Introduction

HOX genes encode evolutionarily conserved transcription factors that control the formation of body segment-specific structures by regulating the transcription of downstream effectors that, in turn, direct the morphogenetic events leading to the complex body forms along the embryonic axes in metazoan [1–2]. Consequently, HOX gene expression pattern requires mechanisms that differentially control HOX transcription in a precise spatio-temporal fashion. This implies an integrated regulation of neighbouring HOX genes achieved through the sharing and the selective use of defined enhancer sequences. The Hoxa5 gene plays a crucial role in lung and gut organogenesis. To position Hoxa5 in the regulatory hierarchy that drives organ morphogenesis, we searched for cis-acting regulatory sequences and associated trans-acting factors required for Hoxa5 expression in the developing lung and gut. Using mouse transgenesis, we identified two DNA regions included in a 1.5-kb XbaI/XbaI fragment located in the Hoxa4-Hoxa5 intergenic domain and known to control Hoxa4 organ expression. The multifunctional YY1 transcription factor binds the two regulatory sequences in vitro and in vivo. Moreover, the mesenchymal deletion of the YY1 gene function in mice results in a Hoxa5-like lung phenotype with decreased Hoxa5 and Hoxa4 gene expression. Thus, YY1 acts as a positive regulator of Hoxa5 expression in the developing lung and gut. Our data also support a role for YY1 in the coordinated expression of Hox genes for correct organogenesis.

Abstract

The Hox gene family encodes homeodomain-containing transcriptional regulators that confer positional information to axial and paraxial tissues in the developing embryo. The dynamic Hox gene expression pattern requires mechanisms that differentially control Hox transcription in a precise spatio-temporal fashion. This implies an integrated regulation of neighbouring Hox genes achieved through the sharing and the selective use of defined enhancer sequences. The Hoxa5 gene plays a crucial role in lung and gut organogenesis. To position Hoxa5 in the regulatory hierarchy that drives organ morphogenesis, we searched for cis-acting regulatory sequences and associated trans-acting factors required for Hoxa5 expression in the developing lung and gut. Using mouse transgenesis, we identified two DNA regions included in a 1.5-kb XbaI/XbaI fragment located in the Hoxa4-Hoxa5 intergenic domain and known to control Hoxa4 organ expression. The multifunctional YY1 transcription factor binds the two regulatory sequences in vitro and in vivo. Moreover, the mesenchymal deletion of the YY1 gene function in mice results in a Hoxa5-like lung phenotype with decreased Hoxa5 and Hoxa4 gene expression. Thus, YY1 acts as a positive regulator of Hoxa5 expression in the developing lung and gut. Our data also support a role for YY1 in the coordinated expression of Hox genes for correct organogenesis.

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YY1 Acts as a Transcriptional Activator of Hoxa5 Gene Expression in Mouse Organogenesis

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participate to the regulation of the collinear expression of Hox genes [16].

Transcriptional regulators of Hox gene expression have been identified [17]. They include developmentally regulated factors like the CDX homeodomain-containing proteins that integrate retinoic acid (RA), FGF and Wnt signaling for the setting of the correct expression domain of Hox genes [10–21]. Hox genes are also directly responsive to RA, which activates retinoic acid receptors that then interact with retinoic acid response elements (RARE) identified near Hox genes mainly from paralogous groups 1 to 5 [22–23]. Hox expression is under the control of HOX proteins themselves involved in auto- and cross-regulation [24–25]. Finally, ubiquitously expressed transcription factors such as the multifunctional Yin Yang 1 (YY1) protein can modulate Hox expression in specific contexts [26–29].

A complex organization of overlapping transcriptional units encompassing the Hoxa5 locus exists, which results from alternative splicing and the use of three promoters, one proximal producing the 1.8-kb transcript and two distal ones giving rise to long noncoding RNAs [12]. Using a transgenic approach, we have identified regulatory elements directing the developmental expression of the Hoxa5 proximal promoter. An 11.1-kb genomic fragment can recapitulate the temporal expression and substantially reconstitute the spatial pattern of the 1.8-kb Hoxa5 transcript in mouse embryos. It includes DNA control sequences, such as the 604-bp brachial spinal cord (BSC) enhancer and a 650-bp temporal control region, both contained in the Hoxa5 5' flanking sequences [30–31]. A 2.1-kb mesodermal (MES) enhancer important for Hoxa5 paraxial and lateral plate mesoderm expression in the cephalo-upper thoracic region of the A-P axis is positioned 3' of the Hoxa5 gene. CDX proteins bind this sequence acting as potential regulators for the regionalization of Hoxa5 gene expression along the axis [32]. A 1.5-kb DNA region that targets Hoxa5 lung and gut developmental expression was also identified in the Hoxa4-Hoxa5 intergenic sequences [33].

Several Hox genes, mainly from paralogous groups 1 to 8, are expressed along the respiratory tract [34]. However except for Hoxa5, the lack of overt lung phenotype in single Hox mutants indicates that Hoxa5 plays a predominant function in lung ontogeny [35]. The prevalent role of Hoxa5 in organ development prompted us to further characterize Hoxa5 lung and gut regulatory sequences. Here, we present evidence that Hoxa5 lung and gut expression is under the control of several DNA elements. Some are shared with the flanking Hoxa4 gene and they bind the transcription factor YY1, which acts as a positive regulator of Hoxa5 gene expression in the developing lung and gut.

Materials and Methods

Ethics statement

All animal experiments were performed according to the guidelines of the Canadian Council on Animal Care and they were specifically approved by the institutional animal care committee (Comité de protection des animaux du Centre de recherche du Centre Hospitalier Universitaire de Québec (CPAC); Permit Number: 2012013-2).

Design of Hoxa5/lacZ transgenes

All Hoxa5/lacZ constructs contain the bacterial lacZ gene inserted into the Sall site of the first exon of the Hoxa5 gene, which allows translation of the lacZ ORF from the HOXA5 AUG [31]. Construct 1 (also named pLJ272) was obtained by adding a 5.20-kb EcoRI-Apel DNA fragment at the 3' end of construct 2 from ref. [33]. Construct 2 (pLJ123) was previously described (construct 6 in ref. [31]). It was used as the backbone for most Hoxa5/lacZ constructs in the present study. Constructs 3, 4, 8–12, 14–15 and 17–21 were obtained by ligation at the 3' end of pLJ123 of genomic fragments from the Hoxa4-Hoxa5 intergenic region. Construct 5 was produced by cloning the 1.5-kb XhoI-XhoI fragment upstream an Xhol-HindIII Hoxa5 genomic fragment carrying a Hoxa5 minimal promoter that cannot direct β-galactosidase expression by itself (pLJ143 in ref. [32]). Enhancer activity was tested in constructs 6, 7, 13, 16 and 22 by cloning Hoxa5 regulatory regions in front of the hsp68lacZ fragment, which contains an hsp68 minimal promoter that cannot direct lacZ developmental tissue-specific expression by itself [31,36]. For constructs 17 to 22, nucleotide substitutions in RARE and YY1 binding sites were produced by an overlapping PCR strategy using synthetic oligonucleotide primers carrying appropriate base changes (Table 1A). Introduced mutations were confirmed by sequencing and disruption of the binding sites was validated with the TFSEARCH (http://molsol1.ebc.iaist.go.jp/research/db/TFSEARCH.html) and TESS (http://www.ebi.ac.uk/cgi-bin/tess/tes2) softwares.

Production of transgenic mice

The microinjected Hoxa5/lacZ and hsp68lacZpA fragments were obtained by a SacI+XhoI digestion and an XhoI+XhoI digestion, respectively, to eliminate plasmid sequences. Transgene microinjection into fertilized eggs from C57BL/6 X CBA F1 hybrid intercrosses were done according to standard procedures [37]. Transgenic founder embryos were recovered from foster mothers, genotyped by Southern analysis of yolk sac DNA using a lacZ specific probe to assess the integrity of the microinjected construct, and analyzed for lacZ expression by β-galactosidase staining. Permanent mouse lines were also obtained for construct 1 and genotyped with tail DNA [31].

Mice, genotyping, tissue collection, histology, immunohistochemistry (IHC) and β-galactosidase staining

To assess the effect of YY1 on Hoxa5 expression, mutant mouse lines for the Yflox and Yflox allele were used (provided by Y. Shi) [38]. For the mesenchymal ablation of Yflox, we used Dermo1Cre mice provided by D. Ornitz [39]. Dermo1Cre mice express the Cre recombinase protein from the endogenous Dermo1 (Twist2) locus. Yflox/lox/Dermo1Cre mice were generated by mating between Yflox/lox/Dermo1Cre and Yflox/lox/Cre mice.

Age of the embryos was estimated by considering the morning of the day of the vaginal plug as embryonic (E) day 0.5. Experimental specimens were genotyped by Southern blot and PCR analyses. Organs were collected from E14.5, E16.5 and E18.5 wild-type (wt), Hoxa5/lacZ and Hoxa5/lacZflox/flox mice. For RNA extraction, tissues were snap-frozen in N2. For histology and immunostaining, tissues were fixed in 4% ice-cold paraformaldehyde overnight, paraffin-embedded and sectioned at 4 μm. Immunohistochemistry was performed as described [11]. The primary antibodies used were a goat antibody against CC10 (1/500; gift for Dr. G. Singh), a swine anti-rabbit antibody against podoplanin (1/2; 1/75; DSHB), and a rabbit antibody against YY1 (1/500; Santa Cruz Biotechnology). The biotinylated secondary antibodies used were a goat anti-rabbit antibody (1/300; Vector Laboratories), a swine anti-goat antibody (1/300; Cedarlane), and a goat anti-sheep antibody (1/300; Jackson Immuno Research).
| Fragment | Sequence (5’-3’ ) | Amplicon (bp) |
|----------|-------------------|--------------|
| **A) Mutagenesis** |
| 259-bp XbaI-BssHII (mRARE) F-GCGCAAAGTCCAAGGCCGCTGCTTA | 46 |
| 259-bp XbaI-BssHII (mYY1) F-GAACCTGTCGCCGCGCGCGCG | 46 |
| 163-bp NcoI-SacI (mYY1) F-CAGTACCTGCCTACCTGCCTGCG | 22 |
| Fragment A | F-CTAGAGAAGTGTTTGAGATAG R-CATTATGGGATGTATTGACTG | 85 |
| Fragment B | F-CAGTCAATACCCCAATATG-GAGGTGACGTACGTTG | 104 |
| Fragment C | F-CAGCGGTCTCAACCAATAATATGTCGGCTGAGGGCGCG | 44 |
| Oligo C3(m1) | F-GCGCCAATCTAATGACATATGTCGGCTGAGGGCGCG | 44 |
| Oligo C3(m2) | F-CAGCGTCTCCACAGGGCCTGAAAGTTCGGCTGAGGGCGCG | 44 |
| Oligo C3(m3) | F-CTAGAGAAGTGTTTGAGATAG R-CATTATGGGATGTATTGACTG | 85 |
| Oligo C3(m4) | F-CAGCGGTCTCAACCAATAATATGTCGGCTGAGGGCGCG | 44 |
| Oligo C3(m5) | F-CAGCGGTCTCAACCAATAATATGTCGGCTGAGGGCGCG | 44 |
| Oligo C3(m1YY1) | F-TGAAAATGTCGGCTGAGGGCGCG | 45 |
| Oligo C3(m2YY1) | F-CAGCGGTCTCAACCAATAATATGTCGGCTGAGGGCGCG | 45 |
| Oligo C3(m3YY1) | F-CAGCGGTCTCAACCAATAATATGTCGGCTGAGGGCGCG | 45 |
| Oligo C3(m4YY1) | F-CAGCGGTCTCAACCAATAATATGTCGGCTGAGGGCGCG | 45 |
| Oligo C3(m5YY1) | F-CAGCGGTCTCAACCAATAATATGTCGGCTGAGGGCGCG | 45 |
| Oligo G1 | F-CATGGGATTTTTGCTATGGCTTGCTTGCA AAGGGAGGCTGTGGAA | 45 |
| Oligo G2 | F-TGAAAATGTCGGCTGAGGGCGCG | 45 |
| Oligo G3 | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G4 | F-GCCGGAATGTCGGCTGAGGGCGCG | 45 |
| Oligo G3(m1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m2) | F-GCCGGAATGTCGGCTGAGGGCGCG | 45 |
| Oligo G3(m3) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m4) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m5) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m1YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m2YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m3YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m4YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m5YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo C3(mYY1) | F-TGAAAATGTCGGCTGAGGGCGCG | 45 |
| Oligo C3(m1YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo C3(m2YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m3YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m4YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m5YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m1YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m2YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m3YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m4YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m5YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m1YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m2YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m3YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m4YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G3(m5YY1) | F-CATAAATCTTGCAGTAAAATGTCAAAATGTCGGTGTGTGAGATAA | 45 |
| Oligo G1 | F-ATGGGAGGCTGCTGAT | 18 |
| Oligo G2 | F-CGCGGCGAATGTCGGCTGAGGGCGCG | 45 |
| Oligo G3 | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G4 | F-CTCCGCTTGCTGGCTGAT | 45 |
| Oligo G3(m1) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m2) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m3) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m4) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m5) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m1YY1) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m2YY1) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m3YY1) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m4YY1) | F-CGGCCCGTCCTGGCTGAT | 45 |
| Oligo G3(m5YY1) | F-CGGCCCGTCCTGGCTGAT | 45 |
| **D) ChIP** |
| C3 | F-ACCTTCAGGTCAGGCGGATCAACCTCTCAGGTC | 161 |
| G3 | F-ATGGTGGAGGCTGCTGAT | 173 |
| 15 kb 3’ | F-TGAAAATGTCGGCTGAGGGCGCG | 140 |
| Sfrs10 | F-TGAAAATGTCGGCTGAGGGCGCG | 175 |
| Rcor3 | F-TGAAAATGTCGGCTGAGGGCGCG | 157 |
| **E) qRT-PCR** |
| Hoxa4 | F-CGGCCCGTCCTGGCTGAT | 95 |
| Hoxa5 | F-CGGCCCGTCCTGGCTGAT | 173 |
| Rp19 | F-CGGCCCGTCCTGGCTGAT | 122 |
| Scgb1a1 | F-CGGCCCGTCCTGGCTGAT | 85 |
| T1a | F-CGGCCCGTCCTGGCTGAT | 70 |
| Yy1 | F-CGGCCCGTCCTGGCTGAT | 117 |

Table 1. Oligonucleotide sequences.
Chromatin immunoprecipitation (ChIP) assays

Electrophoretic mobility shift assay (EMSA) and supershift

...samples were equilibrated for 5 min at room temperature. In supershift assays, 2 µg of rabbit anti-YY1 antibody, rabbit anti-H3 antibody (ab1791; Abcam), or control rabbit IgG (sc2027; Santa Cruz Biotechnology). Immuno-complexes were washed three times respectively with 0.5 ml of low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl) and LiCl immune complex wash buffer (0.25 M LiCl, 1% IGEPA-L-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl pH 8.0). A final wash in 0.5 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) was followed by centrifugation at 3000 rpm at 4°C for 2 min. Protein-DNA complexes were eluted by adding 100 µl of elution buffer (1% SDS, 50 mM Tris pH 8.0, 10 mM EDTA) and then equilibrated at 65°C for 15 min. Cross-links were reversed by adding 200 mM NaCl and equilibrated at 65°C overnight. DNA fragments were purified using a QIAquick gel extraction kit (Qiagen) after a two hr treatment with RNase followed by a two hr treatment with proteinase K. qPCR analyses were performed with specific primers listed in Table 1D. The values for the samples immunoprecipitated by anti-YY1, anti-histone H3 and control IgG were recorded as the percentage relative to input. ChIP results were confirmed by three independent experiments and qPCR was performed in triplicate for each sample. The ChIP efficiency was calculated by dividing the amount of PCR product obtained with the immunoprecipitated DNA by the amount obtained with the input DNA, as described in ref. [42].

Quantitative RT-PCR (qRT-PCR) experiments

Total RNA was isolated from lungs of E18.5 embryos. RNA extraction, cDNA synthesis and qRT-PCR were performed as described [41]. Samples were analyzed in triplicate. The Rpl19 gene was used as control. Eight specimens were used for each genotype tested. Primer sequences are listed in Table 1E.

Statistical analyses

Student's t test was performed for gene expression and ChIP studies. A significance level inferior to 5% (p<0.05) was considered statistically significant.

Results

A 14.5-kb genomic fragment recapitulates the spatio-temporal expression from the Hoxa5 proximal promoter

We have previously shown that the 11.1-kb Hoxa5 genomic region located between positions -3767-bp and +7188-bp (relative to Hoxa5 transcription start site (TSS)) reproduced the temporal expression and substantially reconstituted the spatial profile of Hoxa5 gene. However, it did not recapitulate the correct expression in the central nervous system (CNS) and in developing organs, indicating that additional sequences were required [31]. A 1.5-kb Xhol-Xhol DNA fragment located in Hoxa4-Hoxa5 intergenic sequences at ∼3.0-kb upstream the Hox4 gene (positions +9351-bp to +10816-bp) was able to target Hoxa5 expression in lung and stomach [33]. To assess if a DNA region encompassing the Hoxa5 regulatory sequences identified can recapitulate Hoxa5 expression from the proximal promoter, a 1.5-kb Hoxa5/lacZ
transgene (positions -2128-bp to +12384-bp) was tested (Construct 1; Fig. 1A–B). As reported for the 11.1-kb Hoxa5/lacZ transgene, the onset of expression of construct 1 was ~E8.0-8.25, corresponding to that of Hoxa5 endogenous expression (not shown) [31,43]. At E8.5, staining was mainly detected in the foregut region and in somites 5 to 8, consistent with the Hoxa5 expression domain in the mesoderm along the A-P axis at this age (Fig. 1C) [31]. At E9.5-10.5, transgene expression extended more caudally along the axis and appeared in limb buds (Fig. 1D–E). At E12.5-13.5, staining was detected along the neural tube with an anterior boundary in the posterior hindbrain corresponding to the limit of Hoxa5 endogenous expression in CNS. No staining was seen in the most caudal part of the embryo, which expressed the larger Hoxa5 transcripts from the distal promoters (Fig. 1F–G) [12]. Construct 1 directed transgene expression in lung, stomach and intestine, and the staining was mainly restricted to the mesenchymal layer as Hoxa5 endogenous expression (Fig. 1H–J). The Hoxa5 rostro-caudal gradient present in the developing stomach was reproduced with construct 1 (Fig. 1J) [8]. lacZ staining was detected in the intestine, being stronger in the proximal part of midgut and vanishing towards the hindgut (not shown). Thus, the 14.5-kb Hoxa5/lacZ transgene contains regulatory sequences that largely reproduce the Hoxa5 spatio-temporal expression driven by the Hoxa5 proximal promoter.

**Figure 1. β-galactosidase expression profile of construct 1 during embryogenesis.** (A) Partial restriction map of the Hoxa4-Hoxa6 genomic region. Black boxes represent homeoboxes, shaded boxes correspond to translated regions, and white boxes indicate transcribed regions. The arrow shows TSS from the Hoxa5 proximal promoter. (B) Diagram of the Hoxa5/lacZ construct 1 (also named pLJ272) and summary of transgenic expression analysis at E13.5. The first column presents the number of lacZ-expressing F0 embryos out of the total number of F0 transgenic embryos generated. The number of positively stained embryos for each structure listed is indicated. A, AccI; Ap, ApaI; Bg, BssHII; RL, EcoRI; H, HindIII; K, KpnI; Xb, XbaI; Xh, Xhol. (C–G) Representative transgenic embryos stained for β-galactosidase activity showed the expression pattern of construct 1 during development. Black arrowheads indicate the anterior limit of transgene expression in paraxial mesoderm at somite 5 and prevertebra 3. Open arrowheads point the anterior limit of transgene expression in the neural tube. (H) Whole-mount organs from E13.5 transgenic embryos showed β-galactosidase activity in lung, stomach and intestine. (I–J) Histological sections of lung and stomach, respectively, from E13.5 transgenic embryos stained for β-galactosidase activity. The inset in I shows that expression was restricted to mesenchyme. Black arrow in J shows the gradient of expression of the transgene in the developing stomach. c, caudal; e, epithelium; h, heart; i, intestine; l, lung; m, mesenchyme; nt, neural tube; pv, prevertebrae; r, rostral; s, stomach. Scale bar: 100 μm.

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**Hoxa5 organ-specific expression involves several regulatory sequences**

To delineate the DNA regions involved in Hoxa5 organ-specific expression, we analyzed shorter versions of the Hoxa4-Hoxa5 intergenic region in E13.5 transgenic embryos (Fig. 2A–B). Construct 2 (pLJ123) was used as control. It contains the BSC and MES enhancer sequences. It also drives expression in forelimbs and spleen but not in lung and gut (Fig. 2C, I) [31,33]. Insertion of the 3.7-kb KpnI-ApaI genomic fragment downstream of pLJ123 reproduced the spatial profile of construct 1 at the same age (construct 3; Fig. 2D, J). A similar result was obtained when the 1.5-kb XbaI-XbaI DNA fragment was inserted at the 3’ end of pLJ123 indicating that the 1.5-kb sequence contained the regulatory information necessary for proper Hoxa5 expression in organs (construct 4; Fig. 2E, K). Histological analyses of stained organs from construct 4-positive embryos revealed that expression was restricted to mesenchyme as seen with construct 1 (not shown). To assess if the 1.5-kb DNA region possesses enhancer properties, we put it in front of a Hoxa5/lacZ minimal plasmid (construct 5) and of a heterologous hsp68/lacZ plasmid (constructs 6, 7), both plasmids being unable to direct transgene expression on their own [30,32,36]. Construct 5 targeted a strong transgene expression in organs and CNS reinforcing the notion that tissue-specific sequences for these structures were present in the 1.5-kb DNA region.
the 1.5-kb sequence (Fig. 2F, L). In contrast, when tested in the hsp68/lacZ context, faint or no organ expression was observed while CNS expression was reproduced (Fig. 2G–H, M–N). Thus, the 1.5-kb XbaI-XbaI region encloses several Hoxa5 regulatory elements including a CNS-specific enhancer that directs the appropriate limit of expression in the posterior hindbrain regardless of the promoter used, and an organ-specific sequence that requires a Hoxa5 minimal environment for effectiveness.

To narrow down the organ-specific regulatory sequences, a deletion analysis was undertaken (Fig. 3). A 1.0-kb XbaI-SacI fragment (construct 8) targeted organ and CNS expression similarly to construct 4 suggesting that the 455-bp located at the 3′ end of the XbaI-XbaI sequence were not necessary (Fig. 3C, G). This was confirmed with construct 9 containing the 455-bp SacI-XbaI sequence. The expression profile of construct 9 was identical to that of construct 2 demonstrating the lack of regulatory activity in the 455-bp sequence (Fig. 3D, H). Additional deletion of sequences at the 3′ end of the XbaI-XbaI sequence showed that the 259-bp XbaI-BssHII fragment (construct 10) directed expression in the stomach and intestine in few specimens but did not reproduce the anterior boundary in the hindbrain and the lung expression, suggesting the loss of tissue-specific elements (Fig. 3E, I). The reverse construct carrying the 1.2-kb BssHII-XbaI sequence (construct 11) targeted organ expression as construct 4. However, expression in CNS was not entirely recovered suggesting that neural-specific sequences were spread along the 1.0-kb XbaI-SacI region (Fig. 3F, J).

Figure 2. Characterization of the Hoxa4-Hoxa5 intergenic region in E13.5 F0 transgenic mouse embryos. (A) Schematic representation of the 1.5-kb XbaI-XbaI DNA fragment in the Hoxa4-Hoxa6 genomic region. (B) Diagram of the Hoxa5/lacZ constructs used to generate E13.5 F0 transgenic embryos and summary of transgenic expression analyses. A, AccI; Ap, Apal; Bh, BssHII; Bm, BsmI; RI, EcoRI; H, HindIII; K, KpnI; Mf, MfeI; Nc, NcoI; Sc, SacI; Xb, XbaI; Xh, XhoI. (C–H) Carcass of representative E13.5 transgenic embryos and the associated organs (I–N) stained for β-galactosidase activity showed the effects of the different deletions on the expression pattern. Open arrowheads point the anterior limit of transgene expression in the neural tube. The number in the lower left corner of each panel corresponds to the transgene. h, heart; i, intestine; l, lung; nt, neural tube; pv, prevertebrae; s, stomach; sp, spleen.

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X-Gal staining in organs was similar in transgenic embryos carrying constructs 4, 8 and 11, suggesting that organ tissue-specific sequences were included into the 751-bp \( B_{HII}-Sac_I \) region. This was confirmed with construct 12 that showed expression in the respiratory and digestive tracts (Fig. 4B–C, H). Interestingly, when linked to the heterologous \( hsp68/lacZ \) plasmid, the 751-bp \( B_{HII}-Sac_I \) region targeted expression in organs in contrast to what was seen with constructs 6 and 7, suggesting the presence of repressive sequences in the 259-bp \( Xba_I-B_{HII} \) and/or in the 455-bp \( Sac_I-Xba_I \) DNA fragments (construct 13; Fig. 4D, I). Thus, the 751-bp \( B_{HII}-Sac_I \) fragment contains enhancer sequences that direct organ-specific expression.

Further deletions were performed. The 588-bp \( B_{HII}-NeoI \) sequence in construct 14 did not correctly reproduce the staining in organs pinpointing toward the 163-bp \( NeoI-Sac_I \) sequence as the one carrying the organ-specific enhancer (Fig. 4E, J). This was confirmed with the \( NeoI-Sac_I \) sequence in construct 15, which showed organ expression in transgenic embryos (Fig. 4F, K). A similar result was observed when the \( NeoI-Sac_I \) sequence was tested with the heterologous \( hsp68/lacZ \) plasmid (construct 16; Fig. 4G, L). Thus, organ-specific regulatory elements appear to be dispersed along the 1.5-kb \( Xba_I-Xba_I \) region, some located in the 259-bp \( Xba_I-B_{HII} \) fragment, others in the 163-bp \( NeoI-Sac_I \) sequence.

The RARE sequence is not necessary for \( Hoxa5 \) organ-specific expression

To gain insight into the critical DNA sequences involved in organ-specific expression, we investigated whether the 259-bp \( XbaI-B_{HII} \) and 163-bp \( NeoI-Sac_I \) DNA regions contain binding sites for putative transcriptional regulators. The \( XbaI-B_{HII} \) fragment includes a previously identified RARE-DR5 required for \( Hoxa4 \) RA-responsiveness in CNS and for \( Hoxa4 \) lung and

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**Figure 3. Characterization of the 1.5-kb XbaI-XbaI DNA fragment in E13.5 F0 transgenic mouse embryos.** (A) Schematic representation of the 1.5-kb XbaI-XbaI DNA fragment in the \( Hoxa4-Hoxa6 \) genomic region. (B) Diagram of the \( Hoxa5/lacZ \) constructs used to generate E13.5 F0 transgenic embryos and summary of transgenic expression analyses. (C–F) Carcass of representative E13.5 transgenic embryos and the associated organs (G–J) stained for β-galactosidase activity showed the effects of the different deletions on the expression pattern. Open arrowhead points the anterior limit of transgene expression in the neural tube. i, intestine; l, lung; nt, neural tube; pv, prevertebrae; s, stomach; sp, spleen.

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stomach expression (Fig. 5A) [44]. Genomic sequence comparison with the zebrafish HoxAa cluster also revealed that this RARE was part of a DNA region of high homology (>70%) spanning ~100-bp, suggesting the presence of evolutionary conserved regulatory elements (not shown) [45]. To define if the RARE sequence was involved in Hoxa5 regulation during organogenesis, we performed EMSA with WCE from lung, stomach and intestine of E13.5 mouse embryos and a 259-bp XbaI-BssHII radiolabelled probe. Binding was observed and the specificity was confirmed by competition with a 100-fold excess of unlabelled probe. However, competition was also detected when the RARE site was mutated (Fig. 5B, lanes 1–4). Moreover, no competition occurred with oligos containing wt or a mutated version of the RARE (Fig. 5B, lanes 5–6).

We assessed the RARE regulatory activity in E13.5 Hoxa5/lacZ transgenic embryos by mutating the RARE sequence in the 1.5-kb Xbal-Xbal DNA fragment (construct 17) and in the 259-bp XbaI-BssHII fragment (construct 18; Fig. 6B). Except for the CNS anterior boundary that was not reproduced, construct 17 targeted transgene expression in lung, stomach and intestine similarly as construct 4 (Fig. 6C, I). Likewise, construct 18 presented a comparable staining pattern than construct 10 with expression in the stomach and intestine but not in lung (Fig. 6D, J). Thus, the RARE sequence, found to be necessary to drive Hoxa4 expression in embryonic lung and gut, does not play a key role in Hoxa5 organ expression at E13.5.

The YY1 transcription factor binds to Hoxa5 organ-specific regulatory DNA regions

To identify which DNA region from the Xbal-BssHII radiolabelled probe bound proteins in WCE from organs of E13.5 embryos, the 259-bp sequence was divided into three fragments (A to C) used as competitors. Only fragment C competed binding
Figure 5. Characterization of the 259-bp XbaI-BssHII DNA fragment by EMSA. (A) Restriction map of the 1.5-kb XbaI-XbaI sequence extending from +9.3-kb to +10.8-kb in the 3' half of the Hoxa4-Hoxa5 intergenic region. The box denotes the location of the 259-bp XbaI-BssHII regulatory region. Sequence of the 259-bp XbaI-BssHII DNA fragment is indicated. Fragments A, B and C used as competitor in EMSA are underlined. Boxed nucleotides correspond to RARE-DR5 sequence and YY1 binding sites. Symbols * and @ indicate point mutations into RARE and YY1 binding sites, respectively (see Table 1B for nucleotide sequences). (B) EMSA with WCE from lung/stomach/intestine of E13.5 embryos and the 259-bp XbaI-BssHII radiolabelled probe in presence of unlabelled competitors in 100-fold excess showed that protein binding occurred with the Oligo-C3 fragment via YY1 binding sites (lanes 9, 12, 18–19). No binding with the RARE site was observed (lanes 4–6). EMSA with in vitro-translated YY1 protein and the 259-bp XbaI-BssHII probe showed specific binding that was competed by Oligo C3 (lanes 21–24). (C) The binding of WCE with YY1 consensus binding site and the loss of binding when the YY1 antibody was added confirmed the presence of YY1 protein in WCE (lanes 1–5). (D) EMSA with WCE and Oligo C3 radiolabelled probe showed...
with the XbaI-BsoHI probe (Fig. 5B, lanes 7–9). Moreover, only the 45-bp Oligo C3 located at the 3’ end of fragment C could compete binding with the XbaI-BsoHI probe (Fig. 5B, lanes 10–12). Using a linker scanning approach, we found that an 18-bp sequence in Oligo C3 (Oligo-18(C3)) was responsible for the binding (Fig. 5B, lanes 13–18). Sequence comparison with the TFSEARCH and TESS databases revealed YY1 binding sites in Oligo-18(C3). Mutations of the YY1 sites in Oligo C3 impaired its capacity to compete with the XbaI-BsoHI probe (Fig. 5B, lane 19).

To further establish that YY1 can bind the XbaI-BsoHI fragment, we used a murine YY1 protein produced in vitro. Binding specificity to the XbaI-BsoHI probe was confirmed and competition with Oligo C3 caused YY1 protein binding inhibition (Fig. 5B, lanes 20–24).

Figure 6. Characterization of the RARE and YY1 binding sites in E13.5 F0 transgenic mouse embryos. (A) Schematic representation of the 1.5-kb XbaI-XbaI DNA fragment in the Hoxa4-Hoxa6 genomic region. (B) Diagram of the Hoxa5/lacZ constructs used to generate E13.5 F0 transgenic embryos and summary of transgenic expression analyses. The asterisks in constructs 17–18 and the @ symbols in constructs 19–22 correspond to mutations in the RARE and YY1 binding sites, respectively. (C–H) Carcass of representative E13.5 transgenic embryos and the associated organs (I–N) stained for β-galactosidase activity showed the effects of the mutations on the expression pattern. Open arrowhead points the anterior limit of transgene expression in the neural tube. i, intestine; l, lung; nt, neural tube; pv, prevertebrae; s, stomach.

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We then verified that our WCE contained YY1 protein by performing supershift assays. The specificity of the YY1 antibody was validated by testing an YY1 consensus sequence with WCE [20]. Addition of the YY1 antibody specifically resulted in the loss of DNA-protein complexes (Fig. 5C). A CDX2 antibody was used as negative control. In parallel, we tested the Oligo C3 sequence with WCE in EMSA. Binding was observed and it was competed by an excess of cold Oligo C3, Oligo-18(C3) and the YY1 consensus sequence (Fig. 5D, lanes 1–4, 8). No competition was observed when a non-specific probe was used (Oligo C2) or when several mutations distributed along the YY1 binding sites were inserted into Oligo C3 (Fig. 5D, lanes 5, 11). Presence of localized mutations in YY1 sites allowed competition to happen (Fig. 5D, lanes 6–7). Supershift assays were also performed with the YY1-specific antibody and the CDX2 control antibody. Only the addition of the YY1 antibody led to the loss of Oligo C3-protein complexes (Fig. 5D, lanes 9–10). Thus, the YY1 protein present in WCE from organs of E13.5 mouse embryos specifically binds a site located at the 3’ end of the 259-bp Xba-I-BsoHI fragment.

A similar systematic approach was applied to decipher the regulatory elements of the 163-bp Noa-Saeq sequence. EMSAs combining the 433-bp Mfe-I-Saeq radiolabelled probe with WCE demonstrated specific binding with the 163-bp Noa-Saeq portion (Fig. 7B, lanes 1–5). The latter was further divided into four oligos, leading to the identification of the 45-bp Oligo G3 as the binding sequence. Using the linker scanning approach, an 18-bp sequence in oligo G3 (Oligo-18(G3)) was found to be necessary for protein binding (Fig. 7B, lanes 6–15). Sequence comparisons with databases revealed YY1 binding sites in Oligo-18(G3), which when mutated in Oligo G3 impaired its capacity to protein binding with the 433-bp Mfe-I-Saeq sequence as the non-specific BsoHI-BomI competitor (Fig. 7B, lanes 16–17). We also tested the Oligo G3 sequence with WCE, the YY1-specific antibody and various competitors and we confirmed that the YY1 protein present in WCE from organs of E13.5 mouse embryos can specifically bind the 163-bp Noa-Saeq sequence (Fig. 7C; lanes 1–11).

Functional YY1 binding sites are involved in Hoxa5 organ-specific expression

To establish whether the YY1 binding sites identified in the 259-bp Xba-I-BsoHI and 163-bp Noa-Saeq DNA regions are effective in vivo, a ChIP assay was performed on cross-linked chromatin isolated from either lung or stomach of E13.5 wt mouse embryos. DNA from the immunoprecipitate was subjected to qPCR analyses with specific primers for the 259-bp Xba-I-BsoHI and 163-bp Noa-Saeq sequences, for a reported YY1 target gene used as positive control (Sf gli10), and a known YY1 negative control (Ror3) [46]. A downstream region located at 15-kb of Hoxa5 TSS and devoid of YY1 binding sites was used as a negative control for the locus. In lung and stomach, YY1 was recruited to Hoxa5 C3 and G3 regulatory sequences and to the positive Sf gli10 control whereas no binding was observed with the Ror3 and Hoxa5 locus negative controls (Fig. 8; not shown). Thus, YY1 can bind to Hoxa5 organ-specific regulatory DNA regions both in vitro and in vivo.

We then assessed the contribution of the YY1 binding sites to Hoxa5 organ expression in E13.5 transgenic embryos. Construct 19, which contained mutations in the YY1 binding sites of the Xba-I-BsoHI and the Noa-Saeq regions, correctly directed expression in the CNS as construct 4, while no organ expression was detected (Fig. 6E, K). Similarly, mutations of the YY1 binding sites in the Xba-I-BsoHI sequence in construct 20 and in the Noa-Saeq sequence in construct 21 caused a loss of organ expression when compared to constructs 10 and 15, respectively (Fig. 6F–G, L–M).

A comparable result was observed when the Noa-Saeq sequence with mutated YY1 sites was tested in front of the heterologous hsp68/lacZ plasmid (construct 22; Fig. 6H, N). In summary, YY1 acts as a positive regulator of Hoxa5 lung, stomach and intestine expression during embryogenesis.

Inactivation of Yy1 function in mesenchyme impacts on lung formation and on Hox gene expression

In mice, the complete loss of Yy1 gene function resulted in peri-implantation lethality, while the phenotypes associated with the Yy1 conditional and hypomorphic alleles revealed a critical gene dosage requirement for Yy1 during embryogenesis [38,47]. Indeed, Yy1<sup>flox<sup>/-</sup></sup> mice express 25% of normal YY1 protein levels and a high percentage of newborn pups die at birth from lung defects reminiscent to those observed in Hoxa5<sup>−/−</sup> mice [6,38]. To assess in vivo the requirement of YY1 gene function in Hoxa5 lung expression, we generated Yy1<sup>flox<sup>/-</sup></sup> mouse embryos carrying the Hoxa5/lacZ construct 1 transgene. No change in lacZ expression pattern and Hoxa5 RNA levels was observed in the lungs from E13.5 and E18.5 Yy1<sup>flox<sup>/-</sup></sup>Yy1<sup>flox<sup>/-</sup></sup>/Hoxa5/I-I<sub>lacZ</sub> embryos (not shown). The lack of effect on Hoxa5 expression might result from the remaining 25% of YY1 expression.

As Hoxa5 is specifically expressed in lung mesenchyme, we investigated Yy1 gene function in the mesenchyme using the Dermo1<sup>Cre</sup> deleter mouse line [35,39]. No Yy1<sup>flox<sup>+/+</sup></sup>/Dermo1<sup>Cre</sup> animals were recovered at weaning and monitoring of litters showed that all Yy1<sup>flox<sup>+/+</sup></sup>/Dermo1<sup>Cre</sup> pups were found dead at birth (Fig. 9A). At E18.5, the distribution of genotypes was confirmed to the expected mendelian ratio. Thus, mesenchymal Yy1 deletion causes death at birth.

Comparative lung morphology of E18.5 embryos revealed that Yy1<sup>flox<sup>−/−</sup></sup>Dermo1<sup>+/+</sup> and Yy1<sup>flox<sup>−/−</sup></sup>Dermo1<sup>+/+</sup>C<sup>cre</sup> specimens presented a normal structure with dilated peripheral lung saccules and thin mesenchyme. However, lungs from Yy1<sup>flox<sup>−/−</sup></sup>Dermo1<sup>−/−</sup>C<sup>cre</sup> showed a collapsed appearance with narrow airspaces and thick mesenchyme similar to the Hoxa5<sup>−/−</sup> lung phenotype, a likely explanation for the neonatal lethality of Yy1 mutant pups (Fig. 9B–E). We also assessed the impact of the mesenchymal Yy1 deletion on the integrity of the arterial epithelium by looking at the expression of cell specific markers by qRT-PCR and IHC. A statistically decreased expression of the club cell (Clara cell) marker CC10 (encoded by the Sgk1al gene) was observed in lungs specimens from E18.5 Yy1<sup>flox<sup>−/−</sup></sup>Dermo1<sup>+/+</sup>/Cre by qRT-PCR (Fig. 9Q). IHC analysis showed that the decreased CC10 staining paralleled that seen in Hoxa5<sup>−/−</sup> specimens (Fig. 9F–I) [41]. As well, expression of podoplanin (T1α), a marker of type I pneumocytes, which are participating to gas exchanges with the underlying vascular endothelial cells, was decreased in Yy1 mutant specimens similar to what was previously observed in Hoxa5<sup>−/−</sup> mutants (Fig. 9J–M, Q) [35]. Together, these results indicated that the Yy1 mesenchymal deletion perturbed epithelial cell differentiation along the respiratory tract, a phenotype reminiscent to that of Hoxa5 mutants.

We validated the specific inactivation of Yy1 in lung mesenchyme by IHC analysis. At E15.5 and E18.5, the YY1 protein showed ubiquitous expression in lung epithelial and mesenchymal compartments from control embryos, whereas in Yy1<sup>flox<sup>−/−</sup></sup>Dermo1<sup>−/−</sup>C<sup>cre</sup> specimens, YY1 staining was greatly diminished in lung mesenchyme but unchanged in the epithelium (Fig. 9O–P; not shown). qPCR analysis confirmed the decreased Yy1 expression in lungs from E18.5 Yy1<sup>flox<sup>−/−</sup></sup>Dermo1<sup>−/−</sup>C<sup>cre</sup> embryos (Fig. 9R). We also looked at the impact of the Yy1 mesenchymal deletion on Hoxa5 and Hoxa4 lung expression as the 1.5-kb Xba-XhoI DNA fragment containing the YY1 binding sites was shown to be
necessary for *Hoxa4* lung and gut expression [44]. qRT-PCR expression analysis revealed significantly reduced *Hoxa5* and *Hoxa4* expression levels in lungs from E18.5 *Yy1* flox/flox Dermo1 +/Cre embryos, indicating that an integrated regulation of *Hoxa4* and *Hoxa5* genes prevailed in lung expression through the sharing of YY1-responsive sequences.

**Discussion**

Our search for *Hoxa5* transcriptional regulatory sequences led us to identify a 14.5-kb genomic fragment (construct 1) encompassing the *Hoxa5* gene and starting into the homeobox of the 5′ flanking *Hoxa6* gene up to ~350-bp upstream of the *Hoxa5* TSS. The 14.5-kb transgene reproduces the spatio-temporal expression directed by the *Hoxa5* proximal promoter by targeting lacZ expression to the brachial region of the CNS and to the paraxial and lateral plate mesoderm at the cervico-thoracic level as reported for the shorter version of 11.1-kb in length [31]. In addition, the 14.5-kb transgene recapitulates *Hoxa5* expression in the mesenchymal compartment of the respiratory and digestive tracts, the *Hoxa5* rostro-caudal gradient in the stomach, and the anterior limit of *Hoxa5* expression in the CNS at the level of the floor of myencephalon [6–8,31].

*Hoxa5* expression in the developing neural tube is under the control of the BSC enhancer, which directs expression in the brachial region of the spinal cord from E11 to E13, a subset of the *Hoxa5* endogenous pattern in the neural tube [30]. We now report that sequences distributed in the 1.5-kb XbaI–XbaI DNA fragment replicate the anterior expression domain of *Hoxa5* in the neural tube. However, the onset of *Hoxa5* expression in the developing CNS up to E11 is not reproduced with the 14.5-kb transgene indicating that additional sequences are required [31]. Moreover, mutation of the RARE site located in the 1.5-kb XbaI–XbaI fragment causes a caudal shift of the anterior boundary in the CNS suggesting that RA signaling is involved in the establishment of the correct *Hoxa5* expression domain in the neural tube as shown for other *Hox* genes [48–49]. Interestingly, when tested in a *Hoxa4* context, mutation of the RARE sequence did not impact on the activation of *Hoxa4* embryonic expression in the developing neural tube but it affects *Hoxa4* response to exogenous RA in the neural tube [44]. Thus, the *Hoxa4* and *Hoxa5* genes share a RARE site that positively regulates their respective expression in the...
This RARE sequence is highly conserved between Hox clusters and between vertebrates. It was shown to possess neural enhancer activity for the Hoxd4 gene [49–51]. In the HoxB cluster, the Hoxb5 distal RARE was found to regulate the anterior expression boundary of 5 Hoxb genes in the posterior hindbrain raising the possibility that the RARE site located in the 1.5-kb XbaI-XbaI fragment may play a similar evolutionarily conserved role in the HoxA complex [23].

While the RARE site in the 1.5-kb XbaI-XbaI fragment appears important for Hoxa5 expression in CNS, it is not required for Hoxa5 expression in the developing lung and gut when tested in E13.5 transgenic embryos. In EMSA experiments with the 259-bp XbaI-BstHII fragment, the lack of binding between the RARE sequence and WCE prepared from lung, stomach and intestine of E13.5 mouse embryos further supports the absence of a role for the RARE sequence in Hoxa5 organ expression. In contrast, Hoxa4 expression in lung, gut and metanephros at the same embryonic age is dependent on the functional RARE, suggesting that the action of the RARE site is Hox promoter-specific [44]. We cannot rule out the possibility that the RARE site may be functional in a time-dependent manner regulating Hoxa5 lung and gut expression at earlier embryonic stages as suggested by studies showing that RA deficiency negatively impacts on Hoxa5 expression in the developing lung and stomach at E10.5 [52]. Another RARE located at the 3′ end of the human HOXA5 gene was shown to mediate RA responsiveness of the gene in breast cancer cells [53]. This RARE site is conserved in the mouse genome at position +7.4-kb from the Hoxa5 TSS. Even though our deletion studies did not identify the corresponding genomic region to be involved in Hoxa5 gene regulation, we cannot exclude a role for this RARE in Hoxa5 developmental expression.

Our combined approach of transgenesis and biochemistry revealed that YY1 is a positive regulator of Hoxa5 gene expression in the developing respiratory and digestive tracts, while it does not play a major role in CNS expression. YY1 is a multifunctional zinc-finger-containing transcription factor, identified as the...
Figure 9. Analysis of the lung phenotype in Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> mutants. (A) Ratios of genotypes of litters obtained from matings between Yy1<sup>flox/+</sup> Dermo<sup>1<sup>Cre</sup></sup> and Yy1<sup>flox/flox</sup> mice. (B–E) Comparative lung histology of E18.5 Yy1<sup>flox/+</sup> Dermo<sup>1<sup>Cre</sup></sup>, Yy1<sup>flox/+</sup> Dermo<sup>1<sup>Cre</sup></sup>, Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> and Hoxa5<sup>−/−</sup> embryos. Yy1<sup>flox/+</sup> Dermo<sup>1<sup>Cre</sup></sup> and Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> specimens presented a normal lung structure, whereas lungs from Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> and Hoxa5<sup>−/−</sup> embryos were collapsed. (F–M) Characterization of the respiratory epithelium of E18.5 Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> embryos. (F–I) Detection of club cells by CC10 immunostaining showed decreased labelling in lungs from Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> and Hoxa5<sup>−/−</sup> specimens. (J–M) Immunostaining with T1α, a marker of type I pneumocytes, was reduced in lungs from Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> and Hoxa5<sup>−/−</sup> embryos. (N–P) YY1 immunostaining showed ubiquitous YY1 expression in lung epithelial and mesenchymal compartments in E15.5 control embryos, but an important decreased staining with T1α, a marker of type I pneumocytes, was reduced in lungs from Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> and Hoxa5<sup>−/−</sup> embryos. (Q–S) qRT-PCR analysis for Scgb1a1, T1α, YY1, Hoxa5 and Hoxa4 expression in lungs from E18.5 Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> and Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> embryos. Expression levels were significantly diminished for all genes tested in Yy1<sup>flox/flox</sup> Dermo<sup>1<sup>Cre</sup></sup> specimens. Values are expressed as means ± SEM. *p<0.05, **p<0.01, ***p<0.001.

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homolog of Drosophila Pleiohomoeotic (PHO) protein, the latter recruiting Polycomb group (PcG) proteins to negatively regulate genes. YY1 plays crucial roles in numerous biological processes by selectively initiating, activating or repressing transcription, depending upon promoter contextual differences or specific protein interactions [34]. The role of YY1 in Hox gene regulation is mainly associated with repression as shown for the Hoxd4, Hoxd4, HOXB13 and HOXD11-HOXD12 genes. For instance, YY1 binds Hoxb4 promoter and intron enhancer sequences through overlapping NFY/YY1 sites suggesting that the relative levels of binding of the transcriptional activator NFY and YY1 through the same NFY/YY1 sites are present in the HOXD11-HOXD12 intergenic region in human embryonic stem cells, YY1 recruiting PcG proteins for the transcriptional repression of the distal HoxD genes [29]. So far, only the Hoxb7 gene expression is positively regulated by YY1 in tumor and transformed cell lines [27].

Here, our work established for the first time the positive role of YY1 in Hox gene expression in a normal context, the embryo. Our EMSA and ChIP data obtained with organ preparations from E13.5 embryos demonstrated that YY1 physically interacts with two Hoxa5 regulatory sequences located in the Hoxa5-Hoxa4 intergenic region. Mutations in these YY1 binding sites abolished Hoxa5 expression in lung, stomach and intestine from transgenic embryos. Moreover, the conditional deletion of YY1 function in lung mesenchyme resulted in decreased levels of Hoxa5 transcripts in the developing lung. Altogether, these data demonstrated that YY1 acts as a transcriptional activator of Hoxa5 expression in lung and gut during embryogenesis.

In the trunk of E12.5 mouse embryos, YY1 binding sites are present in Hoxa5 upstream sequences and they co-localize with EED and BMI1 binding sites, two PcG protein members of the PcG repressive complex I. PcG binding is specific to the anterior domain of the trunk and it results in the transcriptional silencing of Hoxa5 in this axial region demonstrating that PcG repression is involved in the establishment of the correct Hoxa5 expression domain in the prevertebral column [56]. Thus, Hoxa5 developmental expression is under the control of several YY1 binding sites distributed along the Hoxa5 locus. Depending on the developmental context, YY1 can mediate PcG repression of Hoxa5 expression via binding sites located in Hoxa5 upstream sequences to finely define Hoxa5 expression domain along the A-P axis [56]. Here, we showed that YY1 can also activate Hoxa5 expression in the developing respiratory and digestive tracts acting via binding sites located in the intergenic Hoxa5-Hoxa4 region and shared with the flanking Hoxa4 gene. Studies have shown that YY1 acts by recruiting co-activators to function on YY1-activated targets [57–61]. This situation may also prevail for the Hoxa5 gene. While some of these coactivators are expressed in the lung (INO80, PRMT1, BAP1, GATA-4, AP-1), no information is available about their potential role in lung development.

Genetic analyses have revealed the crucial role played by YY1 during embryogenesis. YY1+/− embryos die shortly after implantation [47]. The use of the Yfy conditional allele with lineage-specific Cre mouse lines circumvents the early embryonic lethality of Yfy null mutants revealing the large spectrum of YY1 actions throughout life [38,62–65]. A dosage-dependent requirement for YY1 is essential for survival as newborn mice expressing 25% of normal YY1 levels die at birth from respiratory failure [38]. Despite these observations, little is known about the Yfy function during lung morphogenesis. Here, we showed that the specific ablation of Yfy function in mesenchyme via the use of Dermo1−/−Yfy−/− deleter mice causes neonatal death of mutant pups likely due to lung defects. Lungs from Yfy+/+Yfy−/−Dermo1−/−Yfy−/− embryos were collapsed with narrow airspaces and thick mesenchyme. Epithelial cell differentiation along the respiratory tract was also affected as shown by the decreased expression of CC10 and T1α, specific markers of club cells present in the respiratory airways and type I pneumocytes lining the alveolar epithelium, respectively. These lung phenotypes were similar to those observed in Hoxa5−/− embryos, supporting the notion that mesenchymal YY1 action during lung formation is mediated, at least in part, by the control of Hoxa5 expression [6,35,41]. This is further reinforced by the decreased Hoxa5 expression in lungs from Yfy+/+Yfy−/−Dermo1−/−Yfy−/− embryos. However, according to the broad transcriptional activity of YY1, the lung phenotype of Yfy+/+Yfy−/−Dermo1−/−Yfy−/− mutants may also result from the deregulation of other genes.

YY1 was shown to be essential for the transcription of the Sgk1al gene in endometrial cells [66]. In the present case, the decreased Sgk1al expression observed in the respiratory epithelium of Yfy+/+Yfy−/−Dermo1−/−Yfy−/− embryos is a non-cell autonomous phenotype since the Yfy mutation is specific to mesenchyme. Sgk1al expression is also diminished in the respiratory epithelium of Hoxa5−/− mutants [41]. Therefore, Hoxa5 could be a downstream effector of YY1. Alternatively, Yfy and Hoxa5 genes may act on Sgk1al expression via distinct pathways.

Hoxa4 lung expression was reduced in lungs from Yfy+/+Yfy−/−Dermo1−/−Yfy−/− embryos, suggesting that the YY1 binding sites present in the 1.5-kb Xbal-Xbal DNA fragment are involved in the regulation of both Hoxa4 and Hoxa5 genes. No lung phenotype was reported in Hoxa4−/− mutants, but it is possible that Hoxa4 exerts a role during lung development masked by functional redundancy. Even though the decrease in Hoxa4 and Hoxa5 expression was modest in lungs from Yfy+/+Yfy−/−Dermo1−/−Yfy−/− embryos, the combined downregulation of Hoxa4 and Hoxa5 may participate to the lung phenotype observed in Yfy+/+Yfy−/−Dermo1−/−Yfy−/− mutants.

In summary, our search for regulatory sequences that correctly reproduce Hoxa5 developmental expression has led to the identification of additional cis-acting elements present in the Hoxa4-Hoxa5 intergenic region that are important for Hoxa5 expression in CNS, lung, stomach and intestine. Several of these sequences are shared with the flanking Hoxa4 gene supporting the model that coordinated regulatory mechanisms between Hox genes are essential for the precise function of each gene and the correct development of the embryo [13]. We have also unveiled the crucial role of YY1 as a transcriptional activator of Hoxa5 lung and gut expression and the repercussions of the Yfy conditional deletion in lung mesenchyme. Further studies will explore the extent of the regulatory role of YY1 in Hoxa5 developmental expression.

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