Boundary sequences flanking the mouse tyrosinase locus ensure faithful pattern of gene expression

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Control of gene expression is dictated by cell-type specific regulatory sequences that physically organize the structure of chromatin, including promoters, enhancers and insulators. While promoters and enhancers convey cell-type specific activating signals, insulators prevent the cross-talk of regulatory elements within adjacent loci and safeguard the specificity of action of promoters and enhancers towards their targets in a tissue specific manner. Using the mouse tyrosinase (Tyr) locus as an experimental model, a gene whose mutations are associated with albinism, we described the chromatin structure in cells at two distinct transcriptional states. Guided by chromatin structure, through the use of Chromosome Conformation Capture (3C), we identified sequences at the 5' and 3' boundaries of this mammalian gene that function as enhancers and insulators. By CRISPR/Cas9-mediated chromosomal deletion, we dissected the functions of these two regulatory elements in vivo in the mouse, at the endogenous chromosomal context, and proved their mechanistic role as genomic insulators, shielding the Tyr locus from the expression patterns of adjacent genes.

Extensive profiling of genome organization and chromatin folding suggested that gene expression and chromatin topology are highly correlated. Actively transcribed genes reside in large A compartments, mega-base structures characterized by the presence of active chromatin marks and high reciprocal physical interactions. Conversely, inactive genetic material is confined to the B compartment. Genes can switch their respective compartment when they become active during development or differentiation, illustrating the dynamic correlation between gene expression and chromatin topology. At a smaller scale (tens to hundreds of kilobases), highly interacting chromatin regions are organized in topologically associating domains (TADs) characterized by high interdomain interactions. TADs partition chromatin in independent units, delimited by boundaries enriched in CTCF-occupied regions. Disruption of boundaries results in misfolding of TADs, with spurious enhancer-promoter interactions that often lead to disease or developmental defects. Within TADs, enhancers reach their target promoters through intra-TAD chromatin loops, mediated by CTCF, Cohesin, Mediator and other transcription factors.

The tyrosinase (TYR) gene encodes the rate-limiting enzyme in the biosynthesis of the melanin. Mutations in TYR are the molecular cause of the most common type of albinism in Western countries (oculocutaneous albinism type 1, OCA1) a rare disease characterized by severe visual deficits and hypopigmentation in the skin, hair and eyes. In the mouse, mutations at Tyr affect coat and eye pigmentation, with similar alterations in the visual pathway, but without gross detrimental effects on overall physiology and viability. For this reason, Tyr has been targeted in mouse and other organisms to benchmark transgenesis and genome engineering strategies, from oocyte microinjection of DNA to the most recent CRISPR nuclease and base editor approaches. Tyr is expressed in neural crest-derived melanocytes populating the skin, the hair bulbs and numerous other body locations, including the choroid, a pigmented layer found between the retina and the sclera of the eye. In addition to melanocytes, Tyr is also expressed in the retinal pigmented epithelium (RPE), a monolayer of hexagonal and binucleated pigmented cells located at the outer most layer of the retina, derived from the optic cup. Its highly specific expression pattern makes the Tyr locus an excellent experimental model locus to explore the...
mechanisms of gene expression, its relation with chromatin topology and the consequences of genetic perturbations at structurally-relevant sequences. The Tyr promoter contains the minimal information necessary to correctly drive Tyr expression in vivo. This transcriptional specificity is conveyed by two highly conserved motifs, the Tyr initiator (Irn) and the M-box, both occupied by the transcription factor Mitf. A regulatory element 5' upstream in the murine Tyr locus was first identified as a DNAse I hypersensitivity site. The inclusion of this element in a tyrosinase minigene resulted in copy number-dependant gene expression in transgenic mice, suggesting features of a chromatin boundary or a locus control region (LCR). Furthermore, when microinjected into oocytes from albino mice, large yeast artificial chromosomes (YACs) encompassing these Tyr upstream sequences fully rescued coat-colour pigmentation, whereas shorter constructs, lacking far upstream sequences, provided weak and variegated Tyr expression. More recently, we deleted the Tyr 5’ element at the endogenous locus using a CRISPR/Cas9 approach and generated allelic series to identify a 410 bp core enhancer sequence.

Here, using in vivo mouse studies, we resolve the chromatin structure of the Tyr locus and integrate it with functional data obtained by targeting additional putative cis-regulatory sequences at the 3' downstream of the Tyr gene.

**Results**

The Tyr locus is organized into two distinct chromatin loops. The mouse Tyr locus spans over 180 kb on chromosome 7 and its expression pattern is restricted to melanocytes and in cells of the retinal pigmented epithelium. Conversely, genes flanking the Tyr locus manifest completely different expression patterns as evidenced by the ubiquitous expression of Nox4 and the central nervous system-specific expression of Grm5 gene. We used published data to explore the chromatin landscape of the Tyr locus. In melanocytes, but not in fibroblasts, acetylation at lysine 27 of histone H3 (H3K27ac) highlights the gene promoter and regulatory elements upstream of the gene body. Upstream and downstream Tyr, chromatin carries repressive chromatin marks such as tri-methylation at lysine 27 of histone H3 (H3K27me3), covering the genes Nox4 and Grm5, inactive in melanocytes (Fig. 1a). To explore the chromatin structure of the Tyr locus in more details and relate it to known regulatory elements of the locus, we decided to analyze this chromosomal territory by performing chromosome conformation capture (3C) in both Tyr expressing cells (B16 melanoma cells) and non-expressing cells (NIH 3T3 L929 fibroblasts). We selected a 367 bp DpnII fragment containing the Irn and M-box as anchor fragment and interrogated 3C libraries. The Tyr promoter engages in multiple interactions within the Tyr locus. A strong interaction was observed within a fragment 80 kb 3’ downstream (thereafter, Tyr 3’ element) (Fig. 1b) and confirmed by Sanger sequencing (Fig. S1b). The promoter/Tyr 3’ interaction is observed in both B16 and L929 cells, indicating the constitutive nature of the chromatin loop, suggesting a structural role, independently of Tyr expression. No interactions are detected with control sequences immediately upstream and downstream the Tyr 3’ element. A cell-type specific interaction is found ~15 kb 5’ upstream of the Tyr gene, co-localizing with the Tyr 5’ element, analyzed previously, restricted to the DpnII fragment containing the Tyr LCR A and B boxes (Fig. 1b) and confirmed by Sanger sequencing (Fig. S1a). To confirm the Tyr 5’/Tyr promoter interaction, we performed the reciprocal analysis by using a bait primer (anchor) located at the Tyr 5’ sequence. Indeed, when this Tyr 5’ region was used as bait, we could recover signal only at the Tyr promoter confirming the specificity of the interaction (Fig. 1c). We previously showed that inactivation of this sequence in vivo by deletion results in a loss of pigmentation, confirming the relevance of such interaction for gene expression. No interaction is observed between the Tyr 5’ element and far 5’ upstream elements, including the CNS-2, an element previously described to be involved in Tyr expression regulation. No contacts are detected between the Tyr 5’ and Tyr 3’ elements, indicating that these two boundary elements probably compete with the same binding sites in the Tyr promoter and are not binding with each other (Fig. 1c). Finally, the anchor primer was positioned at the CNS-2, a sequence observed to be dispensable for Tyr expression in transgenic mice generated with different YAC Tyr transgenes. Using this fragment as bait we could not observe any interaction within the Tyr locus in both 3C libraries in the cell lines used (Fig. 1d).

The Tyr 5’ boundary element contains also a melanocyte-specific enhancer. The anatomical structure of the eye offers the unique opportunity to interrogate the specificity of Tyr regulatory elements with regard of the cell of origin. In fact, pigmented, neural-crest (NC) derived melanocytes in the choroid are surrounding the retinal pigmented epithelium (RPE), a single layer of cells at the basis of the neuroretina. We previously demonstrated that skin pigmentation relies on the Tyr 5’ element. To address the relevance of Tyr 5’ in the eye, we compared whole-mount retinae and histological eye sections of wild-type pigmented and Tyr 5’ mice. Whole mount retina from Tyr 5’ mice display a choroid-specific loss of pigmentation. The typical distribution of hexagonal RPE cells, with unpigmented nuclei, becomes evident in the retina from Tyr 5’ mice, whereas is largely masked by underlaying pigmented choroid in the retina from wild-type animals. Histological analysis confirmed that loss of pigmentation is restricted to the melanocytes present in the choroid, whereas pigmentation of the RPE appears to be unaffected, suggesting that the Tyr 5’ element activity is specific to NC-derived cells and dispensable in the RPE (Fig. 2b). The core sequence of the Tyr 5’ enhancer is enriched in Sox10 and Mitf binding motifs (Fig. S2a), and it is indeed occupied by Sox10 (Fig. 2c), a NC transcription factor that can activate Tyr in melanocytes, but not in RPE, where it is not expressed. These results indicates that the Tyr 5’ regulatory element, which had been shown to operate as a genomic boundary and be bound by Usf1 factor, which has been associated with insulator activity, also contains and functions as a transcriptional enhancer to drive Tyr expression in neural crest-derived melanocytes in the skin and the choroid, but not in the RPE, in line with previous research that hypothesized the presence of additional RPE-specific regulatory elements.
Figure 1. Chromosome conformation of the mouse Tyr locus. (a) Genomic view of the mouse Tyr locus with ChIP-sequencing data for H3K27ac and H3K27me3 profiles obtained from Melan-a (in red) and NIH-3T3 (in blue) cells. Data obtained from GSE69950, GSM1618720 and GSM2634342. (b) Chromosome Conformation Capture (3C) of mouse B16 melanoma cells (in red) and L292 fibroblasts (in blue). The grey bar indicates the anchor primer on Tyr promoter. (c) Chromatin loops detected when the anchor is located in correspondence of the Tyr 5' element. (d) No interactions are detected when the CNS-2 is used as bait region. Figure prepared with Adobe Illustrator.
The Tyr locus is flanked by chromatin insulators. We previously described and dissected the Tyr 5′ element, that coincides with a peak in our 3C assay and whose inactivation in vivo results in cell-type specific loss of pigmentation. Experiments in transgenic mice and in drosophila and in zebrafish embryos suggested that the Tyr 5′ element has insulator activity, in line with previous studies reporting binding of the boundary-associated Usf1 factor at the Tyr 5′ element. Analysis of the nucleotide Tyr 5′ sequence highlighted a cluster of transcription factor binding motifs including Sox10, Mitf and the boundary-associated Usf-1 (Fig. S2a). Indeed, both the full-length Tyr 5′ element and its synthetic GAB core display enhancer-blocking activity in human cells (Fig. 3c).

At this point, we reasoned that the sequence corresponding to strongest chromatin interaction emerged in our 3C assay—located 3′ upstream the Tyr gene—could also act as a chromatin boundary as well. We hypothesized that two boundary elements, at each end of the locus, would guarantee the strict cell-type specificity of Tyr expression and shield the locus from undesired interactions with neighbouring Nox4 and Grm5 genes (Fig. 3a). Supporting our hypothesis, we observed that the Tyr 3′ region is occupied by CTCF, a protein that exert insulator activity, in several mouse tissues and cell lines (Fig. S3a). Furthermore, a SINEB2 retrotransposon, a class of repeated mobile elements associated with insulator activity, was also found in this Tyr 3′ region (Fig. 3a, Fig. S3b), carrying a CTCF-binding motif (Fig. S3c).

Indeed, the Tyr 3′ element functions as an insulator in vitro: the full-length, 2.5 kb Tyr 3′ element displayed the highest enhancer-blocking activity. Its core component, a 241 bp element containing the SINEB2 retrotransposon including a CTCF-binding site, retains more than 50% of the activity of the full-length counterpart in less than one tenth of the size. Upon mutating the CTCF binding site we could reveal the contribution of the SINEB2 element to the boundary activity, which
Figure 3. The Tyr locus is flanked by chromatin boundaries. (a) Schematics of the Tyr locus with cartoons illustrating the Tyr 3' and Tyr 5' boundaries. (b) Enhancer-blocking assay (EBA) of the Tyr 3' element and its core sequence. Putative insulators are cloned upstream the CMV enhancers (control) or downstream the CMV enhancer (test). Enhancer-blocking activity is expressed as fold-repression values of normalized luciferase activity in test constructs compared to the CMV enhancer construct (ECMV). The full 1.2 kb sequence of the chicken 5' HS4 insulator (cHS4) is used as positive control; a threshold line is indicated in red, corresponding to the enhancer-blocking activity of the cHS4 core sequence (II/III). As negative control a mutated version of II/III was used. Anova-Bonferroni multiple comparison test. Significant p < 0.05. (c) Enhancer-blocking assay (EBA) of the Tyr 5' element and its core sequence. (d) Enhancer-blocking assay in zebrafish embryos. Putative insulators are inserted at the Asp718 site franking a hindbrain enhancer and a somite promoter driving GFP. Insertion of the Tyr 3' core element increases the fluorescence ratio between somites and hindbrain. Mutation of the CTCF binding site decreases the fluorescence ratio. Median test; n = 11–39. Tyr 3' versus control, p < 0.00726; Tyr 3' mut versus control, p < 0.135; Tyr 3' versus Tyr 3' mut, p < 0.591. (e) Representative embryo injected with the empty pCAR48R vector. (f) Representative embryo injected with the Tyr 3' core construct and (g) with the Tyr 3' mutated core construct. The hindbrain location is indicated by a yellow box. Figure prepared with Adobe Illustrator.
was reduced, but not fully abolished (Fig. 3c). This was expected, in the absence of the consensus CTCF binding motif.

In vivo, using an EBA in zebrafish embryos, where we tested the ability of a sequence to interrupt promoter-enhancer interactions, we confirmed the statistically significant insulator property of the Tyr3′ core element (Fig. 3d), that is capable of blocking enhancers in vivo. Likewise, upon introducing mutations at the CTCF site (Fig. S3d), the resulting enhancer-blocking activity is reduced (Fig. 3e–g) thereby proving that the boundary activity is, at least partially, CTCF-dependent. However, the inactivation of the CTCF-binding motif does not result in a statistically significant decrease in insulator activity. We speculate that the residual activity depends on the SINEB2 sequence, and the residual CTCF binding that may occur associated to the partially mutagenized CTCF motif. These results suggested that the Tyr expression domain is flanked by two elements capable of blocking enhancer activity in vitro and in vivo, by two chromosomal insulators.

The Tyr3′ element is dispensable for Tyr expression. In order to functionally assess the role, in vivo, at the endogenous location, of the Tyr3′ element, we used the CRISPR/Cas9 system to produce targeted chromosomal deletions involving this Tyr3′ site. Guide RNAs were picked flanking a 2.8 kb region encompassing the Tyr3′ insulator, including the DpnII fragment highlighted by the 3C assay and the sequences tested in the enhancer-blocking assays. We established two mouse lines carrying deletions of different sizes in homozygosis (TYRINS3#16 and TYRINS3#26; Fig. 4a,b). Coat colour of TYRINS3 mice appeared indistinguishable from that of wild type pigmented mice (Fig. 4c). The melanin contents from skin and eye extracts from both lines were comparable to those of wild-type animals (Fig. 4d,e). Whole-mount retinae of Tyr3′ animals were also indistinguishable from those of wild-type littermates (Fig. 4f,g), and we did not observe delay in the appearance of eye pigmentation in developing embryos (Fig. S4a,b) suggesting that perhaps the Tyr3′ element might not be required for Tyr expression in melanocytes or RPE.

Inactivation of the Tyr boundaries perturbs the transcription of flanking genes in vivo. Insulators have been classically studied by means of reporter assays or by testing for copy-number dependent activity of integrated reporter genes. Such approaches limited our understanding of the actual impact of insulators on local gene expression and that of neighboring genes. Only recently, with the advent of genome engineering techniques, the study of boundary elements at their endogenous context became accessible. This can be done by direct editing of CTCF-binding sites or by introducing large rearrangements that disrupt topological chromatin domains. In this context, our mouse lines with genome-edited insulator sequences represent a...
unique opportunity to investigate the functional role of these two genomic insulators and their impact on the expression of Tyr and the adjacent Nox4 and Grm5 genes at their endogenous locus. For the purpose of gaining a mechanistic insight in the functional organization of the locus, we generated homozygous animal lacking the Tyr 3' element (TYRINS3 mice) or the Tyr 5' element (TYRINS5 mice), and quantified gene expression levels of Tyr and of the two flanking genes Nox4 and Grm5 in different tissues.

In the eye, loss of the Tyr 5' element in TYRINS5 homozygous mice results in 50% reduction of Tyr expression. This finding is in line with previous histological analyses and with its enhancer activity in melanocytes of the choroid (Fig. 2). Deletion of the Tyr 3' element in TYRINS3 homozygous mice does not affect Tyr expression in the eye. Once again, this finding is in agreement with eye melanin measurements (Fig. 4e) and whole mount retina, that are indistinguishable from wild-type mice (Fig. 4f,g). The expression of Nox4 and Grm5 remains unaltered in the eye of TYRINS3 and TYRINS5 homozygous mutant animals (Fig. 5a), respectively.

Loss of Tyr 5' in TYRINS5 homozygous animals almost abolished Tyr expression in the skin, in agreement with their lighter coat colour and previous melanin measurements. Interestingly, deletion of the Tyr 3', the element which separates Tyr and Nox4, leads to perturbation in Nox4 expression as evidenced by significant upregulation of Nox4 transcripts in the skin of TYRINS3 homozygous mice (Fig. 5b). This perturbation in the pattern and strength of Nox4 expression in the absence of the Tyr 3' element, further confirms its function as insulator (Fig. 3B–D).

As previously reported, Tyr is not expressed in the brain of wild-type animals and this pattern does not change upon deletion of the Tyr 3' or Tyr 5' elements. Conversely to Tyr, Grm5 is a neuro-specific gene and is expressed in the brain of wild type mice and both Tyr 5' and Tyr 3' mutants. Once again, our findings clearly show that the loss of the boundary region can perturb expression of genes flanking the boundary. This is evidenced by the significant increase in the brain expression levels of Grm5 upon deleting the Tyr 5' element that separates the Tyr and Grm5 loci, in TYRINS5 mice. Consistent with our model, deletion of the Tyr 3' element separating Tyr and Nox4 genes in TYRINS3 mice has no bearing on Grm5 transcript levels (Fig. 5c).

A model of the Tyr locus organization. In this study we have been able to compare, for the first time, the phenotype of various genetically modified mice produced over 20 years to understand the function of the murine Tyr locus. These mice include the classical YAC Tyr transgenic mice encompassing the entire Tyr expression domain; the parental NMRI albino mice, carrying the known CI03S albino mutation; and the two CRISPR-mediated deletions of the Tyr 5' element (TYRINS5 mice) and the Tyr 3' element (TYRINS3 mice), described here (Fig. 6a,b). The YAC Tyr transgenic mice are indistinguishable from wild-type pigmented agouti mice and from TYRINS3 mice. On the contrary, TYRINS5 mice display a faint gray coat colour indicating a significantly reduced Tyr expression in the skin.

Considering the mouse phenotypes observed, the 3C biochemical analyses conducted and the presence of binding sites for known nuclear factors in these two key regulatory elements at either side of the murine Tyr locus, we propose a model that could explain the altered behaviour of Tyr, Nox4 and Grm5 gene expression levels in a wild-type animal (Fig. 6c), in TYRINS3 mice (Fig. 6d) and in TYRINS5 mice (Fig. 6e).

Discussion

In this work we describe the chromatin structure of the mouse Tyr locus by 3C in mouse melanoma (expressing Tyr) and fibroblast (non-expressing Tyr) cells. We describe mechanistically how the murine Tyr locus functions in these two types of cells. Through our biochemical and molecular studies, we have identified two chromatin loops: one involves the interaction between Tyr 5' enhancer and the promoter, and correlates with Tyr expression; the other, detected in Tyr both type of cells, involves the structural protein CTCF, bound to a 3' far upstream element.

The Tyr 5' enhancer was initially identified in the classical "chinchilla mottled" Tyr mouse mutation (Tyr <sup>-/-</sup>) and, thereafter, its pivotal role confirmed in mouse transgenesis experiments, through the rescue of pigmentation using both small and large DNA constructs, or using reporter lines in oocytes from albino mice. These experiments highlighted with high precision the location and the mechanism of the enhancer, that is transactivated by Sox10 and Mitf44. USF1 transcription factor, usually found in chromatin insulators47, was also reported to interact with this Tyr 5' element, likely explaining the boundary activities described in mice, flies, and zebrafish.46 Due to confounding factors, including overall transgene integrity, copy number and integration site, it remains challenging to assess up to what extent the enhancer is required to drive Tyr expression using classical transgenic mouse models, since even the smallest Tyr promoter tested in transgenic mice (270 base pairs in length) was sufficient to drive Tyr expression in both melanocytes and RPE cells.

More recently, thanks to advances in genome editing techniques, we targeted the Tyr 5' enhancer and boundary elements at the endogenous locus (TYRINS5 mice). The phenotype of mice lacking the endogenous enhancer is less severe compared with what we had observed using engineered YACs, where variegation was regularly reported, however highly reproducible across TYRINS5 mouse lines with similar deletions. Using the TYRINS5 mice, we determined that the Tyr 5' element is absolutely required to drive Tyr expression in neural-crest derived melanocytes, where Tyr gene expression is almost undetectable upon deleting the Tyr 5' element (Figs. 2b, 5b), but dispensable in the RPE (Fig. 2). Mechanistically, this correlates well with differences in gene expression between melanocytes, that express Sox10, and cells of the RPE, that are Sox10 negative.44,45. In fact, in melanocytes, Sox10 binding overlaps the core Tyr 5' enhancer (Fig. 2c) that is enriched in Sox10 binding motifs (Fig. 5a). A RPE-specific enhancer was identified using BAC transgenesis and transient LacZ reporter mouse lines; however, the specificity and relevance of such distant enhancer (absent in some of the YAC Tyr transgenic mouse lines that were indistinguishable from wild-type pigmented mice, where DNA sequences upstream of the 5' element had been deleted) remain to be validated at the native chromatin context, and in relation with the Tyr promoter. These are ongoing experiments whose results will be discussed in a following study.

[References]

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At the 3’ end of the locus, at the intergenic space between Tyr and Nox4, we identified a sequence that interacts strongly with the Tyr promoter in melanoma cells and fibroblasts (Fig. 1b). This sequence contains a SINEB2 retrotransposon and it is bound by CTCF (Fig. S3). These features are typically associated with insulators48,55, a

Figure 5. Inactivation of the Tyr boundaries perturbs the transcription of flanking genes in vivo. (a) Relative mRNA expression of Tyr, Nox4 and Grm5 in the eye of wild-type and homozygous TYRINS5 and TYRINS3 mice. (b) Relative mRNA expression in the skin and (c) brain of wild-type and homozygous TYRINS5 and TYRINS3 mice. N = 5 mice per group; Kruskal–Wallis test. Figure prepared with Adobe Illustrator.
class of regulatory elements able to block enhancer-promoter interactions and to partition chromatin in transcriptionally independent domains. Indeed, we could detect a strong insulator activity, in mammalian cells and in zebrafish embryos at this Tyr3′ element comparable to that of the archetypic chicken cHS4 insulator, both CTCF- and SINEB2-dependant (Fig. 3). This further highlights the role of retrotransposons in the gene insulation, in control of chromatin topology, and in genome evolution. Deletion of this sequence in the mouse does not affect pigmentation or melanin levels in the skin or the eye of two independent mouse lines; Tyr mRNA level in the eye and skin does not depend of the Tyr3′ insulator (Fig. 5). However, when we measured the expression level of Nox4, located further upstream and beyond the Tyr3′ boundary, we found that Nox4 was overexpressed in the skin, suggesting that loss of the Tyr3′ allowed Tyr regulatory elements to abnormally and ectopically transactivate Nox4. Similarly, we profiled gene expression in tissues of mice lacking the Tyr5′ element. Grm5, the gene found 5′ upstream of Tyr and beyond the Tyr5′ boundary, is overexpressed in the brain of animals lacking the Tyr5′. We speculate that rearrangements in chromatin topology in the absence of the Tyr5′ element expose the Grm5 promoter to distal regulatory sequences. Several mouse models (Fig. 6a,b) illustrated the relevance of regulatory regions at the mouse Tyr locus. Combining profiling of chromatin topology and functional study in the mouse, we devised a model for the locus: in melanocytes, the Tyr promoter engages two regulatory elements occupied by Sox10 and CTCF (Fig. 6c). Disruption of the Tyr3′ element, that normally interacts with the Tyr promoter and exert insulator activity, results in overexpression of the flanking gene Nox4, in the skin (Fig. 6d). Perturbation of the Tyr5′ element, that normally transactivate the Tyr promoter and functions as a boundary, results in loss of Tyr expression in gain in expression of the flanking Grm5 (Fig. 6e).

These data illustrate the role of enhancers and chromatin insulators in gene expression at the scale of a given locus and its adjacent genes. They also highlight how mutations at regulatory elements could alter the expression of genes in multiple tissues. For more than 20 years (1990–2012) our ability to functionally and structurally interrogate the non-coding DNA sequences within the mouse Tyr locus was extremely limited. The extraordinarily abundance of DNA repetitive elements, including LINE1 retrotransposons in the vicinity of Tyr, rendered
impossible any attempt to apply standard homologous recombination techniques in mouse embryonic stem (ES) cells. With the advent of the CRISPR/Cas9 techniques applied to mouse functional genomics, it has become possible dissecting the mechanistic role of selected regulatory elements at their endogenous chromosomal location. This study highlights the relevance of investigating the non-coding genome in their native chromosomal context, and the advantage of using mouse models over cellular systems in the study of regulatory sequences.

**Materials and methods**

**Cell culture.** Mouse melanoma cell line B16-F1, mouse fibroblast NIH-3T3 L929 cells and human embryonic kidney HEK 293 cells were grown in DMEM medium (Dulbecco’s Modified Eagle Medium, Gibco) supplemented with sterile-filtered 10% fetal bovine serum (FBS, Sigma-Aldrich), 2 mM L-glutamine (Invitrogen) and 10 mM HEPES pH 7.4 (Invitrogen) under aerobic conditions using a sterile hood (Telstar Bio II Advance). Cells were cultured at + 37 °C, 95% of humidity and 5% CO₂.

**Chromosome conformation capture (3C).** 3C analyses were performed as previously reported. Briefly, 1 × 10⁷ B16F0 mouse melanoma or NIH3T3-L929 mouse fibroblast cells were fixed in 2% PFA/PBS. After quenching, cells were resuspended in 500 μl lysis buffer (10 mM Tris HCl, 10 mM NaCl, 0.3% NP40, 1 × Roche Complete) and kept on ice. Methyl Green-Pyronin (MGP, Sigma Aldrich) was used to monitor the release of intact nuclei. Chromatin was digested overnight with 45 Weiss Units of T4 DNA ligase (Promega) in 7 ml ligation buffer (30 mM Tris–HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP). Finally, the sample was treated with Proteinase K and RNase DpnI, and religated with T4 DNA ligase. Products were amplified by PCR with primers specific for the fragment of interest, and the amplified products were cloned in the pELuc vector in between the enhancer and the promoter (PstI, control). Primer sequences are listed in Supplementary Table S1. Luminescence was measured for each construct in triplicate and used to quantify enhancer-blocking activity. The HSF chicken beta-globin insulator and its minimal core motif (II/III) were used as positive control; a mutated version of the II/III sequence was used as negative control. One-way ANOVA with Bonferroni post-hoc correction test was used.

**Enhancer blocking assay (EBA) in vitro in mammalian cells.** Enhancer-blocking activity was measured as previously reported using transient transfection in HEK 293 cells. Putative insulator sequences were cloned in between a hindbrain-specific enhancer and a somite-specific promoter. Single-copy integration in the zebrafish genome was obtained by Tol2-mediated transgenesis in fertilized zebrafish embryos. GFP fluorescence was acquired at 36 hpf and quantified using Laser Pix (BioRad).

**Enhancer-blocking assay (EBA) in vivo in zebrafish embryos.** Assays were carried as reported. Putative insulator sequences were cloned in between a hindbrain-specific enhancer and somite-specific promoter. Single-copy integration in the zebrafish genome was obtained by Tol2-mediated transgenesis in fertilized zebrafish embryos. GFP fluorescence was acquired at 36 hpf and quantified using Laser Pix (BioRad). Enhancer-blocking activity was measured based on relative levels of somite/CNS GFP expression. Statistical analyses (median test) were calculated with IBM-SPSS v.21.

**CRISPR/Cas9-mediated chromosomal deletion.** A pair of RNA guides flanking the Tyr 3′ downstream element was designed using the crispr.mit.edu online tool and cloned by Golden Gate Cloning into the Esp3I sites of MLM3636 plasmid (Addgene #43860). Cas9 mRNA was prepared from hCas9 plasmid (Addgene #41815) as described and injected into B6D2F2 (Harlan; originated from B6CBAF1/OlaHsd) mouse fertilized eggs. Founder animals were bred to albino outbred HsdWin:NMRI (Harlan) mice, the reference albino genetic background used in all previous Tyr transgenic mouse studies.

**mRNA expression analysis.** Total RNA was isolated from cultured cells and tissues using the RNasy Mini Kit (Qiagen) with on-column DNase treatment. 500 ng of RNA were retrotranscribed using SuperScript III Reverse Transcriptase (Thermo Fisher). qPCR reactions were performed with TaqMan Universal PCR Master Mix (Thermo Fisher) using probes for Tyr (Mm00495817_m1), Ntx4 (Mm00479246_m1), Grm5 (Mm00690332_m1) and Tbp (Mm00446973_m1) as described.
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Author contributions
D.S. and L.M. conceived the study and analyzed data. D.S., A.F. and M.C. performed experiments in mammalian cells and in the mouse. A.F.M. and J.L.G.S. performed experiments in zebrafish embryos. P.P. generated the TTYRINS3 mouse model. D.S. and L.M. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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