse embryos, at stages when Tfap2c and Bmp7 show both shared and specific expression patterns (Fig. 1, S1 Fig.). The two insertions located between Tfap2c and Bmp7 (SB-A1 and -A2) showed very similar LacZ staining in the oro-facial region, the branchial arches, and in the forebrain (Fig. 1B, left). These three expression domains are strikingly consistent with reported expression patterns of Tfap2c [42] and particularly with the Tfap2c LacZ gene-trap allele (Fig. 1B- S1 Fig.). This overlap and agreement in expression suggested that SB-A1 and -A2 were included in the Tfap2c “regulatory domain” [15]. The expression of the reporters showed however different relative intensity between the lateral and medial part of the forebrain: while BA0758 and SB-A1 were preferably expressed in the lateral forebrain, with weaker expression in the medial region, SB-A2 showed the inverse pattern, with a stronger medial than lateral LacZ staining. Such position-effects (the promoter is the same for SB-A2 and SB-A2) are not uncommon within regulatory domains [15,52]. They may reflect the presence in the locus of several forebrain enhancers with distinct medial/lateral activity and different range of action.

These forebrain expression domains were not observed with any of the four insertions located within the 23-kb region at the 3’ end of Bmp7 (Fig. 1B, S1 Fig.), suggesting that the telomeric limit of Tfap2c regulatory domain is upstream of this region. More distant insertions in Bmp7 (SB-B(in); SB-B(up)) showed weak medial-only forebrain expression at E11.5, with no lateral expression detected, as also observed for Bmp7 [44]. None of the six telomeric insertions showed the characteristic oro-facial expression observed
with the Tfap2c-associated insertions. In contrast, they shared several common expression domains not reported by the SB-A1 and -A2 insertions (Fig. 1B). The four insertions at the 3’ end of Bmp7 and SB-B(in) showed all prominent staining for Bmp7 expression in the developing heart (from E10.5 to E12.5), and in the interdigital mesenchyme (at E12.5). SB-B(up) displayed only faint LacZ staining in the interdigital mesenchyme, and no staining in the heart. However, LacZ expression from this position overlapped characteristically with other SB-B insertions in the whiskers, nasal pits, and forebrain (S1 Fig.), defining collectively a regulatory domain distinct from the one associated with Tfap2c. This domain includes Bmp7, and accordingly, several of the reported activities overlapped with known Bmp7 expression domains [47,53]. Some regions of the Bmp7 expression domain were not reflected accurately in the activity of the SB reporters, being either missing or spatially expanded. These differences may arise from the limited range of action of some promoter-proximal enhancers [53], and/or from the different post-transcriptional stability and dynamics of LacZ compared to the endogenous Bmp7 transcripts.

Overall, the regulatory activities detected by the sensor differed significantly between the centromeric and telomeric part of the locus, and highlighted two distinct and non-overlapping regulatory domains, each defined by multiple distinct tissue-specific activities, one domain corresponding to Tfap2c and the other to Bmp7. We focused for subsequent analyses on the forebrain (medial and lateral) and heart, as representative markers of these two domains. In these two tissues, the expression pattern of the different genes is stable from E10.5 and E12, contrasting with the dynamic expression of these genes in the developing limbs and face. Also, for these two expression domains, it is technically possible to dissect from embryos the part where the gene or the enhancer is active, without the contribution of too many non-expressing cells.

Enhancers in the intergenic region control either Bmp7 or Tfap2c

To further characterize the functional relevance of these two domains and associated enhancers, we used in vivo Cre-mediated recombination to engineer chromosomal deletions removing either the telomeric half or the whole of the intergenic region (Fig. 2). Each deletion was produced using a combination of loxP sites in cis and trans [54] in order to keep the LacZ sensor at the deletion breakpoint (see Materials and Methods). With the TAMERE strategy, we also obtained a large duplication, reciprocal to del3 (S2 Fig.). All three deletions led to a complete loss of LacZ expression in the embryonic heart and forebrain (Fig. 2B) suggesting that the enhancers detected by SB-A1 and SB-B(3end) lie in the region encompassed by del1. Dup3-lacZ embryos showed LacZ expression in the heart similar to SB-B(3end), corroborating the presence of the heart enhancer(s) at the 3’ side of Bmp7 (S2 Fig.). These deletions also provided information on the locations of additional enhancers associated with other expression domains (S2 Fig.).

We next determined if the enhancers present in the del1 interval contributed to Tfap2c and Bmp7 expression by whole-mount in situ hybridization and RT-qPCR (Fig. 2C–D). In del1 homozygous embryos, Bmp7 expression was drastically reduced in the heart compared to wild-type littermates, while the very weak expression of Tfap2c in the heart was unaffected (Fig. 2C). In the forebrain, where both genes are expressed, we found an almost complete loss of Tfap2c expression in both the medial and lateral parts of del1 embryos. In contrast, Bmp7 expression was barely affected and showed only a slight reduction in the lateral forebrain (Fig. 2C).

These analyses demonstrated a critical role of elements located within the del1 segment for the specific expression of Tfap2c in the forebrain and of Bmp7 in the heart, respectively. Several peaks enriched for chromatin marks associated with active enhancers (H3K27ac, EP300) have been detected within this region in the forebrain, where both genes are expressed, we found an almost complete loss of Tfap2c expression in both the medial and lateral parts of del1 embryos. In contrast, Bmp7 expression was barely affected and showed only a slight reduction in the lateral forebrain (Fig. 2C).

To confirm that del1 contained enhancers with the expected activities, we cloned FB1, an evolutionarily conserved element enriched for both H3K27ac and EP300 in the forebrain, upstream of the regulatory sensor construct. In this transgenic assay, FB1 drove specific and reproducible LacZ expression in the forebrain in E11–12 embryos (Fig. 2E), including the Tfap2c expression...
domain. However, FB1 appeared broadly and equally active in both medial and lateral forebrain, contrasting with the restricted expression detected by the same reporter gene when inserted in the endogenous locus in either side of FB1. In this context, it showed alternatively preferential expression in the lateral (SB-A1, like \textit{Tfap2c}) or medial (SB-A2). These differences suggested that additional factors – possibly the other H3K27ac-region present in the vicinity (see below) – may modulate FB1 intrinsic activity in a position-dependent manner. Amongst the predicted heart enhancers, one of them (mm75) had been tested previously \cite{58} and reported to have broad enhancer activity in the heart of E11.5 mouse embryos (Fig. 2F).

Taken together, these data demonstrated that the del1 region contained heart-specific and forebrain-specific regulatory element(s) critical for the expression of \textit{Bmp7} in the heart, and of \textit{Tfap2c} in the forebrain, respectively. Importantly, these elements appeared to be dispensable for the regulation of one another’s genes. These selective influences and the separate location of the different enhancers further confirmed the partition of this genomic interval into two distinct regulatory domains containing enhancers which act exclusively on one or the other gene (Fig. 2G).

**Topological organisation of the locus**

We next investigated how the regulatory subdivision of the locus corresponded to its topological organization. Hi-C data available for mouse ES cells and cortex \cite{34} suggests that the locus has a relatively loose topological structure, confined between two prominent topologically associating domains (Fig. 3A, 3B).
To determine the pattern of physical contacts involving *Tfap2c* and *Bmp7*, we carried out circular chromatin conformation capture experiments followed by high-throughput sequencing (4C-Seq) using the promoters of these two genes as viewpoints (Fig. 3). We performed these 4C-Seq analyses on dissected samples where one and/or the other gene were expressed (E11.5 heart, medial and lateral forebrain) and whole body of E11.5 embryos where most cells are non-expressing either of the two genes. We also included samples from E12.5 limbs, which comprised a majority of non-expressing cells.

For both viewpoints, the 4C profiles highlighted a large primary interaction domain characterized by high 4C read counts (Fig. 3B, C). We applied a segmentation algorithm [59] to delineate this primary domain in the different conditions (S2 Table). The calculated primary interaction domains for a given viewpoint were nearly identical across the different tissue samples. The 4C profiles were predominantly similar between samples, with the exception of a moderate increase of the 4C signals over the enhancers associated with each gene in the tissues in which they are active (*for Tfap2c*; FB1 and flanking H3K27ac-enriched regions in the brain samples; for *Bmp7* mm75 and surrounding H3K27ac-enriched regions in the heart sample). We confirmed the increased interactions of *Tfap2c* with FB1 and of *Bmp7* with mm75 in an independent 3C experiment (S3 Fig.). Importantly, the reciprocal 3C experiment with FB1 as a viewpoint showed that it contacted strongly *Tfap2c* in the forebrain, but not in the heart, and had much weaker/rarer contacts with *Bmp7*.

Noteworthy, the *Tfap2c* domain and the *Bmp7* domain end shortly before the edges of the flanking TADs detected in mouse ES cells [34], consistent with the notion that these 4C primary domains corresponded to the structural conformation adopted by the locus. In all samples, the primary contact domains of one gene included the enhancer regions we found associated with it, but excluded the ones associated with the other gene. Nevertheless, we observed a consistent overlap between the two domains, demarcating a region of about 10- to 30-kb region, which we termed the transition zone (TZ). To further characterize this region, we used two additional viewpoints for 4C analysis (Fig. 3D–E). Contacts observed from a viewpoint located just before the centromeric end of the *Bmp7* primary domain showed extensive overlap with the latter, extending broadly over *Bmp7* but not stopping almost abruptly at the TZ (Fig. 3D). Similarly, FB1 showed only weak contact with positions located on the other side of the TZ (S5C Fig.). This asymmetry in the distribution of contacts suggested the TZ indeed corresponds to a conformational transition between two different conformations. Importantly, a viewpoint located in the TZ itself showed prominent contacts extending towards both genes (Fig. 3E), consistent with the strong 4C signals observed over the TZ in the reciprocal 4C experiments.

Next, we performed 4C analyses on del1 homozygous embryos, where the TZ region was deleted together with a larger part of the locus, including the different enhancers (S6 Fig.). In this context, we observed a wide extension of the contacts made by *Tfap2c* (resp. *Bmp7*) in the telomeric (resp. centromeric) region, over distances larger than the size of the deleted region. At the same time, the centromeric (resp. telomeric) profiles remained highly similar between WT and del1. Interestingly, the intervals with frequent contacts by *Tfap2c* and *Bmp7* now largely overlapped, as if they “merged” into one domain only limited by the adjacent TADs (S6 Fig., S3 Table). These new extended contacts supported the notion that the TZ may contribute to delineate two distinct structural domains. However, as del1 also significantly reduced the linear distance between *Tfap2c* and *Bmp7*, we decided to use other types of alleles to challenge the structural and regulatory organization of the locus and to test the influence of the TZ on these.

**Chromosomal rearrangements led to enhancer reallocation**

We used insertions carrying *loxP* sites in the opposite orientation to the one left at the SB-B(3end) position in *cis* to engineer three balanced inversions by CRE-mediated recombination (Fig. 4A, S1 Table). In INV-L1 and -L2, the distance between *Bmp7* and the heart enhancer increased to 5.7 and 1.1 Mb, respectively, whereas the relative order and distances between *Tfap2c*, the enhancers and the TZ region were unchanged (S7 Fig.). In INV-M, the heart enhancer was now equidistant from *Bmp7* and *Tfap2c* (187 and 207 kb, compared to distances of 80 kb and 312 kb in the wild-type allele, with mm75 taken as reference). However, in this allele, the TZ was now located between *Bmp7* and the heart enhancer(s). With each inversion, the *LacZ* reporter remained adjacent to the heart enhancer region and displayed its normal heart expression (Fig. 4B, S7 Fig.), demonstrating that these rearrangements did not disrupt heart enhancer activity. In the three inversions, *Bmp7* expression was strongly reduced in the heart, comparable to levels observed with del1 (Fig. 4C). In contrast, *Tfap2c* expression was enhanced by a thousand-fold in the heart of INV-M animals (Fig. 4D), implying that in this genomic configuration, the heart enhancers now activated *Tfap2c* instead of *Bmp7*. This complete switch of the heart enhancer(s) from *Bmp7* to *Tfap2c* coincided with the new relative position of the TZ. The importance of the position of the TZ in the genomic configuration, the heart enhancers now activated *Tfap2c* instead of *Bmp7*. This complete switch of the heart enhancer(s) from *Bmp7* to *Tfap2c* coincided with the new relative position of the TZ. The importance of the position of the TZ was further supported by a lack of up-regulation of *Tfap2c* in INV-L1 and INV-L2 (Fig. 4D), where its location with regards to the TZ/heart enhancers remained unchanged. In INV-L1, we instead found an up-regulation of *Ptgis* (Fig. 4E), which was now located on the other side of the TZ, next to mm75. As *Ptgis* was closer to the heart enhancer (S7A Fig.) we were unable in this case to fully rule out a possible influence of distance on promoter choice. However, in INV-L2, *Dok5*, the new gene juxtaposed “next to” the heart enhancer(s) opposite to TZ was much further away than *Tfap2c* (1.1 Mb versus 0.3 Mb). In this context, neither *Dok5* (Fig. 4F) nor *Tfap2c* were up-regulated in the heart, ruling
out the possibility that the heart enhancer(s) act simply by default the nearest gene.

Intrinsic asymmetric distribution of interactions around the TZ

To examine at the consequences of these rearrangements on the structural conformation of the region, we performed 4C experiments on INV-M and INV-L2 embryos (Fig. 5, S8–S10 Figs.). In INV-M, as in WT controls, Tfap2c showed robust interactions over a domain extending up to the TZ. Due to the inversion, this domain now included the heart enhancer, which displayed much stronger interaction with Tfap2c than those observed in WT (S8A Fig., pink versus grey arrow), a result consistent with mm75 now activating Tfap2c. Conversely, the new primary interaction domain of Bmp7 stopped at the TZ, with a very reduced 4C signal over the heart enhancer in INV-M when compared to WT (S8D Fig., grey versus pink arrow). The viewpoint located between mm75 and TZ, which was part of the Bmp7 interaction domain in WT, showed in INV-M broad and extended contacts overlapping with the Tfap2c interaction domain, ending at the TZ region (Fig. 5B). Interestingly, the inversion had no effect on the 4C profile of the TZ-associated viewpoint, which extended on both sides in all configurations. Thus, in INV-M as in WT, the locus appeared structurally partitioned at the TZ: instead of maintaining their normal contacts and regulatory preferences, genes and regulatory elements established new interactions, depending on their respective position in relation to the TZ.

In INV-L2 embryos, the 4C profile of Tfap2c appeared generally unchanged and did not expand across the TZ into its new flanking region. The TZ-flanking viewpoint remained still limited by the TZ, but highlighted on the other side a broad domain of nearly 1 Mb in the Dok5-Cbln4 gene desert, which is now adjacent to it. The 4C signal was strongly diminished before reaching the promoter of Dok5, which may explain the lack of up-regulation of this gene in the heart of INV-L2 embryos (Fig. 4F). Again, the TZ itself contacted both flanking regions, the relocated Tfap2c domain, and the new Dok5-Cbln4. Importantly, in INV-L2, Bmp7 showed broad contacts over the region now present at its 3’ end, extending for up to 0.5 Mb further in the Cbln4 locus, supporting the notion that the presence of the TZ limited the extent of the Bmp7 contact range (Fig. 5C, S3 Table).

Remarkably, the new distribution of 4C contacts in the different rearrangements appeared to follow quite strictly the relative position of the TZ. It did not appear to depend on the nature of the flanking sequences themselves. The directional bias of contacts made by the viewpoint flanking the TZ is the same in the different configurations (WT, INV-M and INV-L2) (S10 Fig., on the right), irrespectively of the flanking sequences.

Fine-tuning of Tfap2c forebrain expression across the TZ

The expression and structural changes observed in the heart suggested that the TZ behaved as a simple insulator region. In INV-L1 and INV-L2, the Tfap2c domain was fully maintained and unaffected by the genomic rearrangements. Therefore one would expect little impact on Tfap2c. However, we observed an
up-regulation of Tfap2c in the medial telencephalon in both alleles (Fig. 6A–B). This up-regulation is unlikely to be caused by the juxtaposition of new forebrain enhancers, as the regulatory sensor did not detect any forebrain activity in L1 and L2 position, in either the inverted or non-inverted configurations (Fig. 6C). We noted that in INV-L1 and –L2, Bmp7, which is strongly expressed in the medial forebrain, was relocated away from Tfap2c and its forebrain enhancer. This rearrangement had no effect on Bmp7 expression in the forebrain, suggesting that it was the presence of Bmp7 in cis that negatively influenced Tfap2c. Supporting this hypothesis, we did not observe any up-regulation of Tfap2c in the medial forebrain of INV-M embryos (Fig. 6D), where Bmp7 remained adjacent to the Tfap2c. These observations prompted us to re-examine the 4C profiles. As stated before, the intensity of the 4C signals diminished strongly beyond the TZ region. However, we observed that the 4C contacts made by the Bmp7 promoter, albeit weak, were stronger over the Tfap2c domain than over the region located symmetrically from the viewpoint (S9 Fig., green boxes). Reciprocally, Tfap2c showed weak but consistent interactions with the Bmp7 region in WT and INV-M (S9 Fig., blue boxes), interactions which are not observed with a symmetrically located region, or with the region at the equivalent place in INV-L2. To further test if the INV-L1 and –L2 up-regulation of Tfap2c depended on the removal of Bmp7, we produced INV-Bmp7 which consists in a simple inversion of the gene itself. Consequently, Bmp7 remained adjacent to the Tfap2c domain, but the results confirmed the hypothesis that the up-regulation was due to the removal of Bmp7.

Fig. 5. Redistribution of the interaction domains upon chromosomal inversions. 4C profiles were compared amongst WT control (A), INV-M (B) and INV-L2 (C) alleles for the four viewpoints indicated with black triangles. For inversion plots, the genomic coordinates were reordered to take the genomic rearrangements into account: hence, represented profiles correspond to the actual genomic structure of each allele. Representations of the data aligned on the reference (WT) genome are available in S8 and S9 Figs. Dashed rectangles and light-blue bars represent the regions inverted in the INV-M and INV-L2 alleles. The position of the TZ is marked by pink columns. The heart (mm75) and forebrain (FB1) enhancers are depicted as pink and blue ovals, respectively. The bars below each plot represent the corresponding primary interaction domain.

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and separated from it by the TZ (S11A Fig.). In this configuration, we did not observe significant changes of \textit{Bmp7} or \textit{Tfap2c} expression, with the exception of a small reduction of \textit{Bmp7} expression in the lateral forebrain. Altogether, these results supported that the simple presence of an active \textit{Bmp7} in \textit{cis}, despite the presence of the TZ region, can affect \textit{Tfap2c} expression in the medial forebrain.

We also noted that INV-M led to a significant reduction of \textit{Tfap2c} expression in the lateral forebrain (Fig. 6D), even if the genomic region between \textit{Tfap2c} and FB1 was unaffected. This reduction could result from the relocation to the other side of the TZ of two forebrain-specific H3K27ac-enriched regions included in INV-M. As we observed neither a concomitant up-regulation of \textit{Bmp7} (Fig. 6D) nor changes in the activity reported by the sensor (Fig. 6E), it is possible that these elements may not act autonomously but rather modulate the long-range action of FB1.

**Discussion**

We show here that the neighboring \textit{Tfap2c} and \textit{Bmp7} genes are controlled by distinct set of enhancers acting specifically on one or the other gene. Since we observed in a balanced genomic rearrangement a switch of enhancer-gene preferences, the specificity of these enhancers for one or the other gene cannot result exclusively from differences in their promoter structures, as proposed for other situations [49,60]. In contrast, our results indicate that, for this locus, the regulatory interactions are in a large part determined by the relative position of the different elements, as reported for other complex regions [7,61,62].

Our 4C experiments showed that \textit{Bmp7} and \textit{Tfap2c} lie in genomic domains that share limited physical contacts. These domains were only weakly demarcated in the available Hi-C data in ES cells [34]. Therefore, it is unclear if the \textit{Tfap2c} and \textit{Bmp7} domains correspond to adjacent sub-TADs [41], or weak TADs in a rather unstructured region. However, the distinction between...
these different levels of spatial segregation of the genome may in part be semantic, based on arbitrary thresholds, which may not be pertinent for gene regulation. We showed here that the distinct enhancers that regulate each gene (this work, [53,63]) reside and act within the corresponding conformational domain, further supporting the functional relevance of the structural partition we described in establishing distinct domains of regulation [15]. Furthermore, we showed that a balanced rearrangement exchanging the relative position of genes, enhancers and the TZ region led to a concomitant redistribution of physical and regulatory interactions. The switch of the heart enhancer from Bmp7 to Tfap2c and the patterns of contacts observed in this configuration demonstrate together that the topological separation in two distinct domains is key to allocate distant enhancers to one or the other gene.

We observed extensive similarities in the 4C profiles between the different cell tissues assayed, irrespective of the expression state of the corresponding genes. This indicates that the Tfap2c-Bmp7 locus adopts a rather generic conformation which undergoes limited changes in response to transcriptional activity. Such a constitutive folding has also been described for other loci [34,40,64–66]. It suggests that the structural partitioning of the locus into two domains pre-exists and guides regulatory interactions, instead of deriving from directed interactions between active genes and enhancers.

Our functional dissection of the locus highlights that the transition zone separating the two domains has an important role in organizing this topological subdivision. The fusion of the interaction profiles of the two promoters and the centromeric extension of the Bmp7 interaction domain upon removal of the TZ strongly argue in favor of the TZ preventing interactions between Bmp7 and Tfap2c. The different balanced inversions further demonstrate that the TZ organizes this topological separation irrespectively of the nature of its flanking sequences. Interestingly, the TZ region interacts robustly with both flanking regions, suggesting that the topological segregation between Tfap2c and Bmp7 may arise from its action as an interaction sink or decoy, not as a blocker or repulsive element. TAD “boundaries” often displayed strong interactions with regions flanking them on both side [34], suggesting that this behavior could be a rather general feature of topological transitions. The TZ does not appear to coincide with a region of constitutive transcription, contrarily to a large subset of typical TAD boundaries [34]. It is flanked by and includes several constitutive CTCF sites [38]. CTCF sites have been proposed to anchor long-range interactions and to act, together with cohesion and Mediator complexes, as master regulators of the chromosomal 3D conformation [67,68]. However, as only a subset of CTCF sites act as insulators [15,69], and as depletion of CTCF only mildly impacts chromosomal topologies [70] and long-range gene regulation [71], the precise role of these sequences – and of other regions of the TZ – would need to be directly assessed.

With regard to the allocation of the heart enhancer, the TZ behave similarly to a classical insulator (Fig. 7). However, the analysis of INV-L1 and –L2 indicates that the TZ does not provide complete shielding from external influences, as the presence, beyond the TZ, of an active Bmp7 promoter can interfere with the expression of Tfap2c in the medial forebrain. Although contacts between Bmp7 and Tfap2c and its associated forebrain enhancer(s) are limited and even insufficient to lead to productive interactions (i.e. activation of Bmp7), they are nonetheless present at higher than background level. Our data suggests that they may be frequent and/or strong enough to perturb the regulation of Tfap2c by its forebrain enhancer(s), most probably through promoter competition. Several studies have reported that promoters have a tendency to come into close proximity [40,72,73], particularly when they are co-active and linked. Our analysis indicates that the TZ appears to counteract this generic promoter clustering by limiting admixing of the two domains, but it does not however totally prevent the diffusion of regulatory influences between them. The functional impact of these influences underscores the difficulties of defining functional thresholds for the interaction data obtained with 4C or Hi-C. It also emphasizes that topological domains should not be considered as strict autarchic units: topological separation does not exclude neighborly relationships and semipermeable borders. Transformation of the intrinsically broad forebrain activity of FB1 into the graded expression pattern shown by Tfap2c may involve additional neighboring enhancer elements, as hinted by to the INV-M data. However, our observations suggest that the permeability of the TZ to active Bmp7 may also contribute to this fine-tuning (Fig. 7C). In operational terms, the TZ should be considered as a rheostatic controller rather than as a strict insulator.

Interestingly, a sequence orthologous to FB1 is present between Tfap2c and Bmp7 in the coelacanth, but not in teleosts or sharks (S12 Fig.). This indicates that the origin of FB1 can be traced back to the ancestor of the lobe-finned fishes. In contrast, the sequence of the TZ region is far less conserved, suggesting a more recent origin. Expression of Bmp7 in the forebrain is likely an ancestral feature, as it is shared amongst Bmp7 orthologues and paralogues [44]. Conversely, Tfap2c is the only member of its family expressed in the forebrain [42,45], and the only one directly adjacent to a Bmp gene. The evolution of FB1 as a forebrain enhancer may have been favoured by the pre-existing expression of Bmp7 in this tissue, as suggested for other loci [74–76]. In this scenario, we suggest that Bmp7 may have initially been the primary target of this emerging enhancer. The evolution of a region with insulating-like activity would have made FB1 available to Tfap2c. Interestingly, the forebrain expression of Tfap2c regulates the formation of basal progenitors in the developing cortex in mammals [77] and variations of this expression levels, in space and time, have been proposed to account for the increased number of cortical neurons present in higher primates [77]. Changes in gene expression changes are usually attributed to evolution of enhancers or promoters [78]. Our results indicate that a simple change of the filtering capacity of the TZ may also provide evolution with means of modulating gene expression.

Materials and Methods
(See S1 Text for details)

Generation of the different transgenic lines and chromosomal rearrangements
The initial allele used to produce SB-B(3end) was obtained by homologous recombination in ES cells (E14). The targeting construct comprised: the SB8 transposon [79]; an additional loxP site outside of the transposon; a neomycin resistance gene under the control of the PGK promoter that are flanked by two FRT sequences. The homology arms (chr2:172686051–172689701 and chr2:172689702–172694528 (NCBI37/mm9)) were amplified by PCR and then attached to the targeting construct above. After transformation and selection in ES cells, correctly targeted clones were injected into donor C57BL/6J blastocyst. Germine transmission was obtained from one chimera. The FRT-flanked selection cassette was then removed by breeding with hACTB-FLPe mice, leaving only the transposon and the loxP sequence
outside of it at the site (allele SB-B(3end)). The ES clone BA0758 was obtained from BayGenomics, verified by PCR genotyping, and injected to establish a Tfaq2c-gene trap line.

The SB transposon was remobilised and new insertions were mapped as described before [14]. Alleles carrying the different deletions, duplications and inversions were produced by m vivo genomic engineering [18,54], using the 129S1/Sv-Hprt<Sup>tm1(cre)Mnn</Sup>/J CRE line [80]. Deletions del1 and del3 were obtained by recombination in cis between the static loxP site at the end of Bmp7 and the one moved along with the transposed insertion SB-A1 and SB-Sall4, respectively. To keep the regulatory sensor at the deletion breakpoint, we also produced another version of these deletions, del1-LacZ and del3-LacZ, by CRE-mediated recombination in trans [34], between the loxP site from SB-B(3end) and the one at SB-A1 and SB-Sall4, respectively. For the del2-lacZ allele, we used a recombination in trans, between SB-B(3end) and BA0758. Mice were genotyped by PCR (see Supplemental Experimental Procedures).

Mouse experiments were conducted in accordance with the principles and guidelines in place at European Molecular Biology Laboratory, as defined and overseen by its Institutional Animal Care and Use Committee, in accordance with the European Convention 86/3/1986 and Directives 86/609/EEC and 2010/63/EU.

**Gene and reporter gene expression analysis**

LacZ staining and whole-mount in situ hybridization was carried out following standard protocols. For RT-qPCR, total RNA was extracted from the frozen tissues using RNeasy kit (QIAGEN), and then cDNA was synthesized using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs). The quantitative PCR was performed using StepOne Real-Time PCR System with SYBR green reagent (Applied Biosystems). Gapdh was used to normalize expression level for each sample. The extra-embryonic membranes were used for PCR-genotyping of the embryos.

**In vivo enhancer assay**

We cloned the FB1 enhancer (chr2:172551998–172555000, NCBI37/mm9) upstream of the reporter gene used in SB8, in a lentiviral vector [81]. The transgenic provirus was produced in HEK293 cells as described elsewhere [81]. Briefly, the virus was micro-injected under the zona pellucida of one-cell embryos which were maintained in culture up to the blastocyst stage. Embryos were then reimplanted into foster mothers and, at stage E11.5 or E12.5, stained for LacZ activity and genotyped.

**3C assay, 4C library preparation, sequencing and data analysis**

To prepare the 3C library we dissected out the heart and the lateral and medial forebrains from E11.5 C57BL/6 embryos. The cells were dissociated, fixed and then processed following the protocol in Splinter et al. [82]. The fixed genomic DNA was digested with NlaIII enzyme and subsequently self-ligated. To quantify the ligation products of interest, we conducted qPCR with TaqMan probes. qPCR was performed with four technical replicates, and for each value, mean and standard deviation were plotted.

For the 4C analyses, the 3C libraries were first prepared as described above from the respective tissues with NlaIII enzyme. They were then subjected to digestion by DpnII and ligation. After purification of the circularized DNA, inverse PCR was performed to obtain 4C libraries. Reading primers had 3–6 nucleotides of tag sequence, to allow for demultiplexing of the pooled libraries after sequencing. PCR products were purified, mixed altogether and sequenced on a HiSeq 2000 (Illumina). For data analysis, we first demultiplexed the FASTQ files of the 4C sequencing libraries and then aligned them to the mm9 reference genome using Bowtie version 1.0.0 [83]. To normalize with regard to library size, we divided the counts by the total number of counts on the viewpoint chromosome (chr2) for each library and multiplied these values by 1,000,000 (“RPM normalization”). We then smoothed the counts over adjacent fragments, using a window size of 11 fragments. Details are available in Supplementary Information. Sequencing data of the 4C libraries is deposited at ENA (Study Accession ERP005557).

**Supporting Information**

**S1 Fig** LacZ staining of the transposons in the Tfaq2c-Bmp7 locus. (A) Lateral views of whole embryos stained with X-gal, from E10.5 to E12.5. The scale bar is 1 μm. Numbers at the bottom indicate the corresponding IDs in TRACER database (see S1 Table) [79] (B) Frontal view of SB-B(in) and SB-B(up) embryos at
These observations suggested that the corresponding enhancers may out-compete it in this configuration, or from another type of interaction between **Tfap2c** and **Bmp7** promoter (B, C) and FB1 enhancer (C) are shown. The pink, blue and green plots are the data from the heart, the lateral and medial forebrain, respectively. (EPS)
upregulated in the heart following inversion, is now at the right side together with the heart enhancer.

(EP)

**S11 Fig** An intra-domain inversion of Bmp7 did not impact on the expression levels of either Tja1p2c or Bmp7 in the medial forebrain. (A) A schematic representation of INV-Bmp7. (B, C) LacZ expression pattern of INV-Bmp7, in E11.5 and E12.5 embryos [B]. Close-up views of E11.5 heart and forebrain (C). (D) Quantification of mRNA expression levels for Tja1p2c (left panel) and Bmp7 (right) in the inversion allele. Expression levels of the wild type allele in the lateral forebrain for Tja1p2c, and in the medial forebrain for Bmp7, were normalised as 1, respectively. Error bars represent the s.d. of three biological replicates. Statistical significance was scored by a two-sided Student’s t-test between the wild type and the mutation. *p<0.05; **p<0.01; ***p<0.001. ns: non-significant.

(EP)

**S12 Fig** Synteny conservation of the Tja1p2c-Bmp7 locus. (A) Tja1p2c and Bmp7 genes are adjacent in genomes from mammals to lobe-finned fishes, but not in ray-finned fish or cartilaginous fish genomes. The FB1 enhancer is also conserved in coelacanth, but not in the teleost lineage (blue ellipse). (B) Paralogous genes of Tja1p2c and Bmp7 in mice are not located next to each other, even though they are often on the same chromosome. Arrows indicate the direction of transcription of the genes indicated by the boxes.

(EP)

**S1 Table** List of transposon insertions and associated rearrangements. IDs in the TRACER database [79] corresponding to the line names used in this study are indicated in the left column. The column of “Parental Line” indicates from which transposon line the respective insertions were obtained.

(DOCX)

**S2 Table** Estimated coordinates of the primary interaction domains. Coordinates are on chromosome 2, using the NCBi37/mm9 assembly.

(DOCX)

**S3 Table** Estimated coordinates of the primary interaction domains in rearranged alleles. Coordinates are on chromosome 2, using the NCBi37/mm9 assembly, with those within inverted regions reordered accordingly.

(DOCX)

**S1 Text** Supplemental Materials and Methods.

(DOCX)

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**Author Contributions** Conceived and designed the experiments: FS TT. Performed the experiments: TT KL JG. Analyzed the data: FS TT. Contributed reagents/materials/analysis tools: FAK WH. Wrote the paper: FS TT WH.

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