Related genetics mechanisms of "Hox" function in mammalian limb and gut development

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Summary and scope of the thesis

In vertebrates, along with the development of the main body axis and limbs, *Hox* genes are expressed in a collinear fashion. The implicit temporal and spatial coordination of expression of *HoxA*, *HoxB*, *HoxC* and *HoxD* cluster genes contribute decisively to regional specification of the body due to the differential activity of homeodomain transcription factors produced by each one of the 39 *Hox* genes. A particularly well-studied example is the case of the involvement of the *HoxD* cluster during tetrapod limb development, which recently became complemented with considerable advances in the understanding of the function of these same genes in the development of the gastro-intestinal tract. This thesis aims to summarize my experiments focused on two targeted recombinant alleles of the *HoxD* cluster, called *Del(1-10)* and *Del(4-11)* which significantly contributed to understanding the genetic control of both limb and gut patterning.

Limb bud growth is characterized by two waves of *Hoxd* expression, the first of which occurs at early budding stage and involves all the genes of the cluster (from *Hoxd1* until *Hoxd13*) with 3'end, also called anterior gene transcripts distributed all along the limb bud and 5'end, also called posterior gene transcripts restricted to the posterior limb bud. The second wave is characterized by the predominant expression of *Hoxd9* up to *Hoxd13* such that the now apparent more proximal limb domain is positive for *Hoxd9, Hoxd10* and *Hoxd11* transcripts whereas the distal, or digit domain is labeled for *Hoxd10* up to *Hoxd13*, while expression of *Hoxd12* and *Hoxd13* genes is excluded from the proximal domain. Extensive genetic analysis in mice showed, that the morphogenetic role of any *Hox* gene is most apparent in limb regions, where the gene in question is the more posterior one expressed. For instance morphological defects in mutants of *Hoxd9* are restricted to the proximal limb, the humerus, the role of *Hoxd11* to the lower arm, and *Hoxd13* to the hand, and fingers. The posterior prevalence rule explains the underlying functional hierarchy, by invoking interactions occurring at post-transcriptional level when anterior and posterior HOX protein products occur together in the same cell. Initial examples of such functional hierarchy among HOX products were discovered in *Drosophila*, where they were grouped under the term phenotypic suppression.

During the past decade, a number mouse *HoxD* cluster mutants have been isolated and characterized which support the general outlines of this interpretation. In particular, genetic analysis of limb and gastro-intestinal tract development has provided concordant evidence for the broad applicability of the posterior prevalence rule. A targeted partial deletion of the *HoxD* cluster, which removed genes from *Hoxd1* to *Hoxd10* (*Del(1-10)*), drives premature *Hoxd12* and *Hoxd13* expression at ectopic position in the early limb bud. This affects the symmetry of the forming limb through ectopic induction of *Shh* at the anterior margin of the bud leading to double-posterior identity of digits, and occasional polydactyly. The number and morphology of digits is also controlled by the function of *Gli3* transcription factor, because in extra-toes (*Xt*) mutants, who lack functional *Gli3*, polydactylous limbs develop. The ectopic activation of posterior *Hoxd* genes in anterior limb in absence of *Gli3* function and other data involving ectopic *Hoxd* gene expression suggested that *Gli3* may control the number of digits by downregulating posterior *Hox* function. To assess further the nature of *Hox* and *Gli3* genetic interaction in limb development we took advantage of a number of novel mouse strains where the gene content of the *HoxD* cluster was reduced by targeted deletions due to loxP/Cre mediated site-specific recombination. First we crossed mice carrying the full *HoxD* deficiency, *Del(1-13)*, into the *Xt, Gli3* deficient background. We

obtained late fetuses of all the allelic combinations, and analyzed the morphology and skeletal pattern of the limbs. Unexpectedly the number and morphology of digits remained characteristic of the *Gli3* deficient genotype, suggesting that in *Xt* mice the gain of posterior *Hoxd* genes is not sufficient to cause polydactyly.

Gain of the posterior *Hox* genes, *Hoxd11*, *Hoxd12* or *Hoxd13* in *Del(1-10),* and certain other *HoxD* mutants often caused polydactyly, however. To gain insight into this complicated situation we aimed to assess the impact of *Gli3* over posterior *Hoxd* gain-of-function by breeding *Del(1-10)* and *Xt* compound heterozygous mice in a dihybrid cross panel. It was observed that mice homozygous for the *Del(1-10)* deletion in addition to a symmetric autopod, display shorter humerus. This proximal limb defect got exaggerated further in mice double homozygous for *Xt* and the *Del(1-10)* deletion showing severely truncated forelimbs, where the humerus had disappeared and the rest of the limb displayed only remnants of zeugopod and autopod. Detailed analysis showed that the humerus was progressively shortened along with the reduction of the dose of *Gli3* and the increase of ectopic expression of *Hoxd12* and *Hoxd13*. The extent of limb growth depends on secreted factors *Fgf10* and *Shh* in mesenchyme, and *Fgf8* in the apical ectodermal ridge (AER). The severe limb truncation suggested that in *Xt* and *Del(1-10)* compound mutants the establishment or the maintenance of the AER is strongly compromised, presumably by interfering with mesenchymal factors *Fgf10* and *Shh*. In situ hybridization performed for key genotypes when the AER has just formed, revealed deviations from the normal expression profile of all these genes. Both in *Xt* and *Del(1-10)* forelimbs *Hoxd13* expression was gained in the anterior mesenchyme whereas the *Fgf10* signal was downregulated in the more proximal domain and shifted forward complementary to *Hoxd13*. In forelimb buds of double homozygous *Hoxd13* was transcribed in the very anterior mesenchyme whereas *Fgf10* expression was almost eliminated from the bud. In the same context *Shh* was lost from the posterior limb bud and gained in a group of cells located to the medial-anterior margin. In this particular region the AER was maintained, whereas was absent all along the rim of the limb bud, indicated by dramatic loss of the *Fgf8* signal. Thus premature and ectopic expression of *Hoxd12* and *Hoxd13* in absence of *Gli3* function stops the growth of the limb bud, by interfering with the mesenchymal factors that preserve the integrity of the AER. As a result the outgrowth of the limb along the proximo-distal axis is severely affected.

As well as along other body axes, *Hox* genes are functional in the fetal gastro-intestinal tract, this latter particularly well studied in mice. Many studies contributed to a systematic description of *Hox* expression in embryonic gastro-intestinal tract at early stages of development, but none of them reported a complete profile of an entire *Hox* cluster, so the available information is fragmentary and dispersed trough several works. Nonetheless it was suggested that *Hox* genes confer regional identity to the gastro-intestinal tract, as the mutations of some of *Hoxa* and *Hoxc* genes caused malformations in stomach or intestine. In a similar way it was shown that *Hoxd12* and *Hoxd13* are necessary for the morphogenesis of the anal sphincter. At the mechanistic level, *Hox* genes are held to contribute to the molecular interactions between epithelium and mesenchyme that underlie regional specification of the gastro-intestinal tract and the formation of muscular constrictions along its anterior-posterior axis.

Development of the caecum in mice provides a favorable context to explore *Hox* function in gut regionalization. The caecum is an organ located between the small and large mammalian intestine; its size is considerably big in animals with a diet rich in fibers. The morphogenesis of the caecum in mouse starts at E11 (the eleventh day of embryonic development), when it protrudes out of the abdominal wall in the form of an intestinal hernia. We initially performed a systematic analysis of *HoxD* cluster gene

expression in murine gastro-intestinal tract. We found that most of the *Hoxd* genes are transcribed in caecum mesenchyme; remarkably all the genes from *Hoxd1* up to *Hoxd11* are represented in the forming caecal bud whereas *Hoxd12* and *Hoxd13* are excluded from this region. These observations suggested that the presence or absence of specific *HoxD* gene products in caecum might be important for its development. Indeed mice homozygous for the partial *HoxD* deficiency described above, *Del(1-10),* in addition to the limb defects, displayed caecum agenesis, while heterozygous exhibited a caecum of reduced dimension. To elucidate the molecular factors involved in suppression of caecal outgrowth we performed in situ hybridization analysis on whole mount gastro-intestinal tract using probes detecting the activity of the remaining genes. Both *Hoxd11* and *Hoxd12* were found expressed in caecum of *Del(1-10)* mice with an ectopic limit that reach the ileo-caecal valve. We have isolated and studied a related, but distinct mouse strain named *Del(4-11)* where *Hoxd1*, *Hoxd3* and *Hoxd12*, *Hoxd13*, were left respectively at the 3' and 5' ends of the cluster. Homozygous *Del(4-11)* mice lacked the caecum as well. Consistently, in homozygous and heterozygous mice for this deficiency *Hoxd12* was gained in the caecal area, consistent with its protein product being able to suppress any morphogenetic activity of HOXD1 or HOXD3 in caecum budding.

In search of molecules promoting caecum bud morphogenesis we found that mice lacking the *Fgf10* function had atretic caecum. In our mice the *Fgf10* expression in caecum mesenchyme was almost absent in precisely the same cells where *Hoxd12* was ectopically expressed. Very likely this *Hoxd12* gain-of-function suppresses the growth of the caecum by inhibiting *Fgf10* as a consequence of counteracting the activity of more *Hox* anterior genes. In search of yet other molecules that may be crucial in caecum development and/or agenesis we focused on *Pitx1* transcription factor. We discovered that *Pitx1* transcripts accumulate in caecum bud mesenchyme, but they are absent from this region in *Del(1-10)* and *Del(4-11)* homozygous mice. We thus suggested that the ectopic gain of posterior *Hox* genes in ileo-caecal transition of these lines suppresses both *Fgf10* and *Pitx1* expression in mesenchymal cells. As result *Fgf10* signal amplification is interrupted thereby hampering the growth of the caecum.

To support the hypothesis that during budding development, in limb bud or caecal bud, the developmental growth defects were indeed due to inactivation of anterior *Hox* gene function by the ectopic presence of posterior *Hox* gene products we performed genetic complementation assays. Breeding *Del(1-10)* with other *HoxD* alleles where *Hoxd1*, *Hoxd3*, *Hoxd4*, *Hoxd8*, *Hoxd9* and *Hoxd10* were progressively added we titrated the suppressive posterior ectopic dose with progressively more doses of apparently functionally equivalent anterior products. The outcome was the fully penetrant recovery of caecum in mice with one split *HoxD* haplotype, carrying from *Hoxd1* to *Hoxd10* on one chromosome and from *Hoxd11* up to *Hoxd13* on the other one. In conclusion posterior *Hoxd* genes harm the outgrowth of the caecum by neutralizing the activity of anterior *Hox* genes. This series of experiments therefore provides a striking illustration of the morphogenetic importance of the posterior prevalence rule.

Introduction

1. Homeotic genes

The morphology of the body plan in bilaterians depends on the concerted action of several factors whose activity in space and time is coordinated by a minor number of so-called *homeotic* genes. Homeotic mutants were extensively studied in the fruit fly *Drosophila melanogaster* by Edward B. Lewis, who by 1978 systematically described profound changes in the identity of segments along the anterior-posterior axis of the body of the fly. The products of the homeotic genes turned out to be *homeobox* proteins, which are transcription factors. They share a sequence of 60 amino acids (related to the helix-turn-helix motif) called homeodomain, by which they bind DNA (Scott, and Weiner, 1984). The *homeotic* genes of *Drosophila* are grouped in the homeobox complex (*HOM-C*) that is split into the two groups *Antennapedia* and *Bithorax* (Akam, 1987). In total the HOM-C is composed of 8 genes: *Labial (lb)*, *Proboscipedia (Pb)*, *Deformed (Dfd)*, *Sex comb reduced (Scr)*, *Antennapedia (Antp)*, *Ultrabitorax (Ubx)*, *Abdominal-A (Abd-A)* and *Abdominal-B (Abd-B);* the first five genes belonging to the *Antennapedia* group and the last three to the *Bithorax* group (Kaufman, et al., 1980). Lewis studied mutations in *homeotic* genes that caused the transformation of part of the body of the fly in a completely different one, an event referred as *homeosis*. For example the mutation named *Antennapedia* causes the outgrowth of extra-legs in place of antenna, while the *Bithorax* mutation generates flies having two pairs of wings. These kinds of developmental defects were defined as “homeotic transformations” and in most of the cases were observed to hit a domain correspondent to a segment of the body of the fly. This suggested that a specific mutation had occurred in a selector gene within the progenitor cell of that precise compartment changing the identity and position of the segment along the anterior-posterior body axis.

The acquisition of a different organization of the body in arthropods and chordates likely reflects the variation in number and structure of *homeobox* genes, and their interaction with target genes (Krumlauf, 1994; Carrol, 1995). *Hox* genes represent a sub-group of *homeobox* genes that are represented in all metazoans, like genes related to the *Engrailed (En)*, *even-skipped (Evx)*, *caudal (cdx)*, *paired (Pax)* and POU of Drosophila constitute separates groups involved in control of development (Kessel and Gruss, 1990).

1.1. *Hox* genomic organization and collinear expression

Hox genes know substantial numerical expansion in vertebrates like mouse and human, where reach a total of thirty-nine genes. The latter are grouped in four clusters, from *A* to *D*, which are disposed along the same number of different autosomes. In mammals all the genes of the same cluster share identical 5’to 3’ transcriptional direction and are divided in 13 subfamilies called “paralogous groups”. These are enclosed in a genomic region spanning around 120 kb (Kessel and Gruss, 1990). Due to gene loss not all the *Hox* subfamilies are represented in any given cluster.

The genes belonging to the *HOM-C* and *Hox* complexes may be aligned on the basis of the homeodomain, the homology of their sequence and their relative position along the chromosome. In Vertebrates the paralogous groups 1, 2, 4, 6 and 8 are homologous

to the genes *Labial*, *Proboscipedia*, *Deformed*, *Sex combs reduced, Antennapedia*, *Ultrabitorax* and *abdominal-A* of *Drosophila* whereas from 9 to 13 are related to the *Abdominal-B* gene (reviewed in Krumlauf, 1994). The order by which genes are disposed in the *Hox* and in the *HOM-C* complexes is also preserved. This characteristic is determinant for a property of *Hox* clusters called collinearity. *Hox* are expressed in a collinear fashion, which means that along the anterior-posterior body axis the most 3'end genes are induced to a more anterior level anteriorly than those located 5'end, in correlation with their genomic order within the cluster.

In mouse, segmented tissues like the central nervous system and the paraxial mesoderm the anterior expression boundaries of *Hox* along the AP axis is reminiscent of its position in the cluster (spatial collinearity) (Duboule and Dollé 1989; Kessel and Gruss 1990). Furthermore *Hox* with a more rostral anterior boundary of expression are those induced at earlier times (temporal collinearity). So far the collinearity rule was observed in several embryonic contexts, represented by the neural tube, neural crest, branchial arches, limbs, gut and gonads. A temporal collinearity of *Hox* induction was observed as well in cultured cells (Simeone, et al., 1990). Spatial and temporal collinearity depend on two distinct mechanisms of gene regulation that coordinate *Hox* gene expression. In atropodes and nematodes, characterized by the rapid division of germ band, only spatial collinearity is observed. Conversely whenever the morphogenesis is accomplished by the sequential addition of segments on the posterior end of the body temporal collinearity is apparent as well (Kelsh, et al., 1994).

The organization of *Hox* genes in clusters is probably the result of a process of selection by which the insertion of genomic constraints is indispensable to couple the complex regulation of *Hox* expression with the progression of the embryogenesis. A well documented exception to clustering is represented by the primitive tunicate *Oikopleura dioica*, in which *Hox* are dispersed throughout the genome (Seo, et al., 2004). The recent review of Duboule (2007) well described the genomic organization of *Hox* genes in bilaterian organisms, defining a novel nomenclature defining the structure the cluster as atomized, split, disorganized, and organized, on the base of the compaction or dispersion of genes within a group. The atomized cluster is typical of both the urocordates *Oikopleura dioica* and *Ciona intestinalis*, where *Hox* genes are dispersed all along the genome; and the organized cluster is typical of vertebrates where *Hox* genes are tightly associated within one or more clusters. The appearance of novel structures in vertebrates like vertebral column, limbs and external genitalia, and their degree of complexity, is associated with the organization of *Hox* genes in multiple clusters suggesting the exsistance of a correlation between the clustering and the degree of adaptation of the animal to the environment. Such organized state, is reached a process called cluster "consolidation", which would allow the acquisition of a meta-genic kind of regulation, by which a gene is able to fulfill a determinate task, only if in a group with other genes. Such kind of regulation is successive to the cooption of *cis*-regulatory elements on a specific subset of genes.

1.2. *Hox* evolution

There is an evolutionary view according to which the four *Hox* clusters characteristic of vertebrates arose from events of amplification and duplication of a minor number of ancestral genes (Kappen, et al., 1989; Krumlauf, 1992). Amplification events would have increased the number of nearby genes until reaching the number of thirteen in vertebrates. This number is influenced by the absence of duplication associated to genetic loss of ancestral genes. For this reason a paralogous group often counts less than

four members. Conversely duplications of the cluster would have created a definite number of *Hox* groups.

Apparently the number of *Hox* clusters increases throughout evolution. Amphioxus (a cephalocordate) has a single *Hox* cluster, whereas teleost fishes like Zebrafish have up to seven *Hox* clusters (Amores, et al., 1998; Postlethwait, et al., 1998). In this context *Hox* genes in Teleostei fishes underwent through a complex genomic reorganization ending in the creation of seven to eight *Hox* clusters, where most of the duplicated genes were eliminated. The resulting number of *Hox* genes is slightly higher compared to mouse, but in the genome they span almost the same overall size. The total number of *Hox* does not influence the increase complexity of the body. Conversely, the dispersion of *Hox* genes in multiple clusters brings to a major degree of the organization of each cluster, suggesting why Teleostei are represented by a large number of species, with different morphologic features (Duboule; 2007).

Dispersed in the genome other genes were identified on the base of their high homology sequence with *Hox*. These are often organized in clusters and referred as the group of the *para-Hox* genes. This discovery suggested that both *Hox* and *para-Hox* derive from the duplication of an ancestral complex so-called *proto-Hox* cluster (Brooke, et al., 1998).

1.3. *Hox* induction in early vertebrate development

During gastrulation *Hox* are induced within the posterior streak at around E7.5-8.5 of mouse embryonic development, when the first mesenchymal cells leave the primitive streak, then they spread forward within the endoderm, the mesoderm and the ectoderm of the new-forming axial and paraxial structures (Deschamps and Wijgerde 1993; Forlani et al., 2003; Gaunt, et al., 1986; Kessel and Gruss 1990). The early expression of *Hox* in the posterior part of the streak is an autonomous process, by then *Hox* expression spreads anteriorly because of a mechanism of cell-cell interaction mediated by members of *Wnt* and *Fgf* family (Forlani et al., 2003). Once *Hox* expression is extended in the region anterior and lateral to the anterior part of the streak a second wave of *Hox* induction occurs at early somitic stage. This late phase is clonally transmitted by the precursor to the descendants (Deschamps, and Wijgerde 1993). The expression of most of the *Hox* genes is maintained between E9-E12, in a window of time corresponding to the main events of organ morphogenesis.

Limura and Pourquié (2006) electroporated constructs expressing *Hoxb* genes from *Hoxb1* to *Hoxb9* carrying green or red fluorescents proteins in the early chick gastrula. By observing the synthesis of protein conferring to the cells a different color they visualized cellular migration from the epiblast to the primitive streak and their contribution to the somitic mesoderm. The authors found that the position of clones derived from infected cells with respect to the Hensen's node was dependent on *Hoxb* activation time. Thus *Hoxb1* expressing cells were fast entering the primitive streak and populating the anterior part of somitic mesoderm, while at the same time *Hoxb9* expressing cells were still in the epiblast and contributed later to more caudal domains.

1.4. Maintenance of *Hox* expression

During embryogenesis *Hox* genes are maintained in a repressive or active transcriptional state through several rounds of cell division. Proteins belonging to *Polycomb* and *Trithorax* groups form separate complexes that bind the DNA through so called Polycomb response element (PRE). These groups of proteins are regulators of genomic programming and and modulate gene expression through structural modification of the chromatin. These proteins either create a compacted (inactive) or

conversely a loose (active) chromatin structure changing the methylation and acetylation status of histones (reviewed in Francis and Kingston, 2001; Ringrose and Paro, 2004; Schwartz, et al., 2008). Mutations in members of these gene families are associated to homeotic transformations both in *Drosophila* and Vertebrates.

The oncogene *Bmi-1* (together with *Mel-18*) is homologous to *Drosophila Posterior sex comb* (*Psc*), which controls segmental identity maintaining *Hox* gene in a repressive state. The inactivation of *Bmi-1* in vertebrates leads to hematopoietic and neurologic defects, but additionally is the cause of axial posterior transformations of thoracic into lumbar vertebrae, and of lumbar into sacral vertebrae (Van der Lugt, et al., 1994). Indeed, in absence of *Bmi-1* the anterior expression limit of some *Hox* genes in somitic mesoderm moves toward a rostral direction. Conversely the ectopic *Bmi-1* expression leads to the anterior transformations of vertebrae and to the posterior shift of the anterior expression boundary of the *Hoxc5* gene (Alkema, et al., 1995).

M33 is the murine counterpart of the *Drosophila Polycomb* (*Pc*) (Pearce, et al., 1992) and is able to rescue partially the phenotype of *Pc* mutants (Müller, et al., 1995). Mice lacking *M33* function died shortly after birth and the survivors displayed posterior transformations reminiscent of those conferred by of *Bmi-1* loss of function. In these mice the axis (C1) was absent and the number of sternebras was also inferior compared to *wt* (Coré, et al., 1997). Limb defects were found in the stylopod and at the level of the scapula. In mutants the *Hoxa3* anterior limit was shifted in the cervical region compared to *wt*, but the expression of other *Hox* genes did not change, suggesting that M33 was not critical for the maintenance the state of all *Hox* genes. Double mutants for both *M33* and *Bmi-1* displayed a stronger phenotype in cervical and in thoracic part compared to single mutants. In addition they revealed novel phenotypes in the skull and clavicle suggesting that these proteins work synergistically (Bel, et al., 1998). The expression of *Hoxc8* and *Hoxc9* was anteriorized in the axial mesoderm of mutants, whereas *Hoxb1*, *Hoxd4* and *Hoxd11* expression was not affected.

Kim and colleagues (2006) detected the two different *Polycomb* complexes, one containing Eed and the other Bmi1 proteins, bound on the regulatory regions of *Hoxa5* and *Hoxc8* genes. The impact of these complexes on axis development was evaluated by the analysis of mice where both *Eed* and *Bmi1* were inactivated: it was shown that the posterior transformation of the axis was dependent on the number of genes present, and was maximal in double homozygous mutants.

The murine mixed lineage leukaemia gene (*Mll*), whose human homolog is involved in chromosomal translocations associate to leukemia, is one of the members of the *Trithorax* group. Beside of haematopoietic anomalies, mice lacking one copy of this gene exhibited posterior and anterior transformation of the vertebral column, as well as defects in the sternum. In heterozygous embryos the anterior expression limit of *Hoxa7* and *Hoxc9* was shifted caudally, but the expression of both genes was abolished in homozygous mice, suggesting that *Mll* controls segmental identity and positively regulates *Hox* genes transcription (Yu, et al., 1995). Mice where both *Mll* and *Bmi-1* were deleted, did not show skeletal defects encountered in the correspondent single mutants, and *Hox* gene expression was the same as in *wt* mice, suggesting that *Mll* and *Bmi-1* display antagonistic functions in patterning the axis. Despite the identification of several PRE in Drosophila genome, the corresponding sites in mammals have not been discovered yet. During the earliest stages of embryonic development and in ES cells the Hox repressive state needs to be removed. In the same way their transcriptional state has to be maintained once Hox collinear expression is induced owing the activity of Retinoic acid or other factor such as CDX, WNT and FGF. Recently it has been discovered that the balanced activity of Polycomb and Trithorax proteins is not the only

mechanism of chromatin remodeling. UTX and JMJD3 proteins both contain a histone demetilase activity (Agger, et al., 2007); indeed it has been shown that several *Hox* genes are targets of these proteins and are derepressed once the activity of UTX and JMJD3 is impaired. However some functional in vivo experiment inactivating UTX in zebrafish showed that the Hox were only slightly derepressed, and did not show strong homeotic transformations. These findings revealed that despite the importance of UTX in development there is not a clear relationship between the latter and *Hox* regulation (reviewed in Soshnikova and Duboule, 2008).

2. Genetic analysis of Hox gene function in mice

In attempts to study their function in development *Hox* genes were either inactivated or misexpressed in different parts of the body. To this aim essentially two different approaches: gene targeting in ES cells or random transgenesis (Capecchi, et al., 1989) were used. The molecular mechanism by which *Hox* specify the morphology of a determinate region is unknown, however the genetic analyses suggested two alternatives models, called "combinatorial code" and "posterior prevalence". These two models are briefly presented in the next sections, accompanied by experimental results obtained following *Hox* gene disruption or mis-expression in trunk and limbs.

2.*1.* Combinatorial code, Phenotypic suppression and Posterior prevalence

Two main currents of thought propose different models to explain how *Hox* products confer segmental identity. One is the "combinatorial code" by which the morphogenesis of a given area of the body is determined by the combination of different *Hox* products expressed in the region. The other model is called "phenotypic suppression" in flies and "posterior prevalence" in vertebrates (reviewed by Duboule and Morata, 1994).

In *Drosophila Hox* are organized in the two complexes *ANT-C* and *BX-C* (Lewis et al 1978) and represent a simpler genomic situation compared to mouse. Following the ubiquitous expression of *Ubx* controlled by the *Hsp70* (heat shock, inducible) promoter all the cephalic and thoracic segments of the fly acquired an abdominal phenotype. In contrast the more posterior segments of the fly (telson) where *Ubx* is absent did not change positional identity (Gonzales-Reyes and Morata., 1990). Remarkably the most posterior end of the body was susceptible to *Ubx* ectopic expression whenever *AbdB* function was disrupted. These observations suggested that the specification of each anatomical part of the fly (like head, thorax and abdomen) is subjected to a hierarchical control operated by *Hox* proteins. The "phenotypic suppression" model implies that the function of a *Hox* product is suppressed by that of the gene located immediately posterior. The "posterior prevalence" model in vertebrates is based almost on the same principles and was elaborated after observation of mutants where *Hox* genes were inactivated or expressed outside their original endogenous domain.

Basically after mutagenesis two opposite situations named respectively *loss-of-function* and *gain-of-function* were observed. In general *Hox* inactivation leads to a loss-of-function phenotype, equivalent to the homeotic transformation of posterior structures toward more anterior ones. Conversely the ectopic expression of a *Hox* gene in a area of the embryo were it is normally not active ends up in a gain-of-function phenotype, illustrated by the homeotic transformations of anterior structures into elements having a posterior identity. Both in flies and vertebrates the range of anatomical defects was laying in correspondence of the anterior boundary of the expression domain of the mutated gene. This suggested that the function of posterior genes predominates over the function of more anterior ones in the establishment of segmental identity. The collinear *Hox* expression along the body axis generates various distributions of proteins at different body levels (Kmita and Duboule, 2003). In this view collinearity would protect the body from the gain-of-function of 5'Hox predicted by the posterior prevalence model. In some cases the extent of the anatomical defect spans over a larger area than that represented by the anterior and posterior expression limits of the mutated gene (Kessel and Gruss, 1991) supporting the *Hox* combinatorial code rather than the posterior prevalence model.

2.2. *Hox* function in trunk development

During embryogenesis the extension of the anterior-posterior axis is coupled with the process of somitogenesis. In mouse the first pair of somites originates immediately posterior to the otic vescicle and one cycle of somitogenesis takes about 120 minutes. Once somites are formed, they differentiate in bones, cartilages, tendon, muscles and dermis according to signals coming from adjacent tissues. Each somite acquires a dorsal-ventral polarity by which it is divided into dorsal dermomyotome and ventral sclerotome. The first will form the skeletal muscles and the last will give rise to the bones from which vertebrae will derive. Together with the acquisition of the dorsal-ventral polarity, the regionalization of the somite proceeds along the rostro-caudal axis.

Initially somites seem morphologically identical to one-another; the acquisition of segmental identity is reflected in the relative position of the somite along the AP axes and the associated external organs. The subdivision of the somitic mesoderm in cervical, thoracic, lumbar and sacral regions is early determined and is the result of the expression of *Hox* genes in overlapped domains within the pre-somitic mesoderm (Kessel and Gruss, 1991). *Hox* expression in the axis of vertebrates confers specific identity to the vertebrae (and to the nerves) (Gaunt, 2000). At the end a fixed number of vertebrae will be attributed to the cervical, thoracic, lumbar and sacral regions of the animal determining its "axial formula".

2.2.1. Loss-of-function phenotypes: examples of additive and synergistic effect

The following section is a list of the ensemble of vertebral phenotypes scored in mice where one or more *Hox* genes were simultaneously inactivated. *Hox* expression was perturbed to establish if during axis morphogenesis the products of genes belonging to the same paralogous group are functionally equivalent (redundant), or rather act in a cooperative (or additive) manner. In a great majority of cases the combined inactivation of more genes of the same group increased the severity of the phenotype compared to the inactivation of a single gene. The conclusion is that the severity of the malformation inversely proportional to the dose of genes left intact, suggesting that genes of the same paralogous can functionally replace one for another.

Nonetheless the mutagenic approach revealed new features that were not scored in single mutants, suggesting that the products of multiple genes act cooperatively or in synergy on the same targets within the population of progenitor cells.

Most of the cases reported below concord with the posterior prevalence rule, by which Hox loss-of-function generates the anterior homeotic transformations of vertebrae. Nevertheless occasional exceptions to this model are represented by posterior transformations.

One example of synergistic and quantitative interaction is represented by the disruption of *Hox* paralogous 3. Mice in which *Hoxd3* was disrupted showed an anterior transformation of the first and second cervical vertebrae (the atlas and the axis) into cranial bones (Condie and Capecchi, 1993). Conversely the malformations generated by *Hoxa3* loss of function were principally affecting neural crest and mesodermal derivatives like cartilage and skeletal elements of the throat (Chisaka and Capecchi, 1991). *Hoxb3* and *Hoxd3* single mutants did not show defect in this area (Manley and Capecchi, 1997): indeed the *Hoxb3* targeted inactivation gave a range of trunk malformations different from those observed in *Hoxa3* and in *Hoxd3* single mutants (Manley and Capecchi, 1997). The atlas was absent in double mutants where respectively *Hoxa3* and *Hoxd3* or *Hoxb3* and *Hoxd3* were inactivated, suggesting that the formation of this bone depends from the quantitative interaction of a number of genes expressed in the cervical region rather than from the product of a single gene

(Manley and Capecchi, 1997). Similarly the cartilage of the throat was mildly affected in *Hoxb3*/*Hoxd3* double mutants and severely malformed in *Hoxa3/Hoxd3* and in *Hoxa3*/*Hoxb3* mutants (Condie and Capecchi, 1994; Manley and Capecchi, 1997). Concluding remarks of these works were: 1) the products of *Hox* paralogous group 3 interact synergistically to drive differentiation of a common set of precursor cells in skeleton or cartilage; 2) the severity of given phenotype in neural crest and mesoderm derivatives was proportional to the number of depleted alleles of the *Hox* paralogous group-3.

The inactivation of *Hox* paralogous group-4 generates phenotypes that don't fully concord with the posterior prevalence model. In absence of *Hoxd4* function the basioccipital bone was malformed, C2 was transformed into C1 and both C1-C3 neural arches were abnormal (Horan et al, 1995). On the other hand the disruption of *Hoxa4* caused the partial anterior transformation of C3 into C2 (Kostic and Capecchi, 1994) and the posterior transformation of C7 into a thoracic vertebra (Horan et al., 1994). *Hoxb4*$^{-}$/$^{-}$ mutants showed the partial anterior transformation of the axis into atlas and displayed a longitudinally split sternum owing to the fusion or the closer association of sternebrae. Such phenotype suggested that *Hoxb4* specifies both cervical and thoracic identity (Ramirez-Solis, et al., 1993).

Mice lacking *Hoxc4* showed posterior transformation of C7 into a thoracic vertebra, while T3 and T8 were transformed toward a more anterior fate (Saegusa, et al., 1996).

These results contrast with the findings of Boulet and Capecchi in 1996, who reported the anterior transformation of a big part of the axis, from T2 to T11, following the disruption of *Hoxc4*. However this extended phenotype was due to the interference with the expression of more posterior genes like *Hoxc5* and *Hoxc6*. Indeed the insertion of a *neo* cassette in *Hoxc4* downregulated the expression of *Hoxc5* in the thorax and shifted posteriorly the *Hoxc6* rostral expression boundary (Boulet and Capecchi, 1996).

The effect of *Hox* quantitative interaction on axial morphogenesis was shown by double and triple mutants for *Hoxa4*, *Hoxb4* and *Hoxd4* (Horan and al., 1995). In double mutants lacking *Hoxb4* and *Hoxd4* the C3 and C2 vertebrae were transformed respectively into in C2 and C1, as well as C6 and C7 were changed in C5 and C6. The region of the posterior cervical column touched by the transformation fell in the expression domain of other *Hox* like *Hoxa5* and *Hoxb5* and was reminiscent of the phenotype caused by the loss of *Hoxb5* (Rancourt, et al., 1995). However the observed phenotype did not depend on *Hoxb5* loss of function since the synthesis of HOXB5 in double mutants was comparable to *wt*. In *Hoxa4-b4-d4* triple mutants the severity of the transformation increased along with the number of depleted alleles, since all the segments from C2 to C5 were transformed toward the C1 type. The anterior transformation of C5, C6 and C7 into vertebrae not identical to C1 was in agreement with a quantitative model of posterior prevalence. In this view vertebrae are specified by the quantity of anterior and posterior *Hox* genes that compete for the same targets. Thus in absence of 3'*Hox* the identity of vertebrae depends on the dose of 5' *Hox* expressed in the axis (Horan, et al., 1995).

At stage E9 of development *Hoxa5* was expressed in the pre-somitic mesoderm of the thoracic region (Dony and Gruss, 1987) and at later stages was better defined in the tract of the vertebral column comprised between C3 and L4 (Gaunt, 1988).

Homozygous mice for a mutation inactivating *Hoxa5* function displayed homeosis in the tract of the vertebral column between C6 and L1 and coincident with *Hoxa5* expression domain. In many of these animals C6 acquired more anterior identity while C7 was transformed into a thoracic vertebra (Jeanotte, et al., 1993). Such posterior axial

skeletal transformation occurred in a region positive for other posterior *Hox* and was consistent with the *Hox* combinatorial code rather than with posterior prevalence model.

The targeted inactivation of *Hoxb5* and *Hoxb6* to generate single mutants gave rise to anterior homeotic transformation of C6 in C5 and T1 in C7, like in *Hoxb5*$^{+/-;}$ *Hoxb6*$^{+/-}$ mice. This phenomenon was described as "non allelic non complementation", meaning that both of the genes behave as they belong to the same allele. The *Hoxb5*/*Hoxb6* provides an example of quantitative activity in the specification of a particular anatomic region of the vertebral column (Rancourt, et al., 1994).

The function of *Hoxa7* and *Hoxb7* in axial skeleton was investigated in mutants where these genes were alternatively disrupted. Unexpectedly only *Hoxb7* (and not *Hoxa7*) mutants reported fusions between the first and second pair of ribs. Nevertheless double homozygous mice lacking simultaneously *Hoxa7* and *Hoxb7* showed an increased penetrance of the abnormal phenotype suggesting that the product of these genes interacts synergistically in patterning the thorax. Although the anterior expression limit of *Hoxa7* and *Hoxb7* fall in the cervical region, the skeletal malformation was limited to the vertebrae of the cervico-thoracic area proving that transitions between vertebral types are more sensitive to changes in *Hox* dose genes dose than other regions (Chen, et al, 1998).

The anterior expression boundary of *Hoxb8*, *Hoxc8* and *Hoxd8* along the anterior-posterior axis is sequentially shifted toward the tail. At E9.5 stage of development *Hoxb8* expression limit is anterior to *Hoxc8*, which in turn precedes *Hoxd8*. The expression domain of these genes includes both presomitic mesoderm and neural tube; consistently defects in the expression of these genes had repercussion both in central nervous system (CNS), and vertebral column (Van den Akker, et al., 2001). Mice homozygous for a mutation disrupting *Hoxc8* showed the anterior transformation of L1 into a thoracic vertebra. Some of these animals were characterized by the presence of an additional sternebra, by the anomalous connection between the 8th rib and the sternum, and by ribs of equal size protruding from T12 and T11 (Le Mouellic, et al., 1992; see also Van den Akker, et al., 2001). The inactivation of *Hoxd8* caused malformations of the lower thorax resulting in one additional rib, whereas the disruption of *Hoxb8* led to fusions between ribs 1 and 2, and to the transformation of T3 into T2 (Van den Akker, et al., 2001). In general mutations in *Hox* paralogous group-8 genes had impact on regions where these genes were more abundantly expressed. Each mutant had a different phenotype. Double mutant missing both *Hoxb8* and *Hoxc8* manifested with higher penetrance some of the defects observed either in *Hoxb8*$^{-/-}$ or in *Hoxc8*$^{-/-}$ mice (as the transformation of S1 to L6), though some other malformations in the low thoracic cage were milder compared to *Hoxc8*$^{-/-}$ mice (like the presence of an extra-rib on L1). Mice lacking both *Hoxc8* and *Hoxd8* function showed the same kind of defect as *Hoxc8*$^{-/-}$ with increased severity (T8 to T7 transformation, additional rib on L1, the shift from S1 to L6 phenotype). Triple mutants *Hoxb8*$^{-/-}$; *Hoxc8*$^{-/-}$; *Hoxd8*$^{-/-}$ exacerbated the malformations found in the upper thoracic cage of single or double mutants. In conclusion the vertebral skeleton spanning from the cervico-thoracic transition until the sacrum is patterned by the synergistic functional interactions between the products of these genes (Van den Akker, et al., 2001).

The anterior expression boundary of *Hoxc9* in trunk is posterior to the 9th prevertebra. The targeted inactivation of *Hoxc9* like the *Hoxc8* loss of function, led to anterior transformation of the vertebral column along with malformations of the sternum and ribs. The thoracic vertebrae included from T10 to T13 acquired a T9-like morphology, whereas the sternum was joined to 8-9 ribs instead of 7. This phenotype

was attributed to the shift of *Hoxc8* posterior expression boundary in trunk (Suemori et al., 1995).

Hoxa9 null mice showed an anterior transformation of the lumbar vertebrae from L1 to L5 similarly to *Hoxd9* homozygous mutants where from L3 to L5 and from S2 to S4 were specified through a more anterior fate (Fromental-Ramain 1996). The disruption of *Hoxb9* led to the fusion of the first two ribs before they join to the sternum. This latter displayed a reduced number of intercostal segments contacting an abnormal number of ribs (Chen and Capecchi 1997). The ribs and sternum of *Hoxa9* and *Hoxb9* double mutants were much more severely affected compared to mutants in singles genes, suggesting that they both interact synergistically to pattern the thoracic skeleton (Chen and Capecchi 1997).

The *Hoxd10* inactivation led to the anterior transformation of S2 into S1 and to the association of both the two sacral vertebrae with the pelvis. A number of mutants showed 3-4 sacral vertebrae carrying lateral wing shaped processes, often fused (Carpenter, et al., 1997). The phenotype of mice in which *Hoxd9* and *Hoxd10* function was simultaneously disrupted was similar to *Hoxd10*$^{-/-}$ mice. Therefore there is no genetic interaction between *Hoxd9* and *Hoxd10* in patterning the lumbo-sacral region. Wellik and Capecchi in 2003 generated mice lacking the function of all the members of the *Hox* paralogous *group-10*. The vertebral column of the triple mutants was characterized by the presence of ectopic ribs caudal to T13 (anterior transformation). Despite the apparent disappearance of lumbar region and the malformation of the sacral vertebrae, these mice still formed a structure similar to the sacrum.

Mice with a targeted mutation in *Hoxa11* showed the posterior transformation of T13 into L1 and formed an additional lumbar vertebra in the sacral region (Small and Potter, 1993). This phenotype represents a deviation from the posterior prevalence rule. Mice lacking the three members of the *Hox* paralogous *group-11* the sacral segments did not form, in contrast lumbar region was extended caudally. In spite of the strong homeosis, the bones of the hindlimbs were still connected to the appropriate vertebral segment, marking the presence of the pelvis (Wellik and Capecchi, 2003). The authors concluded that *Hox group-10* controls the morphogenesis of the lumbar region by suppressing the formation of ribs and thoracic cage. Conversely the group *-11* drives the morphogenesis of the sacral vertebrae and the loss-of-function of these genes allows *Hoxa10-d10-c10* to extend the lumbar region (Wellik and Capecchi, 2003).

2.2.2. Loss-of-function: an example of artefactual phenotype

Mice lacking *Hoxb2* exhibited skeletal malformation of cervical region and thorax, owing the partial transformation of C2 into C1 and the splitting of the sternum in two longitudinal structures. The sternal defect was reminiscent of mutations in *Hoxb4* and as expected the *Hoxb4* expression in cervical vertebrae and body wall of *Hoxb2*$^{-/-}$ mice was reduced or suppressed. On the other hand and consistent with the *Hoxb1* loss of function phenotype (Goddard, et al., 1996) *Hoxb2*$^{-/-}$ mice displayed facial paralysis. Further analysis showed that *Hoxb1* expression in neural crest cells was abrogated, explaining why *Hoxb2* loss-of-function was affecting the development of somatic motor component of the VII cranial nerve (Barrow and Capecchi 1996). However the impact on *Hoxb1* and *Hoxb4* expression in *cis* was not specific. The insertion of a *neo* cassette in *Hoxb2* competed at transcriptional level with the expression of adjacent genes. In gene targeting disruptions are often made by insertion of the *neomycin* selection cassette under the control of the *PGK* promoter. Consequently the expression of neighboring *Hox* genes was biased by the strong promoter activity and the analysis of phenotypes was misdirected by the transcriptional interference effect.

To avoid the artefactual misregulation of *Hox* genes surrounding the locus of the inactivation, recently the *neo* cassette was flanked by two *loxP* sites. This way the *neo* gene may be excised by the activity of the *Cre* recombinase. Such control manipulations allowed for the conclusion that some of the unexpected posterior transformations cited above were attributed to the presence of *neo* selection marker in the targeting vector.

2.2.3. Gain-of-function phenotypes

The ubiquitous expression of *Hoxa7* in mouse under the control of the chick β-actin promoter generated cranial and cervical malformations owing the presence of an additional vertebra (pro-atlas) at the cranio-cervical transition (Kessel et al., 1990). This phenotype affected a region of the axis external to the endogenous *Hoxa7* expression domain.

The misexpression of *Hoxd12* in lateral plate mesoderm affected the morphogenesis of the pelvis that was reduced or absent. The sternum was mildly or severely split, affecting the viability of newborns. Anterior ribs were often fused and in some cases only six of them articulated with the sternum, indicating the posterior transformation of the thorax (Knezevic, et al., 1997). Since the transgene was not expressed in somites such malformation was attributed to defects in the lateral plate mesoderm derivatives.

Rarely cases of gain-of-function produced unexpected anterior transformations of the vertebral column. In *wt* mice both CNS and axial mesoderm are positive for HOXC6, in a region of the axis included between T2 and T9 (Oliver, et al., 1989). The insertion of 40 copies of the human *HoxC6* in the mouse genome produced a phenotype reminiscent of *Hoxc8* loss-of-function. These mice displayed the anterior transformation of L1 into T13, of T12 into T10 or T11 and defects in the anatomy of the sternum. In heterozygous the posterior boundary of *HoxC6* greatly extended until the tip of the tail indicating an ectopic expression in the posterior region of the embryo (Jegalian et al., 1992). However neither *Hoxc8* transcription nor the accumulation of its product was affected, suggesting that *HoxC6* protein likely competes with the product of *Hoxc8* for the same target (Jegalian et al., 1992).

2.3. *Hox* and limb development

The appendicular skeleton is represented by fins in fishes and limbs in tetrapods. The presence of fins in aquatic tetrapods or wings in birds results from modifications of the limb pattern conferring a suitable adaptation to locomotion in varying environments. The study of the molecular basis of limb development in vertebrates contributes to decipher the evolution of this organ. Arguably the best characterized example is the case of *Hox* activity in the formation of digits.

In dependence of their anterior or posterior position along the body axis the four limbs are distinguished as forelimbs or hindlimbs showing different morphology. Forelimbs begin to grow at around E9 of mouse development, one day before hindlimbs. Limbs derive from the cellular proliferation of the embryonic lateral mesoderm, whose differentiation will bring about formation of all skeletal components of the limb itself. The margin of the limb bud is covered by a pseudo-stratified epithelium, also called Apical Ectodermal Ridge (AER). The AER controls the outgrowth of the limb bud stimulating the proliferation of the underlying “progress zone” in mesenchyme. Theoretically the dividing cells acquire their positional identity in the progress zone (Summerbell, 1973).

During embryogenesis the limb elongates toward a proximo-distal direction gradually acquiring a dorso-ventral, and anterior-posterior polarity. At the end of their

outgrowth limbs are divided in three domains along the proximo-distal axis that are the stylopod (humerus in forelimbs, femur in hindlimbs), the zeugopod (radius an ulna in forelimbs, tibia and fibula in hindlimbs) and the autopod, corresponding to hands and feet (carpus, metacarpus and fingers in forelimbs; tarsus, metatarsus, and toes in hindlimbs). Limb elongation proceeds in a way that the proximal part of the limb (stylopod) forms before the distal parts (autopod).

2.3.1. HoxA and HoxD clusters

In limb buds genes with the most 3'end position within the *HoxA* and *HoxD* clusters are expressed earlier compared to those located at the most 5' end, such that their expression domain reflects their genomic organization in the cluster (Dollé et al., 1989).

At early stages the most 5' *Hoxa* and *Hoxd* genes are transcribed in a region of the limb bud that is contained within the expression domain of the most 3'end genes reminding a "Russian doll" (reviewed in Duboule, 1992; Haack and Gruss, 1993). Accordingly *Hoxd1* is expressed all over the early limb bud while *Hoxd13* is restricted to a posterior group of cells. The area where the expression domain of *Hoxd* genes overlaps with the ZPA (Tickle and Eichele, 1994 and controls the anterior-posterior patterning of the limb. The graft of the ZPA to the anterior limb bud generates digital duplications associated with ectopic *HoxD* expression in the anterior limb domain (Saunders and Gasseling, 1968; Izpisua-Belmonte, at al., 1991-a).

At later developmental stages, after the establishment of the AER, there is a second wave of *Hox* expression that involves genes from *Hoxd9* up to *Hoxd13.* Whereas *Hoxd9* is expressed uniformly within the proximal domain of the limb bud, *Hoxd10* and *Hoxd11* both show a proximal and a distal expression domain, the latter of which labels the digital plate. The expression of *Hoxd10* and *Hoxd11* in these two regions is biased posteriorly, as well as *Hoxd12* and *Hoxd13* that are transcribed exclusively in the digit domain; clearly this pattern divides the limb in two well defined proximal and distal domains. The early phase of *Hox* induction is important to the establishment of the stylopod and zeugopod and relies on a different molecular mechanism than that which controls the late phase of *Hoxd* activation in limbs, indispensable for the autopod development (Tarchini and Duboule, 2006). In addition unlike from 5'*Hoxd,* the transcripts of *Hoxa9* to *Hoxa13* don't show an anterior-posterior polarity at this later phase, but are progressively confined to the distal limb domain (proximo-distal polarity). This observation suggests that the spatial collinearity of *HoxD* and *HoxA* genes depends on different kind of regulation.

From the point of view of evolution the expression profile of posterior *Hox* gene in fins and in limbs diverges at late stage, at the time when the digits (in tetrapods) are growing. Only limbs express *Hoxd* posterior genes in the distal domain, suggesting a reason why fins in fishes are devoid of structures homologous to digits (Sordino, et al., 1995). The subdivision in temporal phases of *Hoxd* genes induction is observed in chicken (Nelson et al., 1996).

Like in trunk, the perturbation of the *Hox* expression in limbs in a certain window of time and in the space alters the final limb morphology. The following section reviews the selected examples of loss- and gain-of-function reported in literature.

2.3.2. Loss-of-function phenotypes

At early developmental stage *Hoxd9* and *Hoxa9* are both uniformly expressed in mesenchyme of the developing limb whereas at later stages their transcription is restricted around the humeral and femoral condensations (Duboule et Dollé, 1989; Fromental-Ramain, et al., 1996; Haack and Gruss, 1993).

Mice where *Hoxd9* was disrupted displayed a slight forelimb phenotype consisting in the shortening of the humerus characterized by an underdeveloped deltoid crest. In contrast the disruption of *Hoxa9* did not give rise to any limb phenotype. However the humerus in *Hoxa9-/-*;*Hoxd9-/-* double homozygous mutants was shorter and had a severely affected deltoid crest; in addition, but less frequently, ectopic bones developed next to the elbow joint. The severe phenotype of double mutants revealed that *Hoxa9* and *Hoxd9* interact synergistically in the development of the zeugopod (Fromental-Ramain, et al., 1996).

The expression of *Hoxa10* at E14.5 surrounds the distal cartilage of the humerus and femur; however its disruption did not have repercussions on the skeleton of forelimbs: it rather affected the femur and the bones of the knee (Favier, et al., 1996).

Hoxd10$^{-/-}$ mice displayed hindlimbs specific defects conferring a defective locomotion (Carpenter, et al., 1997). The accurate analysis of the skeleton of these mutants showed that the femur and tibia were misaligned, resulting in an abnormally positioned foot. The articulation of the knee was defective as well, following the upward shift of the patella along the femur, and the presence of ectopic sesamoid bones along the anterior margin of the patella (Carpenter, et al., 1997).

The simultaneous disruption of both *Hoxd9* and *Hoxd10* affected more radically the anatomy of hindlimbs. Ectopic sesamoid bones were found on the dorsal side of the forelimbs, around the base of the humerus and in opposite position with respect to the orthotypic sesamoid bone (de la Cruz, et al., 1999). Like in *Hoxd9* $^{-/-}$ mice, the deltoid crest of the humerus of *Hoxd9*; *Hoxd10* double mutants was affected.

Hoxd11$^{-/-}$ mutants displayed major defects in forelimb development, in the length and the structure of the phalanges and carpal bones (some bones in the wrist were fused) and the shape of the distal end of ulna and radius. Conversely hindlimbs were normal (Davis and Capecchi, 1994; Favier, et al., 1995).

Surprisingly the carpal and digital domain in forelimbs of *Hoxa10*$^{-/-}$; *Hoxd11*$^{-/-}$ mice were more severely affected compared to *Hoxd11*$^{-/-}$; furthermore their ulna and radius were truncated and thickened, whereas the proximal part of the ulna was deformed (Favier, et al., 1996). This phenotype accounts for a partial functional redundancy between members of different paralogous groups in forelimb morphogenesis, but not for their full functional equivalence; indeed the phenotype of *Hoxa10*$^{+/-}$*;Hoxd11*$^{-/-}$ mice was less severe compared to *Hoxa10*$^{-/-}$*; Hoxd11*$^{+/-}$ mutants.

The targeted mutation of *Hoxa11* had repercussion on the zeugopod of both forelimbs and hindlimbs, owing to malformation of the ulna and radius, the incorrect joining between the fibula and tibia and the fusion between some of the carpal bones (Small and Potter, 1993).

The role of the paralogous group *11* in morphogenesis of zeugopod has been corroborated by the observations of severe forelimb truncations in *Hoxa11*$^{-/-}$; *Hoxd11*$^{-/-}$ mice. The size of the radius/ulna was strongly reduced (in these mutants also tibia and fibula were shorter). Compounds *Hoxa11*$^{-/-}$*; Hoxd11*$^{+/-}$ and *Hoxa11*$^{+/-}$*; Hoxd11*$^{-/-}$ exhibited an intermediate phenotype accounting for a functional equivalence of these two genes and for their participation in a dose-dependent manner to the morphogenesis of the zeugopod (Davis, et al., 1995).

Mice where the function of the complete *Hox* paralogous groups-*10* and -*11* was disrupted showed defects in the morphogenesis of stylopod and zeugopod. Mice lacking *Hox-10* had slightly affected humerus and a strongly affected femur and knee, the first of which was reduced in length and the latter was lacking the patella. Mice lacking *Hox-11* exhibited a more accentuated defect compared to *Hoxa11/Hoxd11* double mutants,

represented by short and not completely ossified tibia and fibula, and consistent with the absence of *Hoxc11* expression (Wellik and Capecchi, 2003).

At early stage both *Hoxa13* and *Hoxd13* are expressed in the more posterior and distal region of the limb, which correspond to the ZPA (Dollé, et al., 1989; Yokouchi, et al., 1991). At later stages *Hoxd11*, *Hoxd12*, *Hoxd13* and *Hoxa13* are transcribed in the distal autopod (Duboule, 1994), with *Hoxa13* and *Hoxd13* specifically restricted to the finger domain.

In agreement with its expression pattern, the targeted inactivation of *Hoxd13* affected digit development (like the shortening of some bones and the loss of phalanges). The formation of the cartilage of the autopod was delayed (heterochrony) leading to bone fusions (Dollé et al, 1993). The *Hoxd13 loss-of-function* affected neither the radius nor the ulna.

The targeted mutation of *Hoxa13* caused a different modification of the limb compared to the $Hoxd13^{-/-}$ mutants (Fromental-Ramain, et al., 1996). This malformation led to the defective chondrogenesis of the tarsal, of the carpal bones and of digit I (which does not form), associated to the reduction in size and the more-less extended fusion between some of the digits.

The role of *Hoxa13* in digit development was further elucidated by the characterization of the *hypodactyly* allele, which consists in the partial deletion of the first exon of *Hoxa13*. In most of the cases this mutation is homozygous embryonic lethal, and the few survivors had only one digit on each limb. The phenotype of heterozygous mice consisted in the shortening of digit I and in the retarded ossification of the autopod (Mortlock, et al., 1996).

Playing with the dose of *Hoxa13* and or *Hoxd13* were obtained all possible compounds mutants. The loss of one copy of *Hoxa13* on $Hoxd13^{-/-}$ background gave rise to short and polydactylous forelimbs. Vice-versa the lack of a copy of *Hoxd13* on $Hoxa13^{-/-}$ background caused the loss of digit I, like in $Hoxa13^{-/-}$ single mutants, and to the fusion between terminal phalanges as in $Hoxa13^{+/-}$; $Hoxd13^{-/-}$ mice. $Hoxa13^{+/-}$; $Hoxd13^{+/-}$ compounds exhibited a range of defects common to $Hoxd13^{-/-}$, attenuated by the lack of only one copy of *Hoxa13*. Double homozygous $Hoxa13^{-/-}$; $Hoxd13^{-/-}$ were lethal in uterus and displayed strongly truncated limbs with no sign of digit formation. In conclusion *Hoxa13* and *Hoxd13* acts on limbs development in a dose dependent mechanism, nevertheless their function is not fully equivalent and is synergistic since the observed phenotypes was greater than the sum of single mutants phenotypes (Fromental-Ramain, et al., 1996).

Mice where *Hoxd13*, *Hoxd12* and *Hoxd11* were simultaneously removed by a targeted deletion (*Del3*), and replaced by *Hoxd11-lacZ* reporter gene, provided further hints on the role of *Hoxd* posterior genes in digit development (Zakany, et al., 1996). Limbs of homozygous mice developed abnormally, exhibiting short size digits, polydactyly, and interdigital fusions. These observations were consistent with the predominant role of *Hoxd13* product over *Hoxd12* and *Hoxd11* in digit morphogenesis, in agreement with the posterior prevalence model. Polydactyly was scored in fore- and hindlimbs of mice lacking both the copies of *Del3* allele, or simultaneously homozygous for *Hoxd13* and heterozygous for *Hoxa13*. The conclusion was that the dose of *Hox* products in distal limbs correlates with the length of the digits and their number. Conversely the removal of one copy of *Hoxa13* on *Del3* background conferred oligodactyly. Since $Hoxa11^{-/-}$ on *Del3* background resulted in polydactyly, it was suggested that the number of digits is influenced exclusively by the dose of *Hoxa13*, *Hoxd13*, *Hoxd12* and *Hoxd11* products (Zakany, et al., 1997). This malformation in mouse is reminiscent of the human synpolydactyly congenital disorder (SPD), which is

the result of the insertion of stretches of polyalanine within the HOXD13 protein. This dominant negative mutation generates syndactylous hands and feet, characterized by the formation of an extradigit. The SPD syndrome is generated by the loss-of-function of *HOXD13*, owing the competition of the mutated form with *wt HOXD13* and other posterior *HOX* genes (Goodman, 2002).

For the first time the total loss-of-function of the *HoxA* cluster in limbs was produced adopting a conditional approach (Kmita, et al., 2005). Fetuses devoid of *Hoxa* genes showed slight malformations of zeugopod and autopod, consisting in absence of the thumbs and in reduction in size of digits. Coversely compounds mutants lacking *HoxA* and *HoxD* clusters showed drastic forelimbs truncations with the radius and ulna primordium replaced by a single cartilaginous element. This anomaly was the consequence of the premature limb arrest, as illustrated by the lack of distal bony elements. From a general point of view the simultaneous deletion of HoxA and HoxD clusters put in evidence that the product of these genes interact synergistically to promote limb development along the proximo-distal axis. The mechanism by which *Hoxd* and *Hoxa* genes drive limb elongation will be the subject of another paragraph.

2.3.3. Gain-of-function phenotypes

Following injection of a retroviral construct the *Hoxd11* gene was ectopically expressed in the anterior limb bud of chicken, transforming the structure of digit I in that of digit II thus altering the anterior-posterior limb pattern (Goff, and Tabin., 1997; Morgan, et al., 1992). The expression of the same gene in the posterior limb domain did not give alterations of digit identity, since the contemporary presence of posterior genes like *Hoxd12* and *Hoxd13* prevails on *Hoxd11*, exactly like in physiological situation. The zeugopod length was affected by *Hoxd11* misexpression; the tibia was shortened (posterior transformation) suggesting that *Hoxd11* induction out of its domain alters the anterior-posterior pattern of the bones of both autopod and zeugopod. The global conclusions of the two works cited above were that *Hoxd11* acts at early stage in mesenchymal condensation and condrogenesis concerning the formation of digits whereas influences bone elongation only at later stages.

The ectopic expression of *Hoxa13* in chick limbs was been shown to reduce the size of the cartilage of the zeugopod probably owing the homeotic transformation of this domain in more distal cartilage of carpus and tarsus, with consequent reduced cellular proliferation (Yokouchi, et al 1995-a).

The misexpression of *Hoxd13* in chick hindlimbs caused a reduction in length of bones like femurs, tibia and fibula (in regions where this gene is not expressed in normal conditions) at late stage of limb development, in contrast with *Hoxd11* (Goff and Tabin., 1997). The observed limb malformation was explained by the posterior prevalence rule, meaning that the product of *Hoxd13* interferes with the activity of more anterior *Hox* genes like *Hoxd11* and *Hoxa11* for the control of a common subset of target genes involved in the patterning of the proximal and middle region of the limb. Analogously in mouse the accidental misexpression of *Hoxd13* in the zeugopod, secondary to the proximity of a *Hoxd11* transgene, produced a shortening of the radius and ulna (Van der Hoeven, et al., 1996)

In transgenic mice *Hoxd12* was driven ectopically in posterior lateral plate mesoderm by the *Hoxb6* promoter and successfully transcribed in hindlimb mesenchyme to ectopic position (Knezevic, et al., 1997). As expected the anterior part of the hindlimbs autopod was strongly affected by digit duplications. In addition digit I was transformed in digit II or III and tarsal bones acquired posterior identity. Similarly to the zeugopodal defects

observed in *luxoid* mutants (Johnson, et al 1967) the tibial bone was shortened (hemimelia) and the fibula was bent. A number of transgenics developed an ectopic ZPA that might explain the mirror-image phenotype of the limb. Since the expression of *Hoxd11* and *Hoxd12* in cultured cells induces *Shh*, the authors supposed that *Hoxd12* is involved in a feed-back loop that maintains *Shh* expression. The ectopic *Hoxd12* expression is related to the formation of post-axial condensations in the anterior half of the limb of transgenic mice (Knezevic, et al., 1997).

Several studies put in evidence the existence of functional hierarchy between *Hox* genes during limb development by which the identity of each segment along the anterior-proximal axis is established by the activity of a specific group of genes (*Hoxa13* and *Hoxd13* in digit, *Hoxa11* and *Hoxd11* in zeugopod, *Hoxa10-a9*, *Hoxd10-d9* in stylopod). Nevertheless the domain of the protein responsible of posterior prevalence is unknown. In humans was discovered the existence of a missense mutation in the homeodomain of HOXD13, that diminishes the capability of the protein to bind the DNA affecting the expression of only on a certain number of genes (Caronia, et al., 2003). The consequent malformation consists in brachydactyly and polydactily of hands and feet. The infection of chicken limb buds with a virus carrying this mutated form of *HOXD13* generates a shortening of the zeugopodal bones of the leg more severe than an injected *wt* form of *HOXD13*. Given that the mutated form lacks in part the ability to bind its target on DNA, the observed posterior prevalence phenotype relies on regions of the protein located outside the homeodomain, likely involved in protein-protein interactions. Another study, based upon swapping of homeodomains among posterior *Hox* genes, goes in the same direction. The *Prx-1* mediated expression of chimeric *Hoxa9* and *Hoxd11* both characterized by the *Hoxd13* homeodomain did not produce the attended shortening of both the stylopod and zeugopod limb dictated by the posterior prevalence (Williams, et al., 2006). Conversely the substitution of the *Hoxd13* homeodomain with those of *Hoxd11* and *Hoxa9* conferred the attended limb phenotype. This test suggested that the part of the protein that interacts with DNA is not involved in the establishment of posterior prevalence, which depends on other regions of the protein.

2.4. *Hox* and development of the central nervous system

The embryonic central nervous system is represented by the brain and the neural tube. The most posterior part of the brain (hindbrain) is divided in units called rhombomeres. *Hox* expression is excluded from the fore- and the midbrain, but *Hoxa1*, *Hoxa2*, *Hoxa3* and *Hoxd3* are induced in hindbrain, where the cranio-facial nerves form. Briefly the mutation of these genes affects the normal development of the latter (Barrow, et al., 2000; Barrow and Capecchi 1996; Carpenter, et al., 1993; Gendron-Maguire, et al, 1993; Goddard, et al., 1996; Manley and Capecchi, 1997; Rijli et al., 1993; Rossel and Capecchi, 1999). By analogy with the vertebral column, posterior *Hox* like *Hoxd9* and *Hoxd10* are induced in lumbar and sacral regions, as well as in limbs. The misexpression of *Hox* in specific regions of the CNS induces the lack or the malformations of the derived nerves, generating more or less severe paralysis associated to defect in locomotion (Carpenter, et al., 1997; De la Cruz, et al., 1999; Tarchini, et al., 2005; Tiret, et al., 1998).

The *Hox* function in central nervous system is illustrated in a number of articles, however is not central to the aim of this thesis and for this reason will not be further analyzed in detail.

3. HoxD cis-*regulation*

As part of a cluster the expression of a given *Hox* gene depends on the balanced interactions between intrinsic regulatory elements and a mechanism of global regulation (reviewed in Spitz and Duboule 2008). To reveal the nature of the regulatory influences acting on *Hoxd* gene expression the corresponding genomic region was engineered in several ways, mostly by relocations of reporter genes inside or outside the cluster. One notable example was the case of a set experiments involving *Hoxb1* as a reporter. Since *Hoxb1* do not share common expression domains regulatory regions with *Hoxd* genes a *Hoxb1/lacZ* transgene was randomly inserted into the genome (*TgNb1/lacZ* mice) or transposed within the 5' end of the *HoxD* complex (*TgHb1/lacZ* mice) (Kmita, et al., 2000-a). While the *lacZ* of the randomly inserted gene was expressed in hindbrain (reminiscent of *Hoxb1*), the one located upstream of *Hoxd13* was absent from this domain, but gained both in limbs and genitals. The relocation of *Hoxb1/lacZ* in other positions of the cluster changed its expression profile in axis, limbs and genitals according the new insertion site. These results proved that the intrinsic regulation of a gene not belonging to the *HoxD* cluster is silenced and subjected to the activity of regulatory elements characteristic of "acceptor" complex.

3.1. Regulatory elements within the *HoxD* complex

A number of intergenic sequences conserved between mouse and chick (sometimes they are conserved in fishes as well) were found in the proximity of the *Hoxd11* gene and were designated from *RI* to *RXII*. Within the *HoxD* cluster, *RI* to *RIV* were mapped between *Hoxd12* and *Hoxd11* homeoboxes, *RV* to *RIX* between *Hoxd11* and *Hoxd10* (Gérard et al., 1993), *RX* between *Hoxd12* and *Hoxd11* and *RXII* between *Evx2* and *Hoxd13*. Some of these regions were shown to induce the expression of the nearby *Hox* genes in a specific fashion, either autonomously or in cooperation with a long-range enhancer. In the next paragraph a set of *cis*-regulatory elements will be reviewed that drive *HoxD* expression in limbs and the central nervous system.

3.2. Regulatory elements outside the *HoxD* complex

Evx2 gene codes for a homeobox transcription factor whose transcription unit is physically associated to the *HoxD* cluster in vertebrates, like in more primitive organisms. *Evx2* as well as *Hoxd13* are expressed in developing digits and genital bud however *Evx2* function is inherent to the forming interneurons within the neural tube. Despite the proximity of *Evx2* and *Hoxd13* on the chromosome their expression pattern in CNS is strikingly different, suggesting that these two genes are influenced by the activity of regulatory regions that specify their activation in different areas of the body. In order to identify the genomic area involved in *Hoxd* regulation, a *Hoxd9/lacZ* transgene was inserted in two new positions outside the *HoxD* cluster, centromeric to *Evx2*. These new novel *HoxD* alleles were called "relocations" or *rel*0, *rel*I, and *rel*II, according to the position of the transgene with respect to *Evx2*. *rel0* refers to the insertion of the transgene between *Hoxd13* and *Evx2* (Kondo, et al., 1999). In *rel*I and *rel*II mice the transgene was progressively more 5' to *Evx2*. The sites positive for *lacZ* expression in *rel*I and *rel*II mice included distal limbs, genitals and CNS reminiscent of the *Evx2* expression profile. Furthermore the transgene in *rel*II (which is much far away from the *HoxD* complex) was activated at earlier time compared to *rel*I and *rel*0, suggesting that the greater distance make the transgene escape to a temporal repressive

control. To map the genomic region involved in the time-dependent *Hoxd* induction deletions were generated from *Hoxd13* to *Hoxd11* (*Del*0) and from *Evx2* to *Hoxd11* (*Del*II). Both in *Del*0 and *Del*II, *Hoxd9/lacZ* was located 5'to *Hoxd10*. Only *Del*II mice showed posterior homeotic transformations of the cervical column, anterior to the *Hoxd11* expression boundary. Indeed *Hoxd9/lacZ*, *Hoxd10* and *Hoxd4* were activated earlier in these mice compared to *wt*. In conclusion the removal of a genomic region upstream *Evx2* in *Del*II allowed the premature expression of *Hoxd* genes located nearby the 3'end break point, giving rise to the observed axial phenotype.

The organization of *Hox* genes in clusters may reflect their need to remain near shared common regulatory regions however the mechanism by which enhancers select a specific subset of genes within the *HoxD* complex remained obscure.

3.3. *Hox* regulation in trunk and the CNS

Studies on *Hox* regulation demonstrated that regions driving the expression of *HoxD* in limbs, genitals and hernia are not coincident with those that induce expression within the trunk and that the formers are situated outside the cluster. Mice carrying a deletion from *Hoxd13* to *Hoxd11* and transgenics for a human PAC containing the *HOXD3* to *EVX2* subgenomic region developed normal trunk but abnormal limbs (Spitz et al., 2001). While the *HOX* expression pattern in trunk was preserved, *HOXD13* and *HOXD11* were not expressed in limbs and genitals. In addition from *HOXD11* to *HOXD4* were absent from intestinal hernia. It was concluded that enhancers driving *HoxD* expression in limbs, hernia and genital buds are located outside the cluster, whereas those activating *Hox* expression in trunk map within the complex.

RIX contains a potential nuclear receptor response element (*NRRE*) whose sequence is conserved in mouse, chicken and fish. It was shown that this region regulates *Hoxd11* in a negative fashion (Gérard et al., 1993). Transgenic mice carrying *Hoxd11/lacZ* and the deletion of *RIX* had a rostral shift of *lacZ* expression in trunk and the anterior shift of the sacrum of 1-2 prevertebrae (Gérard, et al., 1996). The same phenotype was obtained in mice where the *NRRE* was mutagenized in the context of the native *HoxD* cluster and correlated with the ectopic gain of endogenous *Hoxd11* and *Hoxd10* in a more anterior domain. The obtained results confirmed the repressive role of *RIX* on *Hoxd11* and *Hoxd10* anterior expression during trunk development. An opposite result was obtained in mice where *RVIII* was deleted, which displayed the posterior shift of the sacrum owing the transformation of the first sacral vertebra into the last lumbar (Zakany, et al., 1997). *Hoxd11* and *Hoxd10* transcription in trunk was delayed, proving that their time of activation was crucial for the morphogenesis of the lumbar-sacral transition.

Evx2 is transcribed in brain and anterior spinal cord unlike *Hoxd13,* with whom otherwise it shares the digit expression domain. The targeted deletion of *RXII* did affect neither *Evx2* nor the expression of the remaining *Hoxd* genes, proving that this region does not behave as a boundary (Kmita et al., 2002). A deletion removing *RXII* along with *Hoxd13, RXI*, *Hoxd12*, and *RX* gave rise to the ectopic expression of *Hoxd11* and *Hoxd10* in the anterior CNS. A model of "regulatory redundancy" was proposed to explain this pattern, according to which the same cell uses alternatively two different regions as insulator, so that the removal of a single element has no effect on gene expression.

3.4. *Hox* regulation in limbs

HoxD expression in trunk relies mainly on shared *cis*-regulatory sequences located inside the cluster. Conversely in limbs *HoxD* are regulated by genomic elements located

5'-3' to the complex. These regions control *Hoxd* expression in forearms and in digits likely in a competitive fashion. In order to discriminate between the influence of single and global mechanism of gene regulation, *lacZ* reporter gene was targeted to the 5'end of the *HoxD* cluster, associated to *Hoxd11* and *Hoxd9* promoters. These transgenes were relocated by homologous recombination between *Hoxd13* and *Evx2* (Van der Hoeven, et al., 1996). The *Xgal* staining of transgenic embryos for *Hoxd11-lacZ* relocation (*TgHd11lacZ*) revealed delayed *lacZ* activation in the distal domain of the developing limb, reminiscent of the *Hoxd13* expression pattern. Moreover genital eminences became positive for *lacZ*. The same results were reproduced with the *Hoxd9* transgene (*TgHd9lacZ*). It was concluded that the early expression of posterior *Hoxd* in distal limbs and genitals is controlled by a common mechanism, which affect the genomic region extending from *Hoxd13* to *Hoxd10*. Nevertheless the expression of *lacZ* at later stages resembled to the expression of the endogenous gene.

3.4.1. The early Hoxd activations in limbs is controlled by the ELCR

As already mentioned the *HoxD* expression in limbs occurs in two temporally distinct phases: the first wave of *HoxD* transcriptional activation triggers development of the forearm, while digit morphogenesis is dependent on a second transcriptional wave (Tarchini and Duboule 2006). During the early phase transcripts from *Hoxd1* to *Hoxd9* are uniformly distributed throughout the mesenchyme of emergent limb bud, while from *Hoxd10* to *Hoxd13* are restricted in a group of cells in the posterior margin of the limb, overlapping the ZPA. Enhancers driving *HoxD* transcription at early phase of limb development are different form those acting during late phase.

The inversion as well as the partial deletion of the *HoxD* cluster evidenced a relationship between the early *HoxD* expression and anterior posterior polarity of the limb (Zakany, et al., 2004). In mice carrying an inversion of the *HoxD* cluster the early expression of *Hoxd13* and *Hoxd11* was homogenous along the entire limb bud, and not anymore restricted to the posterior limb bud. The same result was obtained in mice lacking the genomic region comprising *Hoxd10* to *Hoxd1*, where a mini-cluster represented by genes from *Hoxd13* to *Hoxd11* was left behind. In limbs of these mice *Shh* was transcribed in two groups of cells, located symmetrically with respect to the A-P axis. Limbs developed double posterior hands, suggesting that *Hoxd* genes control limb AP polarity inducing *Shh* expression. The inversion and deletion approaches represented two alternative methods to relocate 5' end posterior genes nearby the 3' extremity of the complex. The resulting heterochrony suggested that in early phase *HoxD* transcription is regulated by an early limb control region (ELCR) located in telomeric position and outside the cluster (Zakany, et al., 2004).

The early regulation of *HoxD* in limbs was studied in more detail by targeted deletions respectively within the 5' end or internal to the cluster, and evaluating the expression of genes left nearby the 5' and 3'end breakpoints. In mice bearing internal deletions, *Hoxd10*, *Hoxd11* and *Hoxd13* (left at 5'end) were already transcribed at E9 stage of development, and found throughout the anterior half of the limb bud (Tarchini and Duboule, 2006). Conversely the expression of genes lying at 3' end of the breakpoints started at E12 and was restricted to the digit domain. In mice carrying internal duplications *Hoxd11* and *Hoxd12* expression was delayed and restricted to more posterior limb domain compared to *wt*. This is consistent with the previous conclusion that the early phase of *HoxD* expression is coordinated by the ELCR, which is located in telomeric position far from the cluster.

3.4.2. The collinear Hoxd activation in digits is controlled by the GCR

The comparison of *HoxD* expression in appendages of mouse and teleost fishes revealed the existence of a similar early collinear expression pattern. In contrast the late phase of *Hoxd* activation is characteristic of tetrapods and is absent from fishes whose fins have no autopod (Sordino, et al., 1995). The appearance of digits in tetrapods was attributed to a second wave of *Hox* gene activation triggered by specific genomic elements present in their genome, and absent from fishes (Spitz, et al., 2003).

Lunapark (*Lnp*), later re-named *limb and neural patterns*, is mapped at the 3' end of *Evx2* and centromeric to the *HoxD* cluster (Spitz, et al., 2003). *Lnp* shares the digit expression domain with *Evx2* and *Hoxd13* during late phase of limb development, as well as the expression in genital bud and in some regions of CNS. This suggested that *Lnp* shares with *Evx2* and *Hoxd13* a common enhancer active in the digit domain.

To elucidate this mechanism a piece of 700 kb around the murine *HoxD* locus was tested either using human BACs spanning this region, or inserting randomly *lacZ* reporter gene constructs. A region was identified 40 kb downstream *Lnp* and centromeric to the cluster, able to drive reporter gene expression in distal limbs, genitals and CNS. This multiple enhancer was defined as global control region or GCR, because of its influence on *HoxD* expression in these three domains (Spitz, et al., 2003). Despite its high degree of conservation in tetrapods and fish, the size of GCR is progressively and dramatically shortened across the phyla. Transgenic mice bearing fish GCR, showed *lacZ* expression in CNS but not in distal limbs, suggesting that fish GCR contains neural but not digit enhancer activity.

However *HOXD13* was not able to fully recapitulate the endogenous *Hoxd13* pattern when placed under the control of the GCR. An additional limb specific regulatory sequence, called *Prox,* was mapped within the *Lnp-Evx2* intergenic region, as it was driving the expression of a *lacZ* reporter gene in distal limb and genital bud. The combination of the GCR with the *Prox* enhancer rescued the *loss-of-function* limb phenotype in mice lacking genes from *Hoxd13* to *Hoxd11* (Gonzalez, et al., 2007). However *Prox* was not able to work if located at big distance from GCR, as in case of the huge inversion of *HoxD* complex characteristic of *Ulnaless* mutants, suggesting that its activity is neutralized by the presence of boundary elements in this genomic configuration.

3.4.3. Qualitative and Quantitative collinearity

The collinear expression of *Hoxd13*, *Hoxd12* and *Hoxd11* in digits is accompanied by a gradual decrease in the transcription efficiency of these genes. The mechanism underlying the collinear *Hoxd* expression in limbs was studied in mice were *Hox* genes were deleted or duplicated (Kmita, et al., 2002). In contrast to the polydactylous limb characteristic of *Hoxd13* disruption, the *Hoxd13* deletion conferred a milder phenotype, associated to the gain of *Hoxd12* in digit domain. Conversely mice where *Hoxd13* and *Hoxd12* where deleted were strongly polydactylous compared to mice where these two genes where simply inactivated. In these mice *Hoxd11* was strongly gained in digits, but could not functionally substitute for the absence of *Hoxd13*. Unlike disruptions, deletions were relocating digit enhancers closest to *Hoxd12* and *Hoxd11* promoters, modifying their transcriptional activity.

Region *XII* was mapped between *Evx2* and *Hoxd13*. The excision of *RXII* and *Hoxd13* broke the quantitative collinearity. Thus all the genes from *Hoxd12* to *Hoxd10* were expressed in digit domain with the same efficiency, independently of the distance from the digit enhancer (Kmita, et al., 2002). However the deletion of *RXII* alone or the removal of *Hoxd13* did not show a comparable effect.

Recently a new study proposed a model attempting to explain the quantitative collinear mechanism underlying the activation of *Hoxd* genes in limbs and external genitalia (Montavon, et al., 2008). The authors analyzed mutant strains carrying variations in the number and/or in the order of *Hoxd* genes by quantitative real time PCR analysis and mathematical modeling. The fundamental conclusions of this work were: 1-the quantitative collinearity depends on the position (or rank) of a *HoxD* gene with respect to the regulatory regions (GCR and Prox) located upstream; 2- on the greater affinity of the global enhancers for the promoters located at the 5'end of the cluster. The proposed model integrates two plausible steps: initially the enhancer and the cluster make a loop structure and recognize themselves then the enhancer scans the promoters one by one.

3.5. Limbs/genitals and hernia enhancer are located at the opposite sides of the cluster

Mouse lines bearing the inversion of the genomic region from *Hoxd13* to *Hoxd11* displayed polydactyly and short digits, like mice where these three genes were deleted (Kmita, et al., 2000-b). Indeed *Hoxd10* expression in "inverted" animals was lost from the digit domain, whereas *Hoxd12* expression was reduced. In *wt* mice, genes from *Hoxd1* to *Hoxd11* are expressed in the region of the gastrointestinal tract represented by the intestinal hernia. *Hoxd12* and *Hoxd13* are normally absent from this region, but in animals carrying the inversion *Hoxd12* was transcribed in the hernia, suggesting the presence of a boundary element between *Hoxd13* and *Hoxd12*, whose orientation was changed by the inversion. This element has been called "polar silencer", since in the normal orientation it prevents the interaction of *Hoxd12* with the "hernia enhancer" and allows the interaction of *Hoxd10* with the "digit enhancer", respectively located at the 3'- and at the 5'- end of the cluster.

The *HoxD* complex was split in two pieces by means of a large genomic inversion separating *Hoxd11* from *Hoxd10* (Spitz, et al., 2005). This inversion maintained posterior *Hoxd* genes in the same reciprocal orientation, but far away from anterior genes. In *wt* animals *Hoxd11* and *Hoxd10* are expressed in distal and in proximal limb domains, in the hernia and in genital bud. In the inverted configuration *Hoxd11* expression was limited to the distal part of the limb bud, and it was absent from the hernia. Conversely, *Hoxd10* had a complementary expression pattern being restricted to the proximal part of the limb, and maintained in the hernia, but lost in the digits. This result suggested once more that the enhancers regulating *HoxD* expression are located at either sides of the cluster; the proximal limb and hernia enhancers are telomeric to the complex, whereas distal limb (and genital) enhancers are located in centromeric position.

4. Hox and development of non-segmented organs

So far most of the knowledge on *Hox* function in body patterning concerns the morphogenesis of segmented organ, represented the vertebral column and appendicular system. Nonetheless in the past few years a number of studies showed that *Hox* genes are expressed in visceral organs. The next section is a general overview reporting examples of Hox expression and function in skin, uro-genital system and intestine. The chapter relative to gastro-intestinal tract is particularly ample because it is crucial to the focus of this thesis.

4.1. *Hox* expression in skin and derivates: hairs and mammary glands

The morphogenesis of the skin needs the contact between the epithelium of the developing hair follicle (represented by the undifferentiate cells and keratinocytes) and the mesenchyme of the dermal papilla. The regionalization of the skin to form the body coat rather than vibrissae depends essentially on the origin of the dermis; the process of differentiation is also temporally distinct. The skin on the face starts to differentiate by stage E11.5 whereas the skin of the body differentiates by E14.5 onwards. The hair is a structure with cyclic development and the dynamic *Hox* genes expression is considered to be an integral part of the process of follicle renewal. The transcription of one of more genes of the same paralogous group is regulated in the post-natal hair follicle according to the cyclic phase of growth. For example *Hoxa4* is expressed only during the growth phase of the hair and *Hoxb4* is transcribed all along the growing and the regressing and resting phase (Packer, et al. 2000).

Hox expression is graphically represented in the review of Awgulewitsch (2003) where *Hoxc8*, *Hoxa4*, *Hoxb4*, *Hoxc12* and *Hoxc13* transcripts are shown within the dermal placode and/or the epithelium since early hair germ stage. In situ hybridization experiments showed the expression of some *Hox* genes like *Hoxc8*, *Hoxd9*, and *Hoxd11* relatively to the thoracic and lumbar skin at fetal stage, while *Hoxd13* was restricted to the caudal skin after birth (Kanzler, et al., 1994).

Hoxc13 is expressed at stage E10.5 in tail bud; later on transcripts are detectable in nails and hair follicles of the whole skin up to the vibrissae in the head and persist after birth within the air follicles (Godwin, and Capecchi; 1998). This ubiquitous expression pattern seems not to fit the principle of collinearity, however the *Hoxc13* posterior restriction in the embryo observed early in development did not contradict this rule (Duboule, et al., 1998). Mice lacking *Hoxc13* exhibited alopecia owing the absence of vibrissae and the growth of fragile hairs breaking once reaching the body surface. The expression of both *Hoxc13* and *Hoxc12* was compared and the interesting observation of “reverted collinearity” was made in contrast to the situation of the body axis. Indeed *Hoxc13* was transcribed earlier than *Hoxc12* and within the most proximal part of the hair follicle, overlapping with the latter expression domain only in the upper part of the bulb (Shang, et al., 2002). Thus the regulation of *Hox* expression in body hairs does is not rely on the same mechanism that coordinates their expression in others embryonic contexts.

While previously cited *Hox* genes are mostly expressed within the epithelial component of the follicle, the situation changes in mammary glands where members of *Hox* paralogous group -9 are expressed in the mesenchyme, at both foetal and post-natal stage (Chen, and Capecchi., 1999; Duboule, 1999). Females lacking *Hoxa9 Hoxb9* and *Hoxd9* had hypoplastic mammary glands, owing the proliferative and branching defect of the ductal epithelium. The dysfunction of the mammary gland and its defective

anatomy were evident only after pregnancy, indeed the litters died within the first 48 hours without milk in their stomachs and survived only if fed by foster mothers. The triple *Hoxa-b-d9* loss of function suggested that *Hox* genes are involved in maturation and proliferation of mammary gland in adult animals. At the same time their functional cooperation during development of the mammary gland implies that their transcription is controlled by specific regulatory influences that may not exist in other mammals (Duboule, 1999).

4.2. Hox and development of the uro-genital system

Kidneys derive from the differentiation of the intermediate mesoderm into an epithelial structure, the pronephros, which further extends caudally forming the mesonephros and differentiates in the ureteric bud, from which the ureters and pelvis form. The ureteric bud induces the growth of metanephric mesenchyme, which in turn goes through mesenchymal-epithelial transformation to form nephrons.

Hox genes control kidney development by promoting mesenchyme-epithelial reciprocal induction signals. The analysis of the phenotype obtained by *Hox* gain-of-function or loss-of-function allowed the identification of a number of *Hox* alleles whose activity is central to the morphogenesis of the kidneys.

Both *Hoxa5* and *Hoxb5* are expressed in adult or fetal kidney (Jeannotte, et al., 1993; Krumlauf, et al., 1987). By E12.5 of development to E14.5 *Hoxb7* is expressed in ureters and collecting tubules. The expression of *Hoxb7* under the control of the α-myosin heavy chain promoter caused renal duplications (Argao, et al., 1995).

At stage 10.5 of development *Hoxa11* is expressed in the primordial metanephric blastema, adjacent to the Wolffian duct. By then its expression domain extends to the metanephric mesenchyme surrounding the uretheric bud and in the nephrogenic mesenchyme around the tip of the buds. A similar expression pattern in developing kidney was observed for *Hoxd11* (Patterson, et al., 2001). *Hoxc10* and *Hoxc11* are both expressed in metanephros and developing kidneys (Hostikka, and Capecchi., 1998).

Double mutants for *Hoxa10* and *Hoxd10* displayed a bigger kidney positioned in a rostral position compared to *wt* mice (Lin, et al., 2003). Double mutants lacking simultaneously *Hoxa11* and *Hoxd11* function displayed agenesis of the kidneys or severe hypoplasia, along with the transformation of vas deferens in epidydymis (Davis, et al., 1995). The cause of kidney agenesis in these mice was attributed to failure of the urethra to branch in the medial-ventral part of the embryonic kidney, providing an explanation for an overall renal growth failure (Patterson, et al., 2001). In *Hoxa11-c11-d11* triple mutants the metanephros was not induced at all, and the ureters never bud from the Wolfiann ducts. This aspect suggested that the function of *Hoxa11*, *Hoxd11* and *Hoxc11* is redundant at early stage of kidney development, and triggers the initial phase of metanephric development (Wellik, et al., 2002).

Another case of agenesis of the kidney was observed in mice where *Hoxd9/lacZ* transgene was relocated upstream to *Hoxd13*, and depleted of the *neo* selection marker. In these mice *Hoxd13* expression was ectopically gained at the level of the intermediate mesoderm forming the urogenital system and kidneys were replaced by truncated ureters (Kmita, et al., 2000-a). These observations suggested that misexpression of *Hoxd13* counteracts the function of *Hox11* products affecting kidney morphogenesis in agreement with the posterior prevalence model. To check for the existence of functional equivalence among different *Hox* paralogs another group substituted the *Hoxa11* homeodomain with that of *Hoxa13*, producing a new allele called *A11(13hd)*. In contrast with the observations of Kmita, et al., 2000, mice lacking simultaneously the *Hoxd11* and *Hoxa11* function and bearing the *A11(13hd)* allele displayed grossly

normal kidneys, confirming that in kidney development *Hoxa11* and *Hoxa13* homeodomains are functionally equivalent and probably share a common set of target genes (Zhao, et al., 2001)

Extensive in situ hybridization analysis performed from stage E12.5 to E17.5 of development revealed that kidneys are positive for the expression of 28 *Hox* genes (Patterson and Potter; 2004). Some genes are expressed in mesenchyme along the proximo-distal axis of the ureteric bud. Conversely, other genes are expressed in the epithelium of the ureteric bud (and subsequently restricted to the nephron primordium, or to the urethra), whereas a few are transcribed in both mesenchyme and epithelium. It was concluded that the expression of 3' end and 5' end *Hox* genes respectively in the ureteric bud and metanephric mesenchyme is regulated by a collinear mechanism. In addition, as exemplified by the *HoxA* and *HoxB* clusters, members belonging to the same complex often shared a similar expression profile, whereas genes from the same paralogous group have a divergent pattern. The last observation suggested that the mechanism that regulates *Hox* collinear expression in kidney probably differs from those proper of vertebral column or limbs.

A recent work showed the impact of *HoxD* complex during renal development and their importance relatively to the regulation of the mesenchymal-epithelial interactions between the mesonephros and the ureteric buds in the developing kidneys (Di Poi, et al., 2007). The authors performed the comparative analysis of kidneys derived from mice carrying diverse deletions within the *HoxD* cluster. These lines were called *Del(10-11)*, *Del (4-11)*, *Del(1-13)*, *Del(4-13)* according to the *Hoxd* genes removed in *cis*- by the deletion (i.e in the *Del(10-11)* line *Hoxd10* and *Hoxd11* were absent). In particular both *Del(10-11)* and *Del(4-11)* mice had smaller and hypoplastic kidneys; such malformation was subsequent to the impairment of the ureteric buds to form branches and the underdevelopment of the surrounding mesenchyme. The observed phenotype was attributed to 1-the lack of more "anterior" *Hoxd* genes, like *Hoxd8* and *Hoxd9* within the *Del(4-11)* line (*loss-of-function* phenotype); 2- to the ectopic expression of *Hoxd12* in the metanephric mesenchyme (*gain-of-function* phenotype). The authors proposed a model by which *Hoxd* expression in kidneys is controlled by two enhancers localized external to the cluster, the "bud enhancer" located at the 3'-end and the "mesenchyme enhancer" situated at the 5'-end. The removal of a number of *Hoxd* genes would locate the remaining genes in closer proximity to one or another of the enhancers.

4.3. *Hox* and development of genitalia

The genital tubercle is one of the last structures of the body to be generated, and is recognizable from stage E11 of mouse development. Its differentiation continues after birth in dependence of the presence of sex hormones that drive the development of the genitalia in males and females. At early stage the genital bud is simply constituted by mesenchyme surrounded by epithelium, and its outgrowth along the proximo-distal axis is coordinated by the reciprocal inductive interactions between these two components.

Unlike the main anterior-posterior embryonic axis genitalia are not overtly segmented, still *Hox* gene expression has been observed both in the external, the internal genitalia and the accessory sex glands both during fetal and/or adult development.

The observation that teleost fishes develop neither external genitalia nor digits suggested that the genesis of both these structures is coordinated in a similar way. Indeed genitals share similarities with limbs in the onset of *Hox* gene transcription.

Among posterior *Hoxd* genes *Hoxd10* is the first gene to be detectable in fetal genital tubercle, followed by *Hoxd11* to *Hoxd13*; in contrast genes from *Hoxd1* to *Hoxd9* are

not expressed in genital bud (Dollé, et al., 1991). The expression of 5' *Hoxd* genes declines at stage E17.5. Conversely *Hoxb13* and *Hoxc13* are not expressed in genitals (Peterson, et al., 1994; Zeltser, et al., 1996).

In both sexes the uro-genital apparatus completes its maturation process after birth, and in adulthood the uterine lining goes through cycles of degeneration and regeneration in coordination with the estrus cycle. In some cases *Hox* targeted inactivation had impact on morphology and on the function of genital organs, affecting fertility.

During embryonic development *Hoxa11* transcripts were observed in stromal cells surrounding the Wolffian and Mullerian ducts, whereas gonads and epithelium of the ducts were negative (Hsiuh, et al., 1995). This *Hoxa11* expression persisted in adult animals suggesting that the product of this gene is important at embryonic as well as in adult stage. Females lacking *Hoxa11* function were sterile because of defective blastocyst implantation in the uterus during pregnancy.

The implantation of the blastocysts in the wall of the uterus and the progressing of the pregnancy depend on cytokine production like the leukemia inhibitor factor (LIF), whose secretion is stimulated by ovarian steroids. It was found that the level of *HOXA11* in the stromal cells varied during the phases of the estrus and was high during pregnancy. Females lacking *Hoxa11* had small uterus, morphologically similar to the oviduct; the endometrial glands' number and histology was abnormal as well (Gendron, et al., 1997). As expected the level of uterine LIF was reduced in pregnant specimens compared to wt, halting the stromal cell differentiation in the decidua, thus preventing the implantation of the embryo.

Males were sterile as well, owing to failure of the testis to descend into the scrotum, and defective maturation of spermatocytes. Additionally, part of the urogenital system represented by the vas deferens exhibited a partial anterior transformation in epididymis. Analogously *Hoxa10* mutant homozygous females were sterile or gave birth to small litters (Satokata., et al., 1995). *Hoxa10* is initially expressed in the luminal and glandular uterine epithelium, then is restricted to the stroma were its concentration increases during implantation and transformation of stroma into decidua. Homozygous females carrying a mutation inactivating *Hoxa10* mostly did not complete gestation owing to delayed or absent implantation (Benson, et al., 1996). In addition they exhibited a homeotic anterior transformation of the uterus into oviduct, evidenced by the lack of a distinguishable anatomic transition with the ovary, whose position in *wt* fetal reproductive tracts is specified by the rostral *Hoxa10* expression boundary. This transformation was not the cause of sterility, which was attributed to the altered "molecular character" of the mutant uterus, and to a decrease of the extent of decidualization of stromal cells. Homeosis was also observed in homozygous mutants males, where part of vas deferens and of the epididymis were transformed into more anterior structures reducing the length of ducts.

Hoxd10 is expressed in a domain represented by the epididymis and testis in males and by the oviduct and ovary in females (Dollé, et al., 1991). *Hoxc10* and *Hoxc11* are expressed in posterior urogenital sinus, from which the primordial vagina and urethra arise (Hostikka, and Capecchi., 1998). The lack of *Hoxd11* conferred male sterility, and the simultaneous absence of *Hoxa11* and *Hoxd11* gave anterior homeotic transformation of the male reproductive tract (Davis, et al., 1995). Although *Hoxa10* and *Hoxa11* are members of *Hox10* and *Hox11* paralogous groups, their function on uterine physiology is not equivalent to that of the other genes in the groups. In human endometrial stroma *HOXC10*, *HOXC11*, *HOXD10* and *HOXD11* levels decrease during the secretory phase compared to the proliferative stage of the menstrual cycle. This suggested that these

genes regulate the endometrial proliferation and are not required for embryonic implantation (Akbas, et al., 2004).

During embryonic development *Hoxd13* expression was observed in mesenchyme and epithelium of the Wolffian ducts and in urogenital sinus (Oefelein, et al., 1996). In the pre-natal urogenital system, *Hoxd13* is transcribed within the mesenchymal wall of the bladder, in the prostate, in the seminal vesicles, the posterior ureters and vas deferens in males and in the uterus in females (Dollé, et al., 1991; Podlasek, et al., 1997). Prostate and seminal vesicle morphogenesis continues after birth, and inactivation of *Hoxd13* caused a defect in both these organs, owing to a reduction in epithelial branching or mesenchymal clefting along with the absence of the bulbourethral gland in some specimens.

Homozygous mice in which a deletion inactivated the *Hoxa13* gene (hypodactyly allele = *Hd*) were unfertile, and both sexes showed defects in the genitourinary system. Malformations were affecting bladder, ureters and penian bone in males, and vagina in females (Mortlock, et al., 1996). The phenotype of *Hd* mice is reminiscent of the Hand-foot-genital syndrome (HFG), a human congenital disorder caused by a non- sense mutation, which generates a stop codon within the *Hoxa13* homeodomain. In affected individuals hands and feet are malformed, and often the uterus is split as result of a defective fusion of Müllerian ducts (Mortlock and Innis., 1997).

The loss-of-function of both *Hoxd13* and *Hoxa13* affects genital a well as digit development, as shown by compounds mutants where *Hd* allele was combined with a deletion encompassing the genomic region including from *Hoxd13* to *Hoxd11*, or removing the sole *Hoxd13*. The genitals of animals lacking both *Hoxa13* and *Hoxd13* function did not develop at all (analogously the limbs were severely truncated at the zeugopod), and in general their outgrowth was correlated with the dose of functional *Hoxa13*, *Hoxd13*, *Hoxd12* and *Hoxd11* expressed in this organ (Kondo, et al., 1997). The previous evidence was further confirmed by the measure of the size of the baculum in compounds mutants where one dose of *Hoxa13* and two doses of *Hoxd13*, *Hoxd12* and *Hoxd11*, were removed (Zakany, et al., 1997-b).

4.4. *Hox* and development of the gastrointestinal tract

Hox genes are expressed in gut at early stages of embryonic development, as well as in other associated organs.

The activity of the gastrointestinal apparatus consists in achieving food digestion and in the consequent absorption of simplified organic substances.

Basically, it is constituted by a tube with two open ends located on the opposites sides of the body (the mouth and anus), lined with a mucosa surrounded by a muscular layer. The ensemble of these two layers assures the passage of food through the stomach and intestine after ingestion, and the enzymatic degradation of nutrients followed by their absorption.

After deglutition the food is pushed from the mouth cavity into the esophagus and further inside the stomach. Here the first step of the digestive process is achieved by the enzymatic and muscular activity of the stomach walls. The so-called chyme passes through the first tract of the small intestine or duodenum in turn associated to the gallbladder and pancreas. The products secreted by these two organs are responsible for a second step of enzymatic degradation. Then the mixture of nutrients is transported into the jejunum and ileum where the absorption process takes place, followed by passage of residues into the large intestine and rectum. The elimination of undigested material occurs by passage of the stools through the anus. Certain aspects of the molecular mechanisms that control endoderm and mesoderm specification are evolutionary

conserved in invertebrates and vertebrates. Strong evidence on HOX function in conferring positional identity within the gut was provided by studies in *Drosophila*, chicken and mouse. In general *Hox* genes are expressed prior to epithelial differentiation then become confined to anatomic regions having different function.

4.4.1. Gastrointestinal tract morphogenesis and anatomy

The morphogenesis of the gut and visceral organs begins from the definitive endoderm formed during gastrulation. In mouse the GI morphogenesis starts around E8 of development (Kaufman, 1994), as a consequence of the formation of an anterior pocket that soon becomes the foregut diverticulum. This pocket forms following upon an anterior fold of the endoderm called Anterior Intestinal Portal (AIP) and extends rostrally into the cephalic region of the embryo at the same time the AIP is extending posteriorly.

In the posterior part of the embryo, another fold called Caudal Intestinal Portal (CIP) is moving toward the head, giving rise to the hindgut diverticulum. The AIP and CIP meet each other and join at the level of the yolk stalk.

At around E10.5 stage of development the viscera like lungs, thyroid, heart, liver and pancreas, bud off from the primitive gut. At this time the stomach is already evident and makes recognizable the foregut from the mid-part and terminal part of gut. The subdivision of the GI in discrete domains along its anterior-posterior axes occurs later, at around E14 pre-natal stage. At the end the GI is divided into distinctive regions having different morphology and physiology: the mouth, the oesophagus and stomach, the small intestine, organized into duodenum, jejunum, ileum; and the large intestine that includes the caecum and colon, and the terminal part including the rectum and anus.

Along with the elongation, the GI increases its size in radial direction owing the differentiation of mesenchyme in smooth muscle of the circular and longitudinal outmost layers.

4.4.2. Between the small and large intestine: the caecum

In mammals the conjunction between the small and the large intestine is marked by the growth of an appendage hanging in the abdominal cavity in anti-mesenteric position called *caecum* (Grassé, 1949). The caecum is open only in its proximal end and its lumen is in communication with the lumen of the intestine. The products of digestion passing by the ileum enter into the caecum where they remain for a certain lapse of time in order to complete degradation of the fibers promoted by the activity of the bacterial microflora resident there.

In contrast to birds characterized by the presence of a pair of caeca *Mammals* have only one caecum. There are a few reported exceptions: *Cyclopes didactylus* (belonging to the order of *Xenarthra*) has a small caecum at both ends of the large intestine, but in this case the authors claim that there is no homology between this and the real caecum. Rather, it may be the enlargement of the lumen of the colon from which two diverticuli develop. The *Monotremes* have a caecum (*Ornithorhyncus*), and the *Marsupials* as well, with few exceptions. Some of the mammals don't have it, for instance most of the *Chiropters*, the *Hippopotamus*, the *Cetacean odontocetae*, and some of the carnivores like the *Ursidae*, *Procyonidae*, and *Mustelidae*. In other animals the caecum is spectacularly developed and reaches half the total volume of the intestine (in the small primate *Tarsius spectrum* it reaches 80% of the volume).

In *Lagomorphs* and *Rodents*, like rats and mice, the caecum as an organ is very well developed. In rabbit it is the part of the GI with the biggest volume (ten times more than stomach), here the part of the colon located more proximally to the caecum is also

expanded and is called *sacculus*. It is possible to generalize by saying that in some groups of mammals the size of the caecum is dependent on the diet, indeed is bigger in non-ruminant herbivores, which have a diet rich in fibers (containing cellulose) and a simplified stomach, compared to carnivores. Histologically the structure of the caecum is like that of the colon. So far the position of the caecum along the anterior posterior axis of the GI is maintained, being located always between the ileum and the proximal colon. The *Hyrax* (a small mammal of the size of a rabbit, belonging to the order of the *Ungulates*) has an additional big pocket joined to the small intestine, situated anterior to the caecum. This pocket is in reality a derivate of the vitelline duct.

In most of the mammals the connection between the caecum and the ileum is mediated by a muscular constriction called *ileo-caecal* sphincter, whose contraction controls the passage of the alimentary substances coming from the ileum or impedes their reflux. This sphincter is absent in *Chiropters*, who don't have any caecum. The passage of material trough the caecum and colon is allowed by the presence of the *caecal-colonic* sphincter.

Inside the cavity of the caecum there is a flora composed by bacteria and protozoa that, beside the degradation process of cellulose and starch, synthesize vitamins. The function of the caecum is duplex since beside alimentary function, its wall contains several lymphoid follicles that in some of the *Primates*, including the *Humans*, are organized into the *appendix vermiform*.

4.4.3. The morphogenesis of the caecum in mice

The caecum develops from the embryonic gut starting as a small mesenchymal bud at around E10 of development, along with the morphogenesis of other GI annexes. Its outgrowth may be temporally divided in three main phases: the *initiation,* coincident with the appearance of the bud, the *elongation,* characterized by the proximo-distal extension of the caecum, and *termination* by which the caecum stops growing and reaches its final size.

From the *elongation* phase through the end of its morphogenesis the germinal layers constituting the caecum are both derivate of mesoderm and endoderm; consequently their differentiation occurs in parallel with the specification of the rest of the gut. The caecum stops growing at around E18 of development, when the different components of the intestine have reached their relative and definitive proportions (Fairbanks, et al 2004-a; Burns, et al., 2004).

The extension of the caecal bud along the proximo-distal direction is the result of a molecular cross-talk between the outer mesenchyme and the internal epithelium that ends up in the cellular proliferation of both layers.

A number of studies done in *Drosophila* and in chicken embryos need to be considered before considering *Hox* gene expression and function in mammalian gut. In chick, gain-of-function experiments (relying on the injection of retrovirus containing specific genes) allowed to better elucidate the fundamental mechanisms involved in GI patterning. Essentially the *Hox* gene expression in gut mesoderm precedes the formation of anatomical boundaries along the AP axis of the GI. Epithelial-mesenchymal interactions are crucial in promoting intestinal differentiation. In gut, the first inductive signal arises from the endoderm and the mesoderm replies by inducing *Hox* expression and a number of downstream genes. The mesoderm produces diffusible factors that in turn induce the specification of the endoderm.

4.5 Homeobox and gut development in Drosophila melanogaster

The internal part of *Drosophila* digestive tract is composed by endoderm overlayed by visceral mesoderm, both of which surround the yolk. The stomach is morphologically divided in an anterior part or proventriculus, and in a posterior region, the ventriculus, separated by tubular protuberances called gastric caeca. During larval development the midgut forms transient constrictions along the anterior-posterior axis of the embryo, which later disappear along with gut elongation.

The products of *Sex comb reduced* (*Scr*), *Antennapedia* (*Antp*), *Ultrabitorax* (*Ubx*) and *abdominalA* (*abd-A*) genes was observed in the visceral mesoderm in adjacent and not overlapping domains along the anterior-posterior axis of the gut. In the posterior midgut *abd-A* and *Abdominal-B* (*Abd-B*) are co-expressed (Tremml and Bienz, 1989). In absence of *Ubx* function the expression domain of *Antp* was shifted to a more caudal domain (Tremml and Bienz, 1989). This means that in *wt* embryos the *Ubx* product downregulates *Antp* and determines its posterior boundary of expression. Analogously *abd-A* represses *Ubx* expression (Bienz and Tremml 1988).

The absence of *Scr* caused the agenesis of the gastric caeca, while embryos lacking *Antp* did not form the first of the three constrictions in the midgut (Reuter and Scott 1990). Double mutants for *Ubx* and *abd-A* lacked the second midgut constriction (Capovilla et al., 1994), and mutants for *Abd-B* did not form the third midgut constriction (Tremml and Bienz, 1989). These studies showed that the expression of *homeobox* genes in gastric visceral mesoderm was indeed shaping the endoderm.

4.6 *Hox* and gut development in chicken

The *AbdB* related genes *Hoxa9*, *Hoxa10*, *Hoxa11* and *Hoxa13* are expressed in gut visceral mesoderm in dependence of the embryonic stage. At early time of development genes from *Hoxa9* to *Hoxa13* are transcribed within the hindgut in partially overlapped domains; then, once the pairs of caeca are formed, their expression pattern becomes definitive. *Hoxa9* is expressed in the posterior part of small intestine and, together with *Hoxa10*, in the caecal mesenchyme; *Hoxa11* is transcribed in large intestine whereas *Hoxa13* mRNA is restricted to the cloacal region. The expression domain of *Hoxa10* and *Hoxa11* is partially overlapped in mesoderm of caecal primordia suggesting that the product of these two genes is involved in caecal morphogenesis (Yokouchi et al., 1995-b).

At early stages *Hoxa10*, *Hoxa11* and *Hoxa13* are expressed in gut endoderm although not in a collinear fashion. Among these genes *Hoxa13* is the sole that at later stage retains endodermal expression in caecal eminences and throughout the endoderm of the hindgut up to the cloaca (Yokouchi et al., 1995-b).

Other groups found that at early stages the *Hoxb*, *Hoxc* and *Hoxd Abd-B* related genes in embryonic GI of chick are induced in mesoderm of the Caudal Intestinal Portal in collinear fashion. *Hoxd9* is transcribed earlier then it is the turn of *Hoxd10*, *Hoxd11*, *Hoxd12* and *Hoxd13* (Roberts et al., 1995). During hindgut differentiation these genes maintain the relative position of their anterior expression boundaries, in spite of the widening of their related expression domains. The transcription of 5' end *HoxD* and *HoxA* genes encompasses the pair of swelling caeca. *Hoxd9* and *Hoxd10* transcripts are spread along the whole caeca, while *Hoxc9* mRNA is restricted to the anterior half of caeca and *Hoxd11* to the posterior half. *Hoxd12* is expressed in the large intestine and *Hoxd13* in mesoderm and endoderm of the cloaca.

4.7. *Hox* genes and gut development in mouse

Several studies describe *Hox* gene expression along the anterior-posterior axis of the GI at early stages of fetal development. In foregut, midgut and hindgut *Hox* are differentially represented; however a great number of them are expressed in budding caecum, within a defined region at the boundary between the ileum and colon. This particular aspect suggests that the specification of this area at that time of development requires the simultaneous induction of multiple factors to occur. In the next chapter are reviewed most of the known data relative to *Hox* expression and function in developing GI. The mechanism by which *Hox* genes mediate GI patterning is largely unknown, including their precise function in caecum morphogenesis.

4.7.1. Hox expression profile in GI

During embryonic development *Hoxa3* is expressed both in the esophagus and stomach. Like *Hoxa3*, *Hoxd3* is present in mesenchyme of esophagus, stomach and intestine (Tan, et al., 1996).

At early stage of development *Hoxa4* is detectable in stomach and precaecal gut; at midgestation, once GI regions are more delineated, *Hoxa4* retains its expression in stomach, duodenum and jejunum (the anterior tract of small intestine) then extends to the colon (Kawazoe et al., 2002; Pitera et al., 1999). Like *Hoxa4*, *Hoxb4* is transcribed at E10.5 and E12.5 stages both in stomach and small intestine, with a posterior boundary positioned next to the bud of the caecum (Pitera et al., 1999; Kawazoe et al., 2002). By E15.5 *Hoxb4* expression extends to the colon (Pitera et al., 1999). *Hoxb4* mRNA was observed exclusively in the mesenchymal layer of GI, while *Hoxa4* was detected in both mesoderm and mucosa (Galliot, et al 1989; Gaunt et al., 1988; Geada, et al., 1992; Pitera, et al., 1999). At E11.5 *Hoxc4* is expressed in stomach and hindgut, by E12.5 it extends anterior up to the esophagus and posterior to the small intestine, caecum and anterior colon (Pitera et al; 1999). *Hoxd4* is quite dynamic; at E10 is weakly transcribed in the mesenchymal wall of the stomach and later on it is restricted to the ganglion cells that derive from the neural crest and form the enteric nervous system (Gaunt et al., 1988). In contrast with these reports other studies showed that *Hoxd4* mRNA is absent from foregut and midgut, but is early expressed in caecum and in the anterior part of the large intestine where it persists until E15.5 (Pitera et al., 1999; Kawazoe et al., 2002).

From E10.5 to E12.5 *Hoxa5* is transcribed in stomach mesenchyme, in mesenchymal and epithelial lining of the midgut (Pitera et al., 1999; Kawazoe et al., 2002). From E13.5 onwards *Hoxa5* expression in foregut subsides; in contrast it rises in caecum and in the proximal part of the large intestine. By then it becomes restricted to the external muscular layer of the midgut and hindgut, in cells of the myenteric plexus (Aubin et al., 2002; Dony and Gruss, 1987; Gaunt et al., 1990; Holland and Hogan, 1988; Krumlauf et al., 1987; Pitera et al., 1999).

At early stages *Hoxb5* is detected in the mesenchymal wall of the stomach then it progressively extends through the small intestine and caecum. At later stages it is spread all along the enteric nervous system of the whole gut (Holland and Hogan, 1988; Kawazoe et al., 2002; Pitera et al., 1999).

Former studies showed that at E12.5 stage *Hoxc5* is expressed in the epithelium of the oesophagus, in the anterior epithelium and dorsal mesenchyme of the stomach, and within the mesenchymal layer of the intestine (Gaunt et al., 1990). Recent studies have reported that at early stages *Hoxc5* is expressed in the epithelium of the oesophagus, in the rostral part of the stomach, weakly in midgut, and strongly in hindgut. At around E12.5 stage it become restricted to the small intestine until the caecum and it drops in

colon (Kawazoe et. al., 2002; Pitera et al., 1999). At E16 the intestinal expression is confined to both the epithelial and the muscular layers (Pitera, et al., 1999).

At E12.5 *Hoxa6* is strongly expressed in stomach and much more weakly in the small intestine, in the segment between the ileum and caecum (Kawazoe et al., 2002).

At around E10 *Hoxa7* and *Hoxa9* are represented in both midgut and hindgut mesoderm (Sekimoto, et al., 1998); by E12.5 *Hoxa7* mRNA was detectable in the stomach, in the tract of small intestine comprised between ileum and caecum, including the anterior colon. In contrast the transcription of *Hoxa9* at the same stage is restricted to the caecum, with an anterior limit represented by the ileo-cecal transition.

Early on *Hoxb6* and *Hoxb8* are transcribed in the visceral mesoderm of mid-and hindgut, at later stages their expression becomes visible in the stomach (Eid et al., 1993; Sekimoto, et al., 1998). Their expression domain in GI is discontinuous, as *Hoxb8* transcription in small intestine is posterior to *Hoxb6* in correspondence of the ileum and spreads through the caecum. At early stage *Hoxc6*, *Hoxc8*, *Hoxc9* are all expressed in the mesoderm of the midgut and hindgut. Later on *Hoxc6* is strongly expressed throughout the small intestine and caecum, *Hoxc8* is detectable in the ileum, *Hoxc9* is transcribed in the terminal part of the ileum and in the caecum (Sekimoto, et al., 1998). The inactivation of *Hoxc9* did not lead to gut malformation (Suemori, et al., 1995). A former work (Izpisua-Belmonte, et al., 1991-b) shows that *Hoxd8* is broadly expressed in GI of E12.5 fetuses, but is excluded from the oesophagus and stomach. The expression of *Hoxd8* in the hindgut was observed at early stage, whereas at E12.5 spreads in the tract from ileum to caecum. Like *Hoxd8,* at E10 *Hoxd9* is expressed in the hindgut then it narrows to the bulge of the caecum, dropping in correspondence of the ileo-cecal sphincter. Cross-sections of hybridized GIs showed that all these genes are expressed in the visceral mesoderm.

Hoxd11, *Hoxd12* and *Hoxd13* are expressed in the most posterior part of the body, including the mesenchyme of the terminal part of the gut (Izpisua-Belmonte, et al. 1991-b; Dollé et al., 1991). Among these genes the one having the most anterior expression domain is *Hoxd11* that at E12.5 is transcribed in the mesenchyme of the caecum.

The expression pattern of *Hoxd12* and *Hoxd13* in GI was better visualized replacing *Hoxd12* and *Hoxd13* by the insertion of the *lacZ* reporter gene. In this way *Hoxd12* and *Hoxd13* expression was detected in both the mesenchyme and epithelium of the anus, in contrast the rectal epithelium that was negative for both *Hoxd12* and *Hoxd13* (Kondo, et al., 1996). *Hoxd13* expression colocalizes with *Hoxa13* in both mesenchyme and epithelium of the rectum (Warot, et al., 1997).

More recently was analyzed the profile of known transcription factors in developing GI, between E11 and E17 of embryonic development. The extensive analysis, based on quantitative PCR on fetal stomach and small intestine, was performed at the time when most of the morphological changes occur (Choi, et al., 2006). Basically all posterior *Hox* genes between the paralogous group-*9* up to -*13* resulted not expressed in stomach and small intestine at any of the stages examined. This finding supports the concept of an ordered (collinear) *Hox* genes expression along the AP axis of the gut according which posterior genes are excluded from foregut and midgut and in agreement with previous observations. Nevertheless this work shows that *Hoxa1*, *Hoxa4*, *Hoxb4*, Hoxa5, *Hoxc6*, *Hoxb8* transcripts are absent from either stomach or small intestine in contraposition with some of the studies cited above. This latter aspect arise doubts about the utilization of the quantitative PCR technique as univocal method to obtain a conclusive and valid result. Otherwise *Hoxc4*, *Hoxb6*, *Hoxa7*, *Hoxc8*, show regional specificity, the first two being upregulated in stomach and the latter two in small

intestine. Apart these four genes, all the others *Hox* genes are expressed with a similar pattern and with the same amount independently on the stage and on the segment analyzed.

4.7.2. Hox inactivation and GI malformations

Mice lacking *Hoxa3* showed developmental defects in the components derived from smooth muscles and neural crest of the throat. The esophageal sphincter was absent, as well as the soft palate was shorter and the epiglottis was mis-positioned compared to *wt* mice. Consequently mutants exhibited a bloated phenotype owing to the presence of air in the stomach and intestine (Chisaka and Capecchi, 1991). However defects in stomach morphology were not observed. Defects in GI development secondary to the loss of function of *Hoxd3* were not reported (Condie et Capecchi, 1993).

The over-expression of *Hoxa4* led to abnormalities of the smooth muscle layer of GI and to the defective differentiation and migration of enteric neural cells. This gave rise to an anatomical malformation consisting in the enlargement of the bowel, also called megacolon (Wolgemuth, et al., 1989; Tennyson et al., 1998).

The lack of *Hoxc4* stimulated abnormal proliferation of the esophageal epithelium, which caused the blockage of the lumen. Additional defects perturbed the organization of musculature in the upper tract of the esophagus (Boulet, and Capecchi., 1996).

Hoxa5 seems not to be required for the morphogenesis and cellular organization of the small intestine however its loss perturbs the process of intestinal maturation that occurs at the age of weaning (Aubin, et al., 1999). During weaning the synthesis of the enzyme *sucrase* replaces the production of *lactase* characteristic of the suckling phase, but the lack of *Hoxa5* interferes with this process delaying the postnatal maturation of the intestine.

The loss of *Hoxa5* had much more dramatic consequences on the regionalization of gastric epithelium. At the end of the development the stomach is subdivided into an anterior and posterior part, respectively the forestomach (also called muscular stomach) and hindstomach (or glandular stomach). The epithelium of these two areas, alternatively squamous or glandular, has a different function. The proximal part of the hindstomach is organized in gastric units composed by zymogene cells, which are stimulated to secrete pepsinogen by the activity of enteroendocrine cells located in the same glands. Mice lacking *Hoxa5* had abnormally thin gastric epithelium, following the decrease of both zymogene and enteroendocrine cell populations by the second post-natal week (Aubin, et al., 2002). It was suggested that hindstomach morphology was affected by a partial homeotic posterior transformation (Aubin, et al., 2002). Indeed prior cytodifferentiation the *Ihh* expression domain, usually located between hind- and forestomach, was extended at the expense of the region positive for *Shh*, which in turn was confined to the more anterior forestomach.

Mice lacking *Hoxd12* or *Hoxd13* have a disorganized morphology of the internal anal sphincter. The longitudinal muscle layer of this segment was severely affected, resulting in the absence of the ano-rectal-sphincter and in anal prolapse in adult mice. Such phenotype suggested that *Hoxd12* and *Hoxd13* play a role in sphincter morphogenesis (Kondo, et al., 1996).

The importance of *Hoxd* genes in GI morphogenesis was evaluated by means of the deletion of part of the cluster. Mice carrying a *HoxD* mini-complex, composed by the genomic region from *Hoxd1* and *Hoxd3* left at 3' end and by a *Hoxd11/lacZ* transgene located at 5' end of the cluster were generated. Heterozygous mice for the mini-complex displayed a strong ß-gal staining in correspondence of the ileo-cecal sphincter and rectum. In homozygous mice both the ileo-cecal and the ano-rectal sphincters were

absents suggesting that the products of genes from *Hoxd4* to *Hoxd13* are indispensable to assure sphincter morphogenesis (Zakany and Duboule, 1999).

Knockout mice for *Hoxa13* were embryonic lethal, but did not display defects in GI organization. Conversely mice *Hoxa13*$^{+/-}$, *Hoxd13*$^{-/-}$ survived but showed a range of phenotypes going from the disorganization of the rectal musculature to the malformation of the genitourinary system and sex glands (Warot, et al., 1997). This suggested that *Hoxa13* function is much more important for the development of the genito-urinary system rather than gut, where in order to obtain a consistent defect it was necessary to ablate the two copies of *Hoxd13* together with one copy of *Hoxa13*. Double mutants for *Hoxa13* and *Hoxd13* exhibited a cloaca and did not form the genital bud, implying a synergistic interaction between the products of these two genes for the morphogenesis of the rectum and the formation of external genitalia.

5. Candidate genes and developmental mechanisms as potential targets of Hox gene function

5.1. Principal molecules involved in organogenesis

Organogenesis is the consequence of the growth and differentiation of epithelial and mesenchymal derived tissues that occurs from the beginning of embryonic development. At first the organ primordium bulges from condensations of mesenchymal cells that form around the associated epithelium. Then the epithelium progressively thickens and forms branches (or folds) characteristic for each tissue. The growth of the organ is achieved because of the instauration of a complex net of interactions between its epithelial and mesenchymal components. The basic pathways that drive the initial steps of morphogenesis are common to several organs, like lungs, limbs, kidneys, hairs, teeth, mammary gland etc.

Several studies have been focused to assess the existence of the instructive/permissive interactions between mesenchyme and epithelium. The rudiments of organs can be separated in their epithelial and mesenchymal components, and the interaction between epithelium and mesenchyme can be followed in vitro by the dissociation and re-association of tissues in co-culture experiments (Grobstein 1953).

Finally the signals between the two layers are often reciprocal. Nevertheless they must occur in a precise window of time and developmental stage to be functional.

5.1.1. Fibroblast growth factors

Fibroblast growth factors (FGFs) represent a wide family of polypeptides conserved in nematodes, in insects and vertebrates, which share homology of sequence and gene structure (Ornitz, et al., 2001). The percent of identity of their core sequence among vertebrates and mammals ranges from 80 to 90% (Goldfarb, et al., 1996). FGFs exert their biological function acting on cell survival, proliferation and differentiation.

The cellular response is driven by the interaction of FGFs with their receptors (FGFRs) which are transmembrane proteine kinases characterized by three immunoglobulin-like extracellular domains, used for substrate recognition. In *Drosophila* one *Fgf* gene and two *Fgfr* genes respectively called branchless (*bnl*), breathless (*btl*) and heartless (*htl*) have been isolated. In mammals the alternative splicing of *Fgfr* mRNAs generates a larger number of receptors, in which the extracellular domain is shortened because of the absence of IgI-like domain or where the sequence of IgIII-like domain is variable, giving rise to the isoforms IIIa, IIIb and IIIc having different specificity for the ligand (Johnson, and Williams., 1993; Goldfarb, 1996; Ornitz et al., 1996). Until now up to 7 FGFRs have been characterized, each of which is able to interact with more than one substrate. The binding of FGFR to the ligand triggers the homo- or hetero-dimerization of both ligand and receptor, followed by auto-phosphorylation of the tyrosine kinase residues within the FGFR cytoplasmic domain. The dimerization is promoted by interaction between FGF ligand with respectively heparin or heparan sulfate proteoglican (HSPG) present in the extracellular matrix, and stabilizes the interaction with the receptor. The interaction of FGFR with its substrate stimulates the cell response in autocrine and /or paracrine fashion, activating RAS/MAPK pathway in the cytoplasm and inducing the expression of downstream genes (Goldfarb, 1996).

During embryonic development the tissue specific and temporal activation of both *Fgfr* and *Fgfs* control embryonic and fetal development by sustaining the growth of the tissues and promoting the formation of the organs. Mutations affecting either *Fgfs* and/or *Fgfr* are reflected in defective growth of the skeleton, muscles, blood vessels beside the development of multiple organs in the sensory, nervous and respiratory system (Goldfarb; 1996).

5.1.2. Fgf10

The fibroblast growth factor 10 (*Fgf10*) mRNA was first isolated in rat where it is expressed either in fetal and adult tissues. In spite of the poor homology of its core sequence *Fgf10* is functionally homologous of the *Drosophila* gene *Branchless* (*Bnl*) (Min, et al., 1998). *Bnl* is expressed in the respiratory system of Drosophila in cluster of cells surrounding the tracheal system in a position where the branches will develop (Sutherland, et al., 1996). Misexpression of *bnl* leads the formation of the branches to new positions. In turn *Bnl* interacts with the *breathless* (*btl*) tyrosine kinase receptor, which is the homologous of FGF10 receptors (FGFRIIIb). Similary to the respiratory system of flies *Fgf10* mRNA is relatively abundant in lungs of rat embryos, where it accumulates in sites where new air-branches will form. Other anatomical areas of *Fgf10* pre-natal expression in rats are the duodenum, and pituitary gland, whereas post-natal expression is maintained in lungs and heart (Yamasaki, et al., 1996).

A more detailed analysis of *Fgf10* knockout mutants revealed a greater complexity of abnormalities, like the lack of thyroid, pituitary and salivary glands. Conversely other organs like tooth, inner ear, thymus, stomach, pancreas and kidneys were present but showed dysgenesis (Ohuchi, et al., 2000). For examples the skin of mutants was thinner and showed a reduced number of hair follicles. On the other hand visceral organs like stomach, pancreas and kidneys were smaller, suggesting that *Fgf10* signaling is involved in the permission of the execution of a developmental program rather than in the induction of the growth of a specific organ.

5.1.3. Fgf10 receptor

FGF10 was identified as ligand of FGF receptor “b” isoform (FGFR2b) (Igarashi, et al. 1998), obtained from alternative splicing of *Fgfr2* mRNA that gives rise to *Fgfr2-IIIb* and *Fgfr2-IIIc*. These isoforms are characterized by a different sequence of the third Ig-like domain that confers to the receptor both the ligand and spatial specificity (Miki, et al 1992; Orr-Urtreger, et al., 1993; Yayon, et al., 1992).

Fgfr2b is specifically expressed in epithelial cells and displays its biologic activity binding to FGF1, FGF3, FGF7 and FGF10 (Igarashi, et al 1998; Ornitz, et al 1996). A null mutation in *Fgfr2* led to the abrogation of both the isoforms *Fgfr2a* and *Fgfr2b*, which resulted in perinatal lethality (Arman, et al., 1998). Mice carrying a dominant negative form of *Fgfr2* displayed a wide range of phenotypes represented by cranio-facial and skeletal defects and fore- and hindlimb truncations. Visceral anomalies were found within the gastrointestinal tract (dysgenesis of the glandular stomach), the urogenital system (unilateral kidney), and lungs, which did not form at all (Celli, et al., 1998). The specific inactivation of the *Fgfr2b* isoform led to abnormal phenotypes already observed in *Fgf10* knockout mice, suggesting the existence a functional link between the products of the two genes. Newborns displayed cranio-facial defects, like absence of the anterior pituitary gland, cleft palate, malformation of salivary glands and inner ear, absence of eyelids. In these mice skin and teeth were not correctly developed; both fore- and hindlimbs were absent (De Moerlooze, et al 2000).

5.1.4. Sonic hedgehog

Sonic Hedgehog is a murine homolog of the segment polarity gene *hedgehog* (*Hh*) of *Drosophila*. The *Shh* product is secreted by the cell and displays biological activity as a diffusible morphogen thus modulating the fate of target cells in dependence of its concentration (Wolpert, 1969).

In mouse and human two other *hedgehog* related genes have been isolated: *Indian Hedgehog* (*Ihh*) and *Desert Hedgehog* (*Dhh*) (Echelard et al.1993; Marigo et al., 1995). In mouse *Shh* is expressed in limb buds and in the epithelium of tooth, whiskers, hair, lungs, gut and bladder (Bitgood and McMahon, 1995). In humans *Shh* expression has been detected in fetal intestine, liver, lung and kidney; whereas *Ihh* is expressed in adult tissues like liver and kidney (Marigo et al., 1995). Mice lacking *Shh* function displayed abnormal neural tube morphogenesis, cyclopia, and absence of distal limb structures and of most of the ribs (Chiang, et al., 1996). In addition, the absence of *Shh* compromised the correct development of regions of the body and organs in which it is not expressed. This confirmed that *Shh* codes for an extracellular morphogen whose activity on target cells is crucial for organogenesis.

5.1.5. The SHH receptor: Patched

The receptor of *Hh* is a transmembrane protein localized on the cell surface and encoded by the gene *Patched* (*Ptc*). The latter belongs to the family of segment polarity genes that establish the patterns of segments in *Drosophila* embryo (Alcedo, et al., 1996; Capdevila, et al., 1994; Hooper and Scott 1989; Van Den Heuvel and Ingham, 1996).

At early stage of mouse development *Ptc* is expressed in the headfolds and axial tissues like the ventral neural tube and pre-somitic mesoderm. These domains, located near the *Shh* expressing cells of the notochord, at later stages will be represented by brain, neural tube, and somites (Goodrich et al., 1996). Beside the main body axis *Ptc* is transcribed in foregut and hindgut, branchial arches, hair and whiskers follicles, palate, tongue, tooth buds, limbs and genital eminence.

Both in flies and vertebrates it was shown that without *Shh* the Patched activity down-regulates the expression of *Shh* target genes, which are members of *Wnt* and *Tgfß* families. Such repression is abrogated by the interaction of SHH with PTC and the simultaneous release of the multi-pass transmembrane protein *Smoothened* (*Smo*). The activation of SMO triggers a signal transduction cascade that ends in the transcription of SHH target genes (Stone, et al., 1996; Murone, et al., 1999). In mouse and human a second gene related to *Patched* and called *Ptc2* has been isolated (Carpenter, et al., 1998; Motoyama, et al., 1998-a).

In *Drosophila* the activity of *Hh* is mediated by the segment polarity gene *Cubitus interruptus* (*Ci*), and by the murine homologous zinc fingers transcription factors *Gli1*, *Gli2* and *Gli3* (Hui, et al., 1994). *Ci* is expressed in tissues expressing *Ptc* and in turn induces its transcription (Forbes et al., 1993). Ci level its postranscriptionally elevated by HH (Motzny and Holmgren, 1995) and downregulated by Ptc itself (Johnson et al., 1995). In absence of *Hh* signal the transcription factor *Ci* is cleaved and behaves as transcriptional inhibitor whereas in presence of active *Hh* signaling *Ci* is not processed and has activating function.

In mammals *Gli1* and *Gli3* are transcriptionally and post-transcriptionally controlled by SHH signal (Sasaki et al., 1999) since both GLI2 and GLI3 proteins have an N-terminal repressor domain whose abrogation confers to the proteins strong activator properties.

5.1.6. Pitx transcription factors

The murine *Pitx1* (or *Pituitary homeobox factor 1*, also named *Backfoot*) belongs to a family of three genes (*Pitx1*, *Pitx2* and *Pitx3*) all coding for transcription factors characterized by a homeobox domain (paired) related to the genes *bicoid* and *orthodenticle* of *Drosophila*. *Pitx1* has been shown to play a role in pituitary gland development since it binds to the promoter of the pro-opiomelanocortin gene in precursors cells of the anterior pituitary lobe (Rathke's pouch), before the appearance of differentiated cell lineages (Lamonerie, et al., 1996). In the anterior part of the body, at E8 of development *Pitx1* is expressed in the epithelium of the stomodeum, then it spreads to the epithelium and mesoderm of the first branchial arch, becoming restricted to the developing cranio-facial structures. *Pitx1* null mice show defects in pituitary gland development and in branchial arch derivatives. A homolog of *Pitx1* has also been characterized in *Drosophila* (Vorbrüggen et al., 1997)

A molecular correlation between *Pitx1* and *Hox* has been found during branchial arch development of mice lacking *Hoxa2*. In the head *Hoxa2* is specifically expressed in the mesenchyme of the second branchial arch. Mice null for *Hoxa2* show the second branchial arch developing characteristics of the first one, with consequent malformation of its derivatives (Bobola et al., 2003). *Pitx1* is a downstream target of *Hoxa2* and its expression in mesenchyme of the second branchial arch of *Hoxa2*$^{-/-}$ mice is ectopically gained. Such ectopic expression of *Pitx1* is prevented either by the absence of the epithelium or by the presence of inhibitors of FGF signaling. Hence in wt mice *Hoxa2* expression prevents the second branchial arch to abnormally develop by down-regulating *Pitx1* expression in mesenchyme. The mechanism, by which *Hoxa2* affects *Pitx1* level, is probably due to the interference with *Fgf* signaling. In the hindlimb mesenchyme the *HoxD* and *Pitx1* expression coexists, suggesting a possible interaction between the two classes of proteins in the specification of the hindlimb identity.

6. Mesenchymal-epithelial interaction and limb patterning

6.1. Fgf and limb development

The apical ectodermic ridge (AER) is represented by a pseudo-stratified epithelium overlying the anterior-posterior margin of the limb bud. The ablation of the AER prevents the limb to form distal structures (Saunders, et al., 1948).

Fgf10 is expressed in early limb mesenchyme where it is required for induction of the expression of *Fgf8* in the AER and consequently it promotes limb elongation (Ohuchi, et al., 1997). Accordingly, both fore- and hindlimbs of homozygous *Fgf10* loss-of-function were truncated, owing the disruption of AER and the ZPA (Min, et al., 1998; Sekine et al., 1999). *Fgf8* is expressed in the intermediate mesoderm prior limb budding, where it restricts *Fgf10* expression in the lateral plate mesenchyme of the developing limb buds (Ohuchi, et al., 1997). Subsequently its expression is extended throughout the AER, where promotes limb outgrowth along the anterior-posterior axis maintaining the expression of target genes like *Fgf10* and *Shh* (Lewandoski, et al., 2000; Moon and Capecchi, 2000). Since the *Fgf8* appearance in the intermediate mesoderm precedes that of *Fgf10* in limb mesenchyme it was believed that *Fgf8* was required for the induction of limb outgrowth. However mice lacking *Fgf8* specifically in the intermediated mesoderm were still able to form limbs, without influencing limb bud initiation and elongation. Conversely the ablation of *Fgf8* in the AER affected the survival of limb mesenchyme, giving rise to truncate but not absent limbs. This observation suggested that another molecule in the AER was compensated for *Fgf8* deficiency. This latter is represented by *Fgf4* that is also expressed in the AER. As expected ablation of both *Fgf8* and *Fgf4* specifically in the limb ectoderm blocked limb morphogenesis. Indeed the formation of the bones was precluded by the high apoptosis of the mesenchyme suggesting that the expression of the two factors in the AER is required for the survival of the limb mesenchyme and thus for the limb outgrowth (Sun, et al., 2002; Boulet et al., 2004). In conclusion limb outgrowth relies on the expression of *Fgf10* in mesenchyme that activates *Fgf8* expression in the AER, the latter of which induces proliferation of the mesenchyme.

6.2. Shh in limb development

During embryonic development *Shh* is expressed in the early gut endoderm, in the floor plate of the neural tube, in the notochord and in limbs. In the latter it identifies a group of mesenchymal cells situated in the posterior part of the limbs that is named Zone of Polarizing Activity (ZPA) (Echelard, et al., 1993). The graft of the ZPA to the anterior half of the chick limb induces the growth of additional digits with mirror image with respect of the anterior-posterior pattern of the limb (Saunders and Gasseling 1959). Consistent with this, *Shh* expression in limbs buds colocalizes with the ZPA and when *Shh* is ectopically expressed it induces mirror duplications of digits along the anterior posterior axes of the limb (Riddle et al., 1993). Both the transplant of the ZPA and the *Shh* ectopic induction in chick limbs induce the expression of posterior *HoxD* genes in a sequential pattern reminiscent of that in the posterior region of the limb (Riddle et al 1993).

Mice lacking *Shh* displayed a severe truncated limb phenotype involving both zeugopod and autopod; nevertheless in double *HoxA/HoxD* mutants *Shh* expression was

abrogated in limbs buds, suggesting that early on *Hox* gene dependent activation of *Shh* is mandatory.

Concerning the *HoxD* cluster, only from *Hoxd10* to *Hoxd13* elicit *Shh* transcription in the posterior limb bud. In absence of *HoxD* genes *Shh* is triggered by the products of posterior *HoxA* genes. Mice lacking the full *HoxA* cluster and from *Hoxd9* to *Hoxd13* had almost absent humerus accompanied by agenesis of the autopod, whereas the phenotype was less severe when *Hoxd9* and *Hoxd10* were added back, confirming the function of the formers in zeugopod elongation, and of *Hoxd13* and *Hoxd12* in digits development (Tarchini, et al., 2006). *Hoxd10* and *Hoxd13* activate *Shh*, whereas *Hoxd9* does not, thus the outgrowth of zeugopod and autopod in *Hox* deficiencies is affected by a *Shh* dependent and independent mechanism.

These studies combined with the analysis of limb bud in $Shh^{-/-}$ mice, showed that limb development is characterized by an early phase *Shh* independent and by a late phase, which relies on *Shh*. The limb truncation obtained in absence of *Shh* is less severe compared to the defect observed in absence of *Hoxa* and *Hoxd* function. Indeed at early stage the limb field is pre-patterned by the expression of *Hoxa* and *Hoxd* genes that soon drive the expression of *Shh* within the ZPA. Afterwards the second wave of *Hox* expression is modulated by *Shh* that regulates the digital growth according to the anterior-posterior axis of the limb (reviewed in Zakany and Duboule, 2007). The existence of a functional interaction of *Hox* with a *Shh* limb enhancer, peculiar of the early phase of limb growth, has been documented in mice lacking simultaneously *Pbx1* and *Pbx2*. These mice display severe hindlimb truncation (consisting in the complete absence of the autopod and partial absence of the zeugopodal bones) which is related to the loss-of-function of posterior *Hox* genes and to the lack of *Shh* in the ZPA (Capellini, et al., 2006).

6.2.1. Extra-toes mutation (Xt) and polydactyly

The *Xt* spontaneous mutation has been isolated in mouse, where it was associated with the presence of extra-digits in the anterior side of forelimbs and hindlimbs secondary to additional skeletal elements (Johnson, 1967). When *Xt* mutation is in homozygous configuration it not only affects more severely the limb development (increasing the number of extra-digits) but it has a great impact in the morphogenesis of the brain.

The *Xt* semi-dominant phenotype is reminiscent of a dominant form of polydactyly and cranio-facial defect characteristic of the human syndrome called Greig Cephalopolysyndactily (GCPS) (Winter and Huson 1988). The genomic and morphological link with this pathology was confirmed by the discovery that in affected individuals the *Gli3* gene was disrupted by a translocation (Vortkamp et, al., 1991).

The *Gli3* product is a zinc-finger transcription factor. The analysis of *Gli3* expression pattern in *wt* and in *Xt* embryos substantiated a link between the *Xt* phenotype and the *Gli3* gene function in mouse (Schimmang 1992). In developing limbs GLI3 exist both as full-length protein behaving like transcriptional activator (GLI3A), and in a shorter processed form, with repressive function (GLI3R). The ratio between the quantity of activator and repressor is influenced by *Shh* signaling; the latter in the ZPA counteracts the activity by which GLI3 is processed into the repressive form and favors GLI3 activator (Dai, et al., 1999; Wang, et al., 2000). In this manner GLI3 repressor is confined to the anterior limb domain, where in a feed-back loop inhibits *Shh* expression. *Gli3* is mediator of *Shh* signaling and similarly to *Gli2*, it induces the transcription downstream targets genes like *Gli1*. Unlike *Gli3*, *Gli2* and *Gli1* are not involved in the setting of the anterior-posterior limb polarity.

At stage E10.5 *Gli3* is expressed in the anterior region of both fore- and hindlimbs, excluding the ZPA, and later on becomes restricted to the mesenchymal condensations of digits (Buscher, et al., 1997). In wt limbs a *Gli3* probe gave a strong hybridization signal in mesenchyme of the stylopod, around the head of the humerus, and in the autopod, in correspondence of the digit condensations. In these domains *Gli3* was expressed in the perichondrium, a membrane of mesenchymal cells surrounding the intermediate cartilage, which goes through the process of ossification in bone generation. In *Xt* mutant mice a deletion encompassing the 5'end including the promoter and part of the *Gli3* coding region was abrogating its expression, being the causative effect for the observed developmental defects in these mice (Schimmang, et al., 1992).

Shh activity in limbs is necessary for the development of the AER along the anterior-posterior limb margin and for the formation of the intermediate bones of hands and feet. Accordingly mice lacking *Shh* had no autopod and their zeugopod was poorly developed. Indeed detailed analysis of the mutant limb showed that the elongation of the stylopod as well as the anterior posterior polarity of the humerus and femur was not affected by the lack of *Shh*. In mutants the formation of the AER was induced, but was subsequently disrupted along with the reduction of *Fgf8* expression in the ridge, followed by cell death within the limb mesenchyme. These observations suggested that limbs are pre-patterned before the occurrence of *Shh* signaling (Kraus, et al., 2000; Chiang, et al., 2001).

Differently from *Shh* null mice *Gli3* mutants were characterized by polydactyly and identical digit phenotype; this was initially attributed to the anterior ectopic expression of *Shh* and to the generation of a second ZPA (Masuya, et al., 1995). Further studies showed that in these mice the ectopic expression of *Shh* was associated to the expression of specific genes such as *Fgf4* and *Fgf8* in the anterior part of the AER, suggesting that Shh was at the origin of the polydactyly (Buscher, et al., 1997).

The limb polarization along the anterior-posterior axis depends on the interaction between *Gli3* and *dHand* before the establishment of *Shh* activity. *dHand* is a transcription factor, belonging to the bHLH class of proteins, and is expressed in the posterior part of the developing limb prior of *Shh* and in a domain including ZPA. Beside heart congenital defects, *dHand* null mice had undeveloped limbs and did not express *Shh*; conversely the ectopic *dHand* expression induced pre-axial polydactyly, consisting in mirror-image duplication of digits with posterior identity. This phenotype was attributed to the ectopic *Shh* expression, accompanied by transcription of *Hoxd11* in the anterior limb mesenchyme (Charité, et al., 2000). *dHand* function consists in limiting *Gli3* transcripts within the anterior part of limb bud, whereas that of *Gli3* is to restrict *dHand* expression in the limb posterior mesenchyme. At the same time *dHand* restricts the expression of *Gremlin*, a BMP antagonist that relays the *Shh* activity to the *Fgf* signaling in the AER to the posterior limb mesenchyme (Te Welscher, et al., 2002a).

Double mutants lacking both *Shh* and *Gli3* function were generated in order to establish the genetic interaction between *Gli3* and *Shh* in the determination of limb AP polarity (Litingtung, et al., 2002; Te Welscher, et al., 2002b). Differently from the absence of digits $Shh^{-/-}$ mice (Chiang, et al., 2001), both the zeugopod and autopod of $Shh^{-/-}$; $Gli3^{-/-}$ were well developed; in addition the kind of polydactyly was identical to that observed in $Gli3^{-/-}$ mice, suggesting that the gain of *Shh* (and *dHand*) expression in the anterior part of the limb was not the cause of extra-digits phenotype in these mice. Furthermore the removal of a genetic dose of *Gli3* in $Shh^{-/-}$;$Gli3^{+/-}$ mice restored the presence of digits, indicating that the morphogenesis of the autopod is controlled by both *Shh* and *Gli3*, the first of which influences the gradient of GLI3 repressor along the

anterior-posterior axis of the limb bud, and the latter that constrains the number of digits to five. The gain of expression of posterior genes like *Hoxd12*, *Hoxd13* and *Hoxa13* in the digit domain of mice lacking *Gli3* function indicated that the *wt* number of digits is indeed limited by the downregulation of *Hox* genes transcription exerted by the GLI3 repressive form (Litingtung, et al., 2002; Te Welscher, et al., 2002b).

Beside digit number, the limb anterior-posterior identity depends on the concentration of GLI3 activator modulated by the gradient of *Shh*, thus posterior digits form in correspondence to high *Shh* concentration, while anterior ones form in a limb domain where *Shh* concentration is lower (Lewis, et al., 2001). At the same time *Gli3* acts on *Hoxd* gene expression and in $Gli3^{-/-}$ mice, *Hoxd11*, *Hoxd12* and *Hoxd13* are ectopically activated (Zuniga and Zeller 1999), however digits are dysmorphic and don't have morphological identity. The mild limb phenotype characteristic of $Gli3^{+/-}$ mice, consisting in extra-digit I, was worsened by the ectopic expression of *Hoxd12* that led to the growth of an additional digit II, as was shown in transgenic mice for *Hoxd12*, hemizygotes for *Gli3* (Chen, et al., 2004). Unlike $Gli3^{-/-}$ mice, extra-digits mutants were clearly recognizable suggesting that in these mice the interaction between *Hoxd12* and *Gli3*, was affecting the amount of *GLI3* repressor and /or activator. Transfection experiments suggested that the interaction between *Hoxd12* and *GLI3* was converting GLI3 repressor in activator of target genes, leading to the elaboration of a quantitative model by which the ratio between GLI3 and HOX at late stage affects digits morphology by activating *Gli3* downstream genes in interdigital spaces.

6.3. Pitx1, Tbx and limb anterior-posterior identity

Pitx1 is expressed in the posterior lateral plate mesoderm in the hindlimb region but it is excluded from the area giving rise to forelimbs (Szeto, et al., 1996; Lanctôt, et al., 1997). Consistent with these mice null for *Pitx1* developed hindlimbs morphologically similar to forelimbs, although their molecular posterior identity was preserved (Lanctôt, et al., 1999; Szeto, et al., 1999).

Two other genes belonging to the T-box family, *Tbx4* and *Tbx5* are expressed in hindlimb and forelimb mesenchyme, respectively (Gibson-Brown, et al., 1996). In terms of time *Pitx1* is expressed prior of *Tbx4*. Whenever *Pitx1* is ectopically targeted to the chick wing bud mesoderm, it promotes the induction of *Tbx4*, partially transforming the identity of the wing to a leg (Logan, and Tabin., 1999; Szeto, et al., 1999). On the other hand, the application of FGF soaked beads on the flank of the chick embryo promotes the outgrowth of ectopic limb buds (Cohn, et al., 1995). *Pitx1* together with *Tbx4* expression was triggered in the mesoderm of the ectopic buds having leg identity (Logan, et al., 1998).

6.4. Other putative *Hox* targets

Despite the knowledge reached about the multiplicity of factors that participate in anterior-posterior and proximo-distal limb patterning, still their hierarchial position with respect *Hox* in limb bud is not clear. HOXA13 and HOXD13 interact with the *Eph7* promoter, activating the transcription of the gene (Salsi, et al., 2006). A more recent work showed that HOXD13 recognize and binds in vivo the genes, *dHand*, *Meis1* and *Meis2*, *Bmp2* and *Bmp4*; Salsi, et al., 2008). The injection of *Hoxd13* in chicken limb bud affected the transcription of these genes, showing that the physical interaction of HOX with their target sequences indeed has a functional effect on gene activity. *Bmp2* and *Bmp7* genes were reported to be downstream *Hoxa13* during limb development (Knosp, et al., 2004).

In order to identify *Hox* downstream genes, Cobb and Duboule in 2005 used a sophisticated approach, based on microarrays of RNA derived from embryonic limbs of specimens lacking the entire *HoxD* cluster. Indeed these mice develop a smaller autopod because of the reduced size of digits owing the absence of the posterior *Hoxd* genes. This screening allowed obtain candidate genes that were upregulated, or downregulated, following the *HoxD* cluster removal. With great surprise and in contrast with the former works none of the gene identified with this method was part of the main developmental pathways as BMP, WNT, SHH, FGF. On the other hand it is worth to remind that the impact of the full *HoxD* cluster deficiency on downstream target genes in these mutants (unlike in *Hoxd13*$^{-/-}$; *Hoxa13* $^{-/-}$ mice) is buffered by the function of *Hoxa* genes as shown by the presence of digits in the distal limb. This work is far to be conclusive, but provide an interesting starting point for further investigations.

7. Mesenchymal-epithelial interaction and gut patterning

A number of studies done in *Xenopus*, *Zebrafish*, chicken and mouse showed that the early endoderm is specified in anterior and posterior domains just after gastrulation, once that endodermal cells leave the primitive streak. In mouse the genes *Cerberus-like*, *Otx1*, *Hesx1* are expressed in the anterior endoderm; conversely the *intestinal fatty acid binding protein* (*IFBAP*) and *Cdx2* are posterior markers. The anterior endoderm gives rise to the ventral foregut, which is precursor of liver and lungs, whereas the progressively more posterior endoderm forms the esophagus, the stomach, pancreas and duodenum, small and large intestine (Wells and Melton, 2000). As mentioned above, along with the formation of somites and neural tube the gut develops like a tubular structure, which elongates in parallel to the growth of the embryo. At the beginning the GI anatomy is very simple, and consists in an inner epithelial layer of cuboidal cells overlaid by an outer layer derived from splanchnic mesoderm. Several works were aimed to decipher how the endoderm aquires positional identity. Two of the organs better studied in this context are the liver and the pancreas, representing an example of interaction between the embryonic germ layers and organogenesis. In both the cases the induction of the organ is orhestrated by signals emanating from the mesoderm. There is a difference between the permissive interactions and instructive interactions, in patterning the gut. In the first case the mesoderm is not able to reprogram the endoderm. This is the case of signals coming from the notochord that are unable to induce pancreatic fate in the endoderm unless the latter has been pre-patterned in order to induce pancreatic genes (Kim, et al., 1997). Similarly the cardiac mesoderm is not able to induce liver when associated with another endoderm area. In absence of cardiac mesoderm the pancreas forms in place of liver (reviewed in Grapin-Botton., 2005). Conversely the pancreas is induced by signals produced by the lateral plate mesoderm that induces the expression of *Pdx1* and other pancreatic genes like *insulin* and *glucagon* re-specifying the anterior endoderm toward a more posterior fate (Kumar, et al., 2003).

The nature of these signals has been partially revealed. At the end of the gastrulation FGF4 is expressed in the mesoderm adjacent the midgut and hindgut endoderm. FGF4 is necessary for the establishment and the maintenance the expression of genes like *Pdx1* and *CdxA* in chicken midgut and hindgut (Dessimoz, et al., 2006).

In mouse, the early development of intestinal epithelium is not exclusively determined by signals coming from mesenchyme but is directed by reciprocal interactions between the two components (Haffen, et al., 1987; Kedinger, et al., 1990; Stallmach, et al 1989). In other words each region of the intestinal epithelium acquires secretory or absorbent characteristics because of the balance between the instructive and permissive interaction occurring between endoderm and mesoderm. Several experiments perfomed on gastrointestinal tract of chicken embryos elucidated the nature of exchanges regulating epithelial morphogenesis. For example gastric epithelium formed intestinal villi when combined with intestinal mesenchyme.Furthermore, the origin of mesenchyme was crucial to drive gastric epithelial specification into cells of different regions of the stomach like gizzard or proventriculus (reviewed in Yasugi, 1993).

However at later stages the fetal intestinal endoderm follows an autonomous potential of differentiation that is not anymore determined by the mesenchyme (Duluc, et al., 1994). The changes in the enzymatic properties of the small intestine showed that

the development and differentiation of the GI is a dynamic process, which continues after birth, and is modified by the period of the weaning.

7.1. Fgf10 in GI development

Within the derivatives of the gastrointestinal tract, *Fgf10* is expressed in the foregut mesoderm in correspondence of the dorsal and ventral buds of the pancreas. *Fgf10* knockout mice still formed the pancreatic rudiments, but they did not grow further. In *Fgf10* $^{-/-}$ mice, progenitors of pancreatic epithelium went through the program of differentiation but their proliferation was strongly reduced, limiting epithelial branching and the development of the organ. The lack of FGF10 did not influence the early presence of *Pdx1* positive cells but interfered with the maintenance of the expression of this gene, as shown by the numeric reduction of the population of progenitor cells (Bhushan et al., 2001).

At early stage of development *Fgf10* is expressed in the mesenchyme surrounding the foregut, which contains gastric precursor cells (Bhushan, et al., 2001; Burns et al., 2004). *Fgf10* knockout mice had stomachs of small dimension (Spencer-Dene, et al., 2006) confirming the presupposition that *Fgf10* is involved in the development of the gastric part of the GI. Gross analysis of GIs dissected from these mice showed that the hindstomach, but not the forestomach, was underdeveloped; in addition the epithelium of both "antrum" and "corpus" region of hindstomach (the first of which is located nearby to the duodenum) did not show the usual glandular organization. The histological analysis of markers that are normally restricted to anterior or posterior regions of the stomach showed that *Shh* was expressed ectopically in the epithelium of the hindstomach whereas *Ihh* mRNA disappeared from the same area, suggesting that the specification of gastric epithelium was affected by the absence of *Fgf10* (Spencer-Dene, et al., 2006). An additional feature associated to the lack of *Fgf10* was duodenal atresia in about 40 % of the homozygous mice (Kanard, et al., 2005).

In the early midgut, at the conjunction between small and large intestine, *Fgf10* gene is specifically expressed within the mesenchyme overlying the apex of the caecum (Burns, et al., 2004). The mutual interaction between FGF10 with its receptor FGFR2B promotes the elongation of the caecum by stimulation of epithelial layer proliferation through the apical mesenchyme (Burns, et al 2004). The abrogation of *Fgf10* led to atresia of the caecum, which in homozygous mice was replaced by a small mesenchymal bud devoid of epithelial cells (Fairbanks et al., 2004-a; Burns et al 2004).

Within the early hindgut *Fgf10* is expressed in mesenchyme of distal colon where it sustains the survival and proliferation of epithelial cells populating the crypts but is not essential for their differentiation (Fairbanks, et al., 2005; Sala, et al., 2006). In a number of cases the absence of *Fgf10* was correlated to colonic atresia (Fairbanks, et al., 2005).

In the posterior hindgut *Fgf10* is expressed within mesenchymal cell associated to the epithelium of the distal rectum (Fairbanks, et al., 2004-b). While in normal mice the epithelium of the rectum and anus are in continuity, in *Fgf10* deficient mice there was no physical communication between rectal epithelium and surface of the body. The resulting phenotype resulted in genitalia abnormally positioned close to the tail owing the absence of anal opening (imperforate anus).

7.2. Fgfr2b and GI development

Within the gastrointestinal system *Fgfr2b* is expressed in the epithelium of the esophagus, stomach and intestine (Orr-Urtreger, et al., 1993; Sala, et al., 2006); in the latter the stimulation of FGFR2b was shown to induce the growth of the epithelial intestinal cells in the adult (Housley et al., 1994).

Concerning GI organogenesis, newborns mice in which the *Fgfr2b* isoform was specifically abrogated displayed an atretic caecum, owing the interruption of the pathway that links FGF10 to the proliferation of the epithelium (Burns et al., 2004). In addition to the atresia of the caecum, null mutants exhibited dysgenesis of the stomach as previously shown in *Fgf10* knockout mutants (De Moerlooze, et al., 2000; Spencer-Dene, et al., 2006). Moreover the lack of *Fgfr2b* function was traduced in colonic atresia (Fairbanks, et al. 2005) with a penetrance of 100%. This phenoptype was due to a dramatic decrease of the proliferation and conversely to the increase of the apoptosis in the intestinal epithelium in colon.

7.3. Shh and GI development

In chicken *Shh* is expressed in the GI epithelium throughout embryonic development. After stomach differentiation the *Shh* expression pattern is redefined being excluded by the epithelium of the proventriculus where forced expression of Shh was shown to suppress the gland formation (Fukuda and Yasugi, 2002; Narita et al., 1998).

At early stage of chick embryonic development the hindgut endoderm is positive for *Shh* transcription whereas the adjacent visceral mesoderm express the most 5'end *Hox* genes. The injection of a virus expressing *Shh* in the midgut endoderm was accompanied by the ectopic *Hoxd11* and *Hoxd13* expression in the mesenchyme; this observation provided a link between the inductive signal represented by *Shh* in the endoderm and the patterning of the visceral mesenchyme resulting by the *Hox* expression (Roberts, et al., 1995). The influence of the endoderm on GI mesodermal differentiation is not univocal. Indeed the forced *Hoxd13* expression in midgut mesoderm induced the differentiation of the overlying epithelium as it was belonging to the posterior hindgut (Roberts, et al., 1998).

In mouse GI *Shh* is expressed both during fetal and postnatal development, concomitant to the GI differentiation. Like in chicken, the early expression of *Shh* and *Ihh* spreads throughout all the GI epithelium whereas at late stages *Shh* becomes restricted to the forestomach (including the esophagus) and *Ihh* to the hindstomach. At E14.5 stage *Shh* mRNA is located preferentially in the foregut at the base of the duodenal crypts, whereas *Ihh* is maintained all along the length of the gut (Bitgood and McMahon, 1995).

The analysis of null mutants revealed that *Shh* plays a functional role in morphogenesis of both foregut and airways; where *Shh* transcription becomes visible at around E13.5 of development at the level of the tracheal epithelium. $Shh^{-/-}$ mice did not form the separation between esophagus and trachea, represented by the tracheo-esophageal septum (Litingtung, et al., 1998).

The function of *Shh* in foregut morphogenesis was evidenced by mice lacking *Gli2*, in which the lungs were hypoplastic, the esophagus had a small lumen and reduced muscular layer; the trachea was narrower and showed defects in the cartilaginous rings. In these mice the transduction of the signal through *Shh* is compromised (Motoyama et al., 1998-b), secondary to the reduction of *Ptc* and *Gli1* expression. The effect on the phenotype is much more dramatic in $Gli2^{-/-}$ and $Gli3^{+/-}$ compound mutants, where the trachea and the esophagus constitute a single tube. In $Gli2^{-/-}Gli3^{-/-}$ double mutants the lungs, trachea and esophagus don't form at all (Motoyama et al., 1998-b). *Gli3* has a partially redundant function with *Gli2* in foregut morphogenesis.

Additional observed visceral defects consisted in intestinal transformation of the stomach, stenosis of the duodenum and anus imperforatus (Ramalho-Santos and McMahon, 2000). Unlike *Ihh*, *Shh* expression in the stomach persists in adult mice (Van Den Brink, et al., 2001).

In contrast with the developing GI *Shh* expression is absent from the endoderm of both ventral and dorsal pancreatic buds. Ectopic expression of *Shh* in pancreas primordium driven by the *Pdx1* promoter changes the fate of the pancreatic mesoderm versus intestinal smooth muscle (Apelqvist, et al 1997).

7.4. Shh in stomach and intestine

The glandular stomach is organized in tubular units that are arranged along its cephalo-caudal axis to form two defined territories, respectively the corpus (zymogene glands producing pepsinogen) and the antrum (mucous glands). In the adult, both in human and mouse, the *Shh* mRNA is expressed in the fundic glands of the stomach and at the base of the crypts in the small intestine and colon (Van Den Brink et al., 2002). The inhibition of *Shh* signaling by treatment with cyclopamine enhanced gastric epithelial proliferation suggesting that one of the functions of *Shh* in the adult stomach is the control of epithelial homeostasis and glands renewal trough the downregulation of target genes like *Bmp4* that inhibit cell proliferation (Van Den Brink et al., 2001). *Shh* controls specifically the epithelial differentiation of fundic glands. In specimens with intestinal metaplasia of the stomach *Shh* expression was lost in the transformed epithelium; conversely in Barrett's esophagus where the squamous epithelium is replaced by an intestinal or gastric metaplasia the differentiation in gastric epithelium was accompanied by the gain of *Shh* expression (Van Den Brink et al., 2002).

As in absence of *Shh*, *Gli3* $^{Xt/Xt}$ mutant mice display overgrowth and intestinal transformation of the glandular stomach, suggesting that *Shh* acts trough *Gli3* (and not *Gli2*) in the control of the epithelial growth (Kim et al., 2005).

In the small intestine, both *Ihh* and *Shh* are expressed in the epithelium whereas the targets *Ptc* and *Gli1* are confined to the mesenchyme, the respective proteins in newborn mice are detectable adjacent to the epithelium of the crypts. The over-expression of the Hhip protein (Hedgehog interacting protein, that complexes with Hh antagonizing its function) blocked both *Ihh* and *Shh* signaling in the small intestine epithelium and altered the morphology of villi that appeared flattened owing to excessive epithelial proliferation (Madison et al., 2005). In these mice the mesenchymal expression of the target genes *Ptc* and *Gli1* was up-regulated showing that Hh acts in a paracrine way along the crypt-villus axis (Madison et al., 2005). Instead to be located next to the crypts the intestinal myofibroblasts were misplaced in the proximity of the proliferative epithelium of the tip of villi, forming ectopic crypts.

During gut elongation the GI diameter increases owing the superposition of mesenchymal concentric layers that, starting from the lumen and progressing outwardly, are the lamina propria, the muscularis mucosa, the submucosa, and the lamina muscularis. This last is composed by smooth muscle and by cells of the enteric plexus deriving from the neural crest. *Shh* mimics the inhibitory activity of the epithelium toward the morphogenesis of smooth muscle layer and enteric nervous system, suggesting that it plays a role in the establishment of the radial symmetry of the GI (Sukegawa et al., 2000).

Shh has a role in the morphogenesis of the distal part of the GI, indeed mice null for *Shh* or *Gli2* display ano-rectal anomalies, like the persistence of the cloacae and the presence of anus imperforatus. *Gli3*$^{-/-}$ mice exhibit a milder phenotype such as anal stenosis; however double null mutants for Gli2 and Gli3 showed a severe cloacal phenotype, similar to *Shh*$^{-/-}$ mice (Mo, et al., 2001).

General outline

These two works concern *HoxD* function in development of two different systems, represented by the limb and the gastrointestinal tract. From the point of view of evolution limbs are innovation of tetrapods that allow locomotion out of the sea, while specialization of the gastrointestinal system reflects the adaptation to a special dietary need in connection with the resources of the environment. Despite the obviously different anatomy and function of limb and caecum, we show that *Hox* dependent processes are crucial in early specification and morphogenesis of both. By using mutants mice carrying targeted deficiencies in the *HoxD* cluster we show that *Hoxd* de-regulation has a disruptive impact both on limb and caecum by controling similar developmental mechanisms.

First we show that the limb elongation along its proximo-distal axis depends on *Hoxd* genetic interaction with *Gli3*. In absence of the latter *Hoxd* ectopic expression has a deleterious effect on limb elongation that manifests as degeneration of the stylopodal structure and the absence of the zeugopod. We show that in *Xt/Xt* mice the severity of limb defect increases along with the dose of gained *Hox* genes in early limb bud. We conclude that in limb the gain-of-function of posterior *Hox* genes counteracts the activity of anterior ones (including *Hoxa*) in the specification of proximal segment of the limb. Simultaneously, *Del(1-10)* mice display a malformation of their GI that consists in the agenesis of the caecum. We show that *Hoxd11* and *Hoxd12* are ectopically gained in the tract at the ileo-colonic transition and that the agenesis of the caecum correlates with the increase in dose of posterior *Hoxd* gained in this area. Through isolation and analysis of an independent mutant line, *Del(4-11)* we corroborated this finding, and demonstrated that ectopic expression of *Hoxd12* alone was a sufficient condition of cecum aganesis. In limbs *Gli3* protects the stylopod and zeugopod from the effect of *Hox* posterior prevalence, whereas caecum morphogenesis seems indifferent to the absence of this factor. Thus both our studies show that *Hoxd* gene interactions obey the posterior prevalence rule, by which the function of 5' genes overcome 3' ones in determination of the fate of a body structure.

A second common point to these two works is the finding that while both limb and caecum budding relies on *Fgf10* signaling, the latter is downstream *Hoxd* activity in both cases. On the one hand, in absence of *Gli3*, *Hoxd* gain-of-function almost completely knocks down *Fgf10* expression in limb bud mesenchyme, interrupting the feed-back loop with *Fgf8* in the AER. The elongation of the limb is prevented because the AER degenerates. As the growth of the limb relies on interaction between the AER and mesenchyme, caecum morphogenesis depends on interactions between the mesenchyme and the epithelium. On the other hand, in GI the *Hoxd* gain-of-function blocks the growth of caecum by suppressing *Fgf10* induction in caecum bud mesenchyme. Consequently the epithelium at the ileo-colonic transition does not invade the mesenchyme resulting in agenesis of caecum bud. In view the high number of *Hox* genes transcribed in the cecum bud, the ectopic *Hoxd* expression likely counter-balances the activity of *Hoxa*, *Hoxb* and *Hoxc* genes in caecum growth. In large part the prevalent effect of the gained 5' *Hoxd* was rescued by providing 3' *Hoxd* gene products in our genetic complementation assay, strongly supporting the posterior prevalence interpretation.

In conlcusion with these two works we provide evidence that similar genetic mechanisms coordinate the early development of two organs that both form by a budding mechanism. Since *Hox* posterior prevalence has a negative impact both on limb and cecum budding, trough *Fgf10* downregulation, together these two studies provide

strong genetic evidence that beside *Shh*, *Fgf10* is an important target gene of *Hox* gene mediated developmental control.

Paper 1:

Interaction between *HoxD* and *Gli3* genes control the limb apical ectodermal ridge via *Fgf10*

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Interactions between *HOXD* and *Gli3* genes control the limb apical ectodermal ridge via *Fgf10*

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Abstract

The development of the vertebrate limb is dependent upon two signaling centers, the apical ectodermal ridge (AER), which provides the underlying mesenchyme with essential growth factors, and the zone of polarizing activity (ZPA), the source of the *Sonic hedgehog* (SHH) product. Recent work involving gain and loss of function of *Hox* genes has emphasized their impact both on AER maintenance and *Shh* transcriptional activation. Here, we describe antagonistic interactions between posterior *Hoxd* genes and *Gli3*, suggesting that the latter product protects the AER from the deleterious effect of the formers, and we present evidence that *Fgf10* is the mediator of HOX-dependent AER expansion. Furthermore, the striking similarity between some of the hereby observed *Hox/Gli3*-dependent morphogenetic defects and those displayed by fetuses with severely altered retinoic acid metabolism suggests a tight connection between these various pathways. The nature of these potential interactions is discussed in the context of proximal–distal growth and patterning.

Keywords: *Hox*; *Gli3*; *Fgf10*; Limb development; Posterior prevalence; Collinearity; Mouse

Introduction

Genetic studies in mice have shown that limb growth and patterning critically depends upon *Hox* genes belonging to paralogy groups 9 to 13 of both the *HoxA* and *HoxD* clusters. Despite structural homology and genomic neighborhood, individual representatives of the different groups have distinct roles in the formation of particular limb regions. For example, in the absence of both *Hoxa13* and *Hoxd13* function, autopods (hands and feet) mostly fail to develop (Fromental-Ramain et al., 1996b; Kondo et al., 1997). Similarly, severe truncations of the zeugopod (forearm, or lower arm) were seen when removing group 11 function (Davis et al., 1995) and, likewise, group 9 deficit mostly affected the stylopod (humerus) (Fromental-Ramain et al., 1996a; Wellik and Capecchi, 2003). These analyses uncovered anatomical defects generally corresponding in space and time to the expression domains of the genes concerned. In developing limbs, both the timing of expression and the position of the functional domains of the various *Hox* genes reflect their linear order along the chromosome (Kmita and Duboule, 2003). However, at the most proximal end of the stylopod *Hox* gene function seems to be somewhat dispensable (Kmita et al., 2005).

The importance and necessity for such a strict temporal-spatial distribution of gene expression domains along the proximo-distal limb axis has been illustrated by several approaches. Extensive rearrangements in the *HoxD* cluster induced limb anatomical defects due to the abnormal expression of *Hox* genes, rather than to their loss of function. When group 13 products were ectopically expressed in growing zeugopods, these segments were strongly affected, reminiscent of group 11 functional deficits. Related examples of forced expression of group 13 or 12 products in developing chick or mouse limbs resulted in similar patterning defects (Goff and Tabin, 1997; Williams et al., 2006). These observations gave support to the existence of functional interactions between *Hox* gene products, following the rule of

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posterior prevalence (Duboule, 1991; Duboule and Morata, 1994), whereby a 'posterior' or 'distal' gene product (e.g. HOXD13) can abrogate the function of a more 'anterior' or 'proximal' gene (e.g. group 11), likely at the post-transcriptional level (Herault et al., 1997; Spitz et al., 2003; van der Hoeven et al., 1996). During limb development, posterior prevalence has been documented at rather late stages, i.e. at times and in domains corresponding to distal pieces of the appendages, and the functional relevance of excluding distal *Hox* gene products from the early limb bud, such as to prevent distal structures to form at proximal locations, has not been assessed.

At the molecular level, posterior prevalence may result from interactions between HOX proteins either with various HOX partners, or with other gene products, leading for instance to the modulation of their functional activities and concurrent impact upon the regulation of target genes (Williams et al., 2006; Zappavigna et al., 1994). Among the few confirmed protein partners of HOX products (Capellini et al., 2006; Chen et al., 2004), the zinc finger domain transcription factor *Gli3* is of particular interest in this context. The *Gli3* gene product is critical for proper limb development, mainly through its antagonistic genetic interaction with *Shh*, as the stimulation of *Shh* signaling prevents the default processing of GLI3 from an activator to a repressor form (Litingtung et al., 2002; te Welscher et al., 2002; Wang et al., 2000), thereby up-regulating *Shh* target genes. In addition to this involvement in *Shh* signaling, *Gli3* and *Hoxd* genes were reported to interact during early limb development, in two different contexts. First, genetic evidence suggested that GLI3 acts as a negative regulator of several *Hoxd* genes, such as *Hoxd13* and *Hoxd12* during early limb budding (Buscher et al., 1997; Zuniga and Zeller, 1999). Secondly, GLI3 was shown to physically interact with the HOXD12 protein during distal limb patterning. In this latter case, the GLI3/HOXD12 interaction modified digit patterning, likely as a consequence of direct protein/protein contacts (Chen et al., 2004).

Mice carrying the *Extra-toes* (*Xt*) mutation lack the function of *Gli3*. These mice have a range of anomalies, among which a severe polydactyly of both fore-and hindlimbs, likely due to the de-repression of *Hox* genes, and concurrent ectopic expression of *Shh*, at the anterior margin of the developing limb (Buscher et al., 1997). In order to assess whether the wild-type pentadactyly was indeed due to a *Gli3*-dependent anterior repression of *Hox* genes, in other words whether the polydactyly observed in *Xt* mutant mice is dependent upon the gain of *Hox* gene function(s), we crossed *Xt* mice with mice carrying either a full, or a partial, deletion of the *HoxD* cluster (Zakany et al., 2001, 2004). Here, we show that removing all *Hoxd* gene function, in addition to *Gli3* in the developing autopod, does not significantly reduce the number of digits when compared to mice mutant for *Gli3* alone.

In striking contrast, however, the combination of the *Gli3* mutant allele with a partial deletion of the *HoxD* cluster (deletion of *Hoxd1* to *Hoxd10* included) gave mice with heavily truncated limbs, a situation drastically different from the phenotype observed with the same deletion, but in the presence of *Gli3* function. In this latter case, gain of function of the remaining 'posterior' *Hoxd* genes lead to an ectopic *Shh* domain anteriorly and consequent bilateral symmetry of an otherwise weakly truncated limb (Zakany et al., 2004). This observation indicates that widespread and early expression of *Hoxd13* and *Hoxd12* can severely impair stylopod development, but only when *Gli3* function is either reduced or removed, suggesting that *Gli3* function protects against the prevalent function of posterior genes over their more anterior neighbors. Such severe limb truncations involved defects in the apical ectodermal ridge (AER), likely due to a dramatic decrease of *Fgf10* expression in limb bud mesenchyme. We discuss the potential roles of these various players in the growth and patterning of the limbs.

Materials and methods

Mouse stocks, crosses and genotyping of mid-gestation embryos and near-term fetuses

The mouse lines carrying the *HoxD* cluster alleles used in this study were produced by *loxP/Cre*-mediated site-specific recombination. *del(1–13)* is an approximately 100-kb large deletion encompassing from the *Hoxd1* to the *Hoxd13* loci. In this deletion, the entire *HoxD* function is lost (Zakany et al., 2001). *Del(1–10)* was generated by targeted meiotic recombination (Herault et al., 1998) using *del(1–13)* as one of the parental alleles to produce an approximately 70-kb large targeted deletion from *Hoxd1* to *Hoxd10* included (Zakany et al., 2004). The two deficiencies have the same breakpoint near *Hoxd1*. All *HoxD* alleles were genotyped in a multiplex PCR reaction, using the 5′-CCACCCTGCTAAATAAACGCTG-3′ *Hoxd11* forward primer, and the 5′-GGTTGCCTCTTTTCCTCTGTCTC-3′ *Hoxd10* reverse primer for wild-type and the 5′-CTATTCAAAGGTGGGGAGCAGTC-3′ *Hoxd1* reverse primer for mutant allele. *Gli3 XtJax* allele was genotyped with the 5′-TACCCCAGCAGGAGACTCAGATTAG-3′ forward and 5′AAACCCGTGGCTCAGAGCAAG-3′ reverse primers, while the *Gli3* wild-type allele with the 5′-GGGTGAACAGCATCAAAATGGAG-3′ forward and 5′-ATAGCCATGTGGTGGTGCCCATG-3′ reverse primers.

Heterozygous males or females of either *HoxD* deficiencies were crossed over *Xt* heterozygous males or females to obtain compound heterozygous *Xt/+;del(1–13)/+* and *Xt/+;Del(1–10)/+* males and females. Both compound mutants were obtained in near Mendelian proportions, and most individuals of both genotypes displayed characteristic digit defects in forelimbs: oligodactyly in *Xt/+;del(1–13)/+*(Fig. 1E) and polydactyly in *Xt/+;Del(1–10)/+*(Fig. 1F). As *del(1–13)* homozygous animals are semi-lethal post-natally and both *Del(1–10)* and *Xt* homozygous animals are lethal at birth, we collected the F2 progeny from *Xt/+;del(1–13)/+* and *Xt/+;Del(1–10)/+* parents on the 18th day post-fertilization (E18) in order to minimize losses of individuals with compound genotypes. Genomic DNA was extracted from tail biopsies or yolk sac (E10, see below) and genotyped by PCR reactions, using the specific primers indicated above.

RNA in situ hybridization

To evaluate early limb development in the various genotypic classes, F2 embryos were collected on the morning of the 10th day of development (E10) and processed for whole mount RNA in situ hybridization following standard procedures (see e.g. www.eumorphia.org/EMPReSS/servlet/EMPReSS Doc. Number: 13_003). Yolk sac samples were collected individually and genomic DNA was isolated for genotyping, whereas individually fixed embryos were stored at minus 20 °C in methanol. Once genotypes were established, representatives of the selected genotypes were grouped and processed together for any given probe. Forelimb buds of all specimens were photographed and the same magnifications are shown. Probes were as originally described: *Fgf8* (Crossley and Martin, 1995), *Fgf10* (Bellusci et al., 1997), *Gli3* (Hui and Joyner, 1993), *Hoxd13* (Dolle et al., 1993), *Meis1* (Saleh et al., 2000) and *Shh* (Echelard et al., 1993). After the in situ hybridization patterns were documented, the embryos were homogenized, genomic DNA was extracted and the genotypes were further verified.

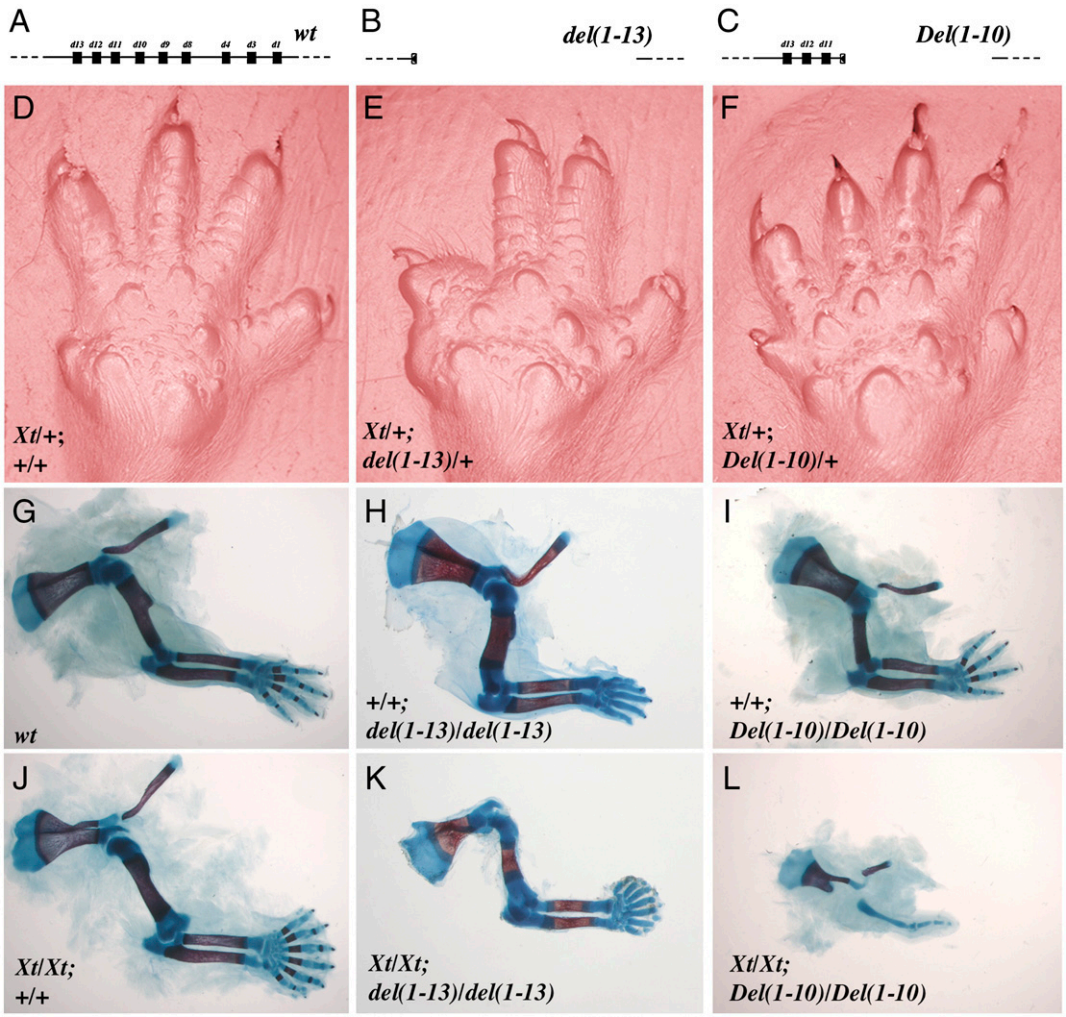


Fig. 1. Forelimb phenotypes of mice carrying either of two mutant alleles of the *HoxD* cluster *del(1–13)* or *Del(1–10)*, and the *Xt* allele at the *Gli3* locus. (A–C) On top line diagrams are shown depicting the respective *HoxD* cluster configurations: wild-type (A), fully deleted (B) and partially deleted (C), with only *Hoxd11*, *Hoxd12* and *Hoxd13* left. The genotypes of the depicted limbs are indicated inscribed in the panels under the cluster diagrams. Below, palm clay prints of heterozygous *Gli3* mutants are shown, carrying two wild-type haplotypes of the *HoxD* cluster (D, *Xt/+;+/+*), one wild-type haplotype and one copy of the *del(1–13)* allele (E, *Xt/+;del(1–13)/+*) or one wild-type haplotype and one copy of the *Del(1–10)* allele (F, *Xt/+;Del(1–10)/+*). The pattern shown in panel D is not significantly different from wild-type and represents also the vast majority of *Del(1–10)* heterozygous (*+/+;Del(1–10)/+*) specimen. Digit pattern of *del(1–13)* heterozygous (*+/+;del(1–13)/+*) differs only marginally from wild-type, with slightly reduced digit 2 and digit 5 lengths (not shown). (G–L) Full forelimb skeletal preparations of late prenatal fetuses (E18) of the F2 generation derived from crosses of either *Xt/+;del(1–13)/+*(H, K) or *Xt/+; Del(1–10)/+*(G, J and I, L) parents are shown below. Humerus reductions in either the normal (I) or the *Gli3* deficient background (L) are much stronger in the presence of the *Hoxd11*, *Hoxd12* and *Hoxd13* genes; compare *+/+;Del(1–10)/Del(1–10)* and *Xt/Xt;Del(1–10)/Del(1–10)* with wild-type. Note also that the massive polydactyly that characterizes the *Gli3*-deficient background (J) remained in the absence of all *Hoxd* genes (K), whereas one to three digits form only in the presence of two doses of the *Del(1–10)* allele (L).

Skeletal preparations

Fetuses were collected by cesarean section on E18, photographed, tail biopsied, eviscerated and skinned for the Alizarin red and Alcian blue standard skeletal staining procedure (see e.g. www.eumorphia.org/EMPReSS/servlet/EMPReSS Doc. Number: 12_005). Forelimb skeletons were dissected off, equilibrated into 80% glycerol, flat-mounted and photographed at identical magnification.

Results

Protective role of Gli3 against posterior prevalence

We crossed *Xt* mice (Figs. 1A, D) with mice either lacking the full *HoxD* cluster (Figs. 1B, E) or carrying a deletion from *Hoxd1* to *Hoxd10* (Figs. 1C, F) in order to assess whether the polydactyly induced by the null mutation of *Gli3* (*Xt/Xt;+/+*, Figs. 1G, J) is due to the de-repression, in time and space, of *Hox* gene function. Mice double homozygous for *Xt* and the *del(1–13)* allele (full deletion) were obtained and still displayed a severe polydactyly, as exemplified by forelimbs bearing seven to eight digital rods (Figs. 1H, K). This polydactyly was induced by the removal of *Gli3* function, as forelimbs of animals homozygous for the *del(1–13)* allele alone were mostly pentadactylous (Fig. 1H). In addition to the digit phenotype, forelimbs of double mutant animals were significantly and globally smaller than controls, the reduction including distal as well as proximal limb segments (Fig. 1K). This was unexpected as the absence of the entire *Hoxd* cluster (*del(1–13)/del(1–13)*)

produced only a minor reduction of the proximal limb (Fig. 1H). Furthermore, *Xt/Xt* mice also show such a reduction in the length of their hindlimbs (Barna et al., 2005; Chen et al., 2004).

A reduction in the length of the limbs was also scored in the second stock used in these crosses. We had previously reported that mice homozygous for the *Del(1–10)* allele displayed a double posterior digit pattern (Zakany et al., 2004), as a consequence of the establishment, at the anterior margin, of a second zone of *Shh* expression. This ectopic domain was triggered by the widespread expression of the remaining *Hoxd11*, *Hoxd12* and *Hoxd13* genes, likely under the transcriptional control of regulatory sequences located in 3′ of the cluster, which upon deletion would influence the expression of these genes (Tarchini and Duboule, 2006; Zakany et al., 2004). This gain of function phenotype showed some variability and a fair proportion of these limbs displayed both a reduced number of digits (oligodactyly) and a more or less severe limb shortening (Fig. 1I). Limb shortening was particularly evident in the stylopod (the humerus) and was substantially less pronounced in *del(1–13)* than in *Del(1–10)* mutant animals (Figs. 1H, I), supporting a gain of function effect as the causative factor. Altogether, these observations indicated that the abnormally early and proximal expression of *Hoxd11*, *Hoxd12* and *Hoxd13*, in *Del(1–10)* mice, impacted upon the growth potential of the limbs, in addition to the loss of function effect following the deletion of several *Hoxd* genes.

Surprisingly, animals carrying the partial deletion of the *HoxD* cluster *Del(1–10)*, associated with the absence of *Gli3* function, almost completely lacked their forelimbs (Fig. 1L). This drastic phenotype was not scored with full deletions of the *HoxD* cluster (Fig. 1K), which suggested that a gain of function of the remaining *Hoxd* genes was likely involved. In such mice, the stylopod was completely lost and a single and truncated cartilage model, in the worst case, was observed at the position of the zeugopod. The autopods were just as severely reduced, displaying a single digit in the continuation of the zeugopod cartilage (Fig. 1L).

Because *Xt* homozygous mice, with or without a *HoxD* cluster, do not display such defects, we concluded that neither *Gli3* nor the *HoxD* cluster are strictly necessary for proximal limb development, even though their combined absence generated somewhat smaller limbs, suggesting a genetic interaction between *Gli3* and *HoxD* genes. In those mice where the absence of *Hoxd1* to *Hoxd10* was associated with ectopic expression of *Hoxd11*, *Hoxd12* and *Hoxd13* in the early limb bud (Zakany et al., 2004), subsequent growth was dramatically dependent on the presence of the *Gli3* product. This genetic analysis thus identified GLI3 as a factor protecting the developing limb from the deleterious effect of an early gain of function of 'posterior' *Hoxd* genes.

To document this interpretation, we looked at the level of both *Gli3* and *Hoxd13* transcripts in E9 limb buds of *Del(1–10)/Del (1–10)* mice. Indeed a number of *Del(1–10)/+* animals display hindlimb polydactyly (Zakany et al., 2004) and the majority of *Xt/+;Del(1–10)/+* mice (Fig. 1C) show anterior polydactyly, reminiscent of fetal *Xt/Xt* forelimbs, suggesting that *Gli3*

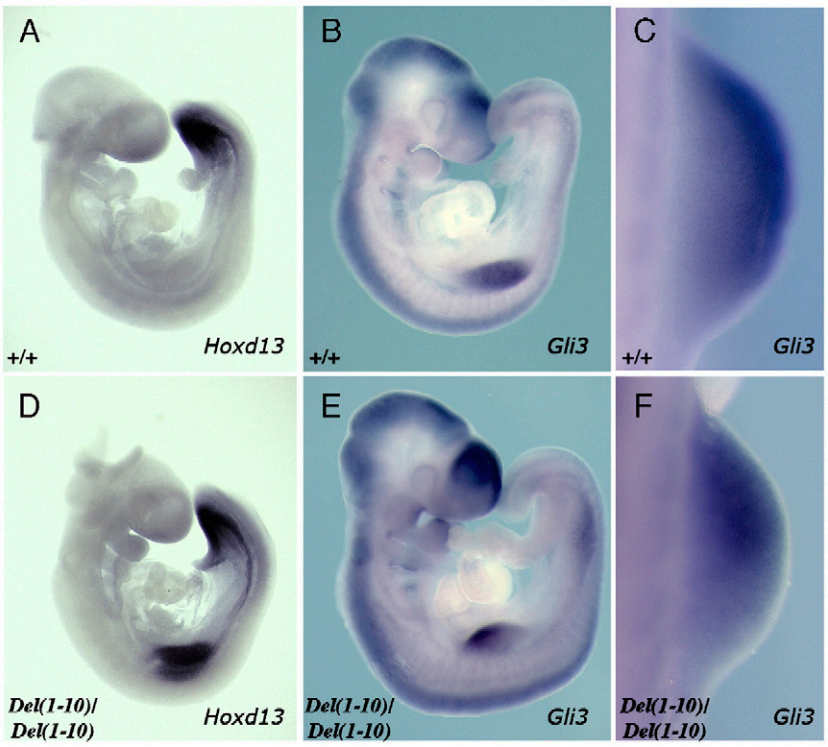


Fig. 2. Expression patterns of *Hoxd13* (A and D) and *Gli3* (B, C, E and F) in wild-type (A–C) and *Del(1–10)* homozygous (D–F) mid-gestation (E9) mouse embryos. All these specimen are wild-type at the *Gli3* locus. (A) *Hoxd13* expression is not yet detectable at this stage in wild-type, while the entire limb bud shows strong ectopic and premature *Hoxd13* transcript accumulation in *Del(1–10)* homozygous (D). Expression pattern of *Gli3* does not differ appreciably between wild-type and *Del(1–10)* homozygous (compare panels B to E and C to F).

expression may be modified in these stocks. The comparison between Figs. 2A and D demonstrates the ectopic and premature expression of *Hoxd13* in *Del(1–10)/Del(1–10)*, with a pattern including the entire incipient forelimb bud. At the same stage, *Gli3* transcript accumulation is comparable in these two genotypes (compare Figs. 2B, C and E, F), suggesting that the effect of ectopic posterior *Hoxd* gene expression is not mediated by a transcriptional suppression of *Gli3*. Furthermore, the co-expression of *Gli3* and posterior *Hoxd* genes in the limb bud mesenchyme make potential direct molecular interaction between these gene products possible.

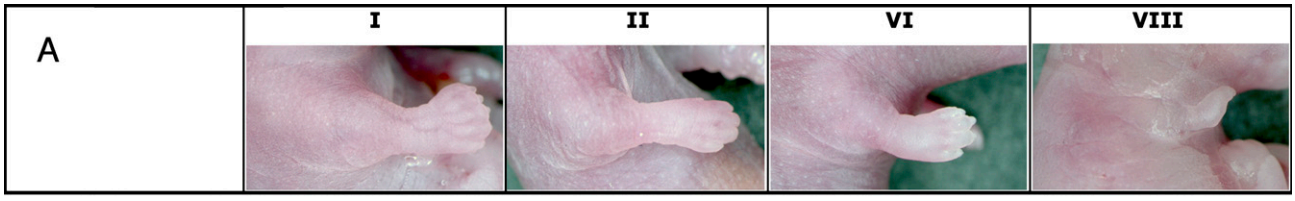
Quantitative interactions

The GLI3-dependent suppression of *Hoxd13* and *Hoxd12* gain of function clearly depended upon the doses of both *Gli3* and the remaining *Hoxd* genes, as shown by the phenotypic distribution detailed in Fig. 3. Forelimbs either lacking only a single dose of *Gli3* (*Xt/+;+/+*) , or having a weak *Hoxd* gain of function in the presence of a full complement of *Gli3* (*+/+;Del (1–10)/+*) generally displayed a wild-type phenotype (Fig. 3; group II). Forelimbs lacking two doses of *Gli3* were polydactylous, but their humeri were of normal size (Fig. 3; I). Reduction in the size of the stylopod started when trans-heterozygous animals were considered, i.e. those with only one dose of *Gli3* and one dose of gained *Hoxd11*, *Hoxd12* and *Hoxd13* (Fig. 3; III, IV). This latter reduction was close to that routinely seen in the homozygous *Del(1–10)* mutants, in the presence of two doses of *Gli3* (Fig. 3; IV).

Animals with three mutant alleles displayed slightly but significantly different phenotypic outcomes. In the presence of two doses of ectopic posterior *Hoxd* alleles combined with the loss of one dose of *Gli3*, the humerus was either severely reduced (7 out of 10; Fig. 3; V, VI) or virtually absent. The situation was similar when only one dose of ectopic posterior *Hoxd* alleles was combined with a complete absence of *Gli3*. This constitution also increased the severity of the phenotype (6 out of 10). Finally, animals lacking both doses of *Gli3* and harboring two doses of ectopic posterior *Hoxd* alleles had no trace of humerus, even though the remnants of both a zeugopod and an autopod were recognizable (Fig. 3; VI–VIII).

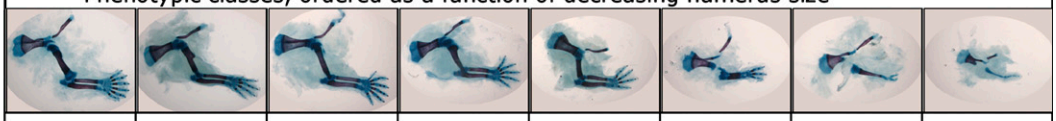
Effect upon the AER

The extent of skeletal truncations suggested that mutant limb development was compromised from a very early stage.



B

Phenotypic classes, ordered as a function of decreasing humerus size



	I	II	III	IV	V	VI	VII	VIII
Xt/Xt;+/+	10							
Xt/+;+/+		11	3[a]					
+/+;+/+		8						
+/+;Del(1-10)/+		7	1[b]					
Xt/+;Del(1-10)/+			12[b]	12				
+/+;Del(1-10)/Del(1-10)				19[c]	4[c]	1		
Xt/+;Del(1-10)/Del(1-10)					7	3		
Xt/Xt;Del(1-10)/+					4	6		
Xt/Xt;Del(1-10)/Del(1-10)						2	3	1

Fig. 3. Variety of forelimb defects in *Xt, Del(1–10)* and compound mutant near-term mouse fetuses. (A) Gross morphology of *Xt* homozygous, wt, *Del(1–10)* homozygous and double homozygous forelimbs. *Xt* homozygous (*Xt/Xt;+/+*) forelimbs were easily recognized by the typical six or seven digit polydactyly. By contrast, the rest of the limb appeared relatively normal. A proportion of *Del(1–10)* homozygous (*+/+;Del(1–10)/Del(1–10)* showed very short four- or three-digit oligodactylous limbs. All compound homozygous *Xt/Xt;Del(1–10)/Del(1–10)* displayed short oligodactylous limbs, including an extreme case of monodactyly. Roman numerals on the top (I, II, VI, VIII) indicate the phenotypic class allocations of the pictured newborns, as explained under (B). (B) Genotype distribution of skeletal patterns listed as eight phenotypic classes recovered in this stock. A representative picture is shown (I to VIII), and the classes are ordered from left to right according to decreasing limb proximal to distal length, reflecting the severity of the defect. In class I, both the humerus and overall limb size are normal, but with a polydactylous autopod. In classes II to V, a progressive reduction in the lengths of the humeri can be seen. In classes V to VIII, the humerus is completely absent. In class VI, the middle bony elements are identified as radius and ulna, although they are more tightly apposed to one-another. In classes VII and VIII, a single skeletal element is found between the strongly reduced scapula and digit(s). This element can hardly be identified with anything present in normal limbs. In class VIII, no ossification is apparent distal to the scapula. When the genotype of the *Gli3* locus is kept identical, increasing the dose of the *Del(1–10)* allele increased the severity of the phenotype. Likewise, when keeping the mutant genotype at the *HoxD* locus identical (either *Del(1–10)/+*, or *Del(1–10)/Del(1–10)*), increasing the dose of wild-type *Gli3* reduced the severity of the alterations.

Accordingly, we collected F2 embryos at E10 from *Xt/+;Del(1–10)/+* compound heterozygous parents. Genotyping and anatomical examination revealed that all possible genotypes were represented in near Mendelian proportions and that all mutant embryos showed the clear presence of limb buds. Therefore, initial limb budding occurs in compound mutants, indicating that size reductions mostly developed later, during and after E10, a stage corresponding to the establishment of the apical ectodermal ridge (AER).

Several genetic activities are necessary for the formation, maintenance and function of the AER, such as the mesenchymal factors *Fgf10* and *Shh*, or ectodermal factors like *Fgf8*. These genes are normally expressed at the time when ectopic *Hoxd11*, *Hoxd12* and *Hoxd13* expression is scored in the *Del(1–10)* allele, which also induces an ectopic *Shh* domain. We examined the expression of *Hoxd13*, *Fgf10*, *Shh* and *Fgf8* in the various genotypic classes described in Fig. 3. Representative forelimb buds of four of these genotypic classes are documented in Fig. 4. Examples of the corresponding skeletal patterns at E18 are included for direct comparison.

In wild-type limb buds, *Hoxd13* and *Hoxd12* expression is first restricted to the most posterior part of the budding limb (early phase in Tarchini and Duboule, 2006). Soon after, expression of these genes *de novo* appears in the future digit domain, at the postero-distal part of the outgrowing bud, under a different transcriptional control (Tarchini and Duboule, 2006). Consequently, E10 limb buds show both this emergent domain (Fig. 4A; arrow), as well as more proximal weakly expressing cells, remnant of the early posterior domain seen at E9 (Fig. 4A; arrowhead). An important effect of this early and posterior expression domain is to trigger *Shh* expression, which will thus be confined to posterior cells (Fig. 4C; (Tarchini et al., 2006). At

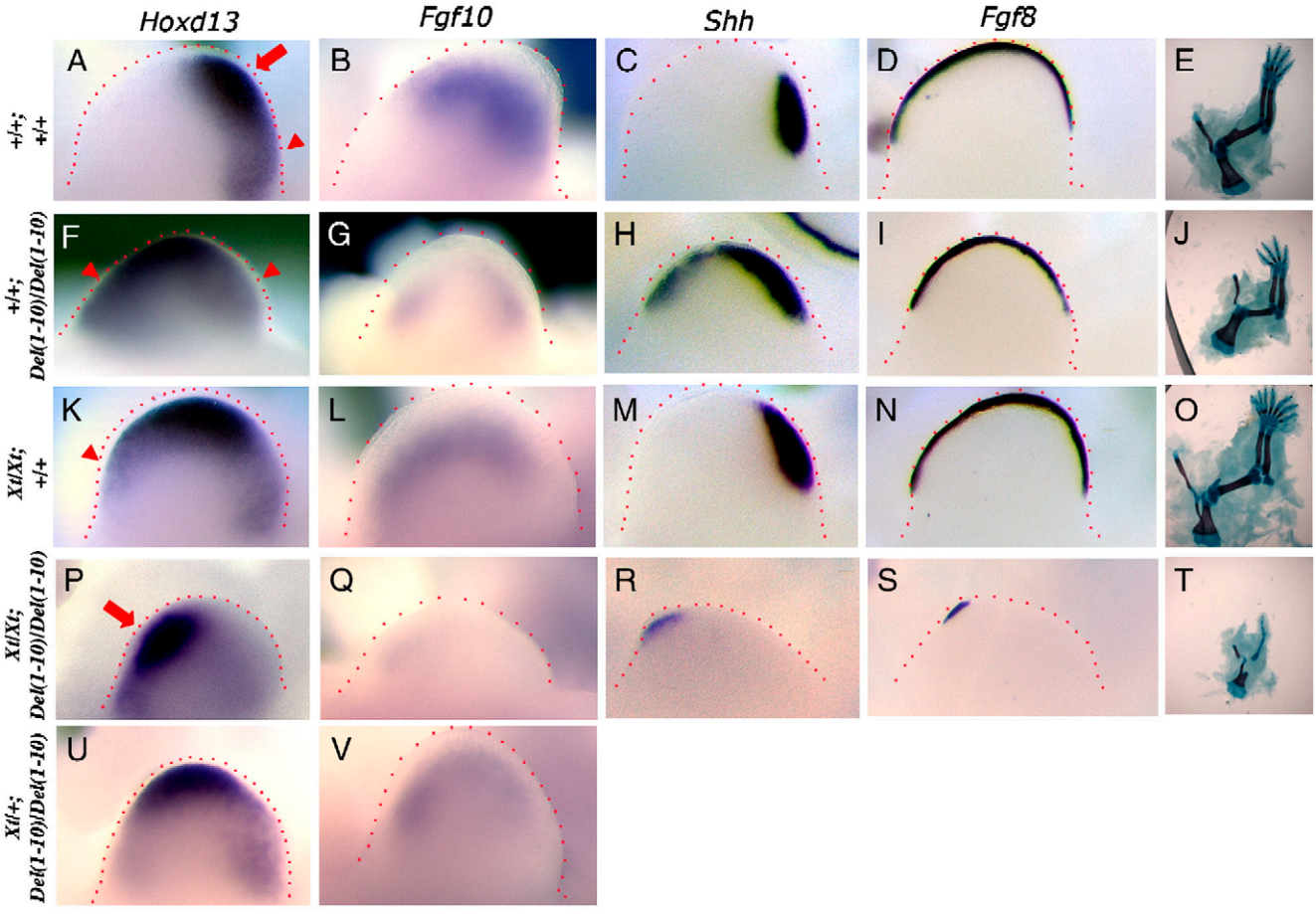


Fig. 4. Expression analyses of *Xt, Del(1–10)* and compound mutant forelimb buds. Whole mount *in situ* hybridization using *Hoxd13*, *Fgf10*, *Shh* and *Fgf8* probes were carried out on wild-type, *+/+;Del(1–10)/Del(1–10)*, *Xt/Xt;+/+*, *Xt/Xt;Del(1–10)/Del(1–10)* and *Xt/+;Del(1–10)/Del(1–10)* E10 embryos. Genotypes are indicated on the left, probes on the top. Panels in the right are representative skeletal E18 preparations to indicate the fate of those buds shown on the left panels. In *+/+; Del(1–10)/Del(1–10)* embryos, the expression patterns of *Hoxd13* (compare panels A and F), *Fgf10* (compare panels B and G) and *Shh* (compare panels C and H) were extended into more anterior regions, whereas *Fgf10* level was reduced and *Fgf8* seemed relatively normal (compare panels D and I). In *Xt/Xt;+/+Hoxd13* (K), *Fgf10* (L) and *Shh* (M), transcript profiles were extended into more anterior regions as well, whereas *Fgf10* level was somewhat reduced (compare panels B and L) and *Fgf8* seemed relatively normal, if not slightly increased (N). In *Xt/Xt;Del(1–10)/Del(1–10)* embryos, expressions of *Hoxd13 (P)*, *Fgf10* (Q) and *Shh* (R) were scored only in anterior regions. Interestingly both *Hoxd13*, *Fgf10* and *Shh* transcripts were hardly detectable in their normal domains and *Fgf8* was confined to a very tiny cluster of cells overlying the *Hoxd13*, *Fgf10* and *Shh*-positive domain (compare panels Q, R and S). Overall, limb buds from *+/+;Del(1–10)/Del(1–10)* and *Xt/Xt;Del(1–10)/Del(1–10)* genotypes appeared smaller than wild-type, which was consistent with the subsequently observed reduction in the size of these limbs. As compared to *Xt/Xt; Del(1–10)/Del(1–10)* (P and Q) in *Xt/+;Del(1–10)/Del(1–10)* embryos the limb bud size (U and V) and *Fgf10* signal intensity (V) were significantly rescued by the presence of one wild-type copy of *Gli3* in presence of a well detectable ectopic *Hoxd13* signal throughout the anterior limb bud (U).

E10, *Fgf10* is expressed with a medial-posterior specificity, in a rather large domain. Positive cells nevertheless are excluded from the most distal parts of the bud (where *Hoxd13* appears stronger), as well as from most anterior cells (Fig. 4B). Throughout limb budding and early outgrowth, *Fgf8* expression extends along the anterior to posterior rim of the limb bud (Fig. 4D), indicating the presence of a well-established AER (Lewandoski et al., 2000).

Mutant limb buds showed clear deviations from these expression patterns. In *Del(1–10)* homozygous, general accumulation of *Hoxd13* transcripts in E9 buds (Zakany et al., 2004) leads to a visible anterior ectopic domain, mirroring wild-type posterior expressing cells (Fig. 4F; arrowheads). The late domain appeared in a more distal position, pre-figuring the bilateral symmetry of the limb. Following the de-localization of *Hoxd13* and *Hoxd12* expression domains, *Shh* appeared along the entire distal rim of the bud (Fig. 4H), to be subsequently split into two opposing domains, leading to the double-posterior morphology (Zakany et al., 2004). Expression of *Fgf10* in these mutant buds was clearly down-regulated, and scored both anteriorly and posteriorly, whereas mostly non-detected in the distal part where the late *Hoxd13* domain was visible (Fig. 4, compare F and G). Here again, expression matched the bilateral symmetry subsequently observed. Consistently, *Fgf8* expression did not substantially change, albeit the overall length of the AER was slightly reduced, consistent with the general reduction in the size of the limb bud (Fig. 4I).

Interestingly, *Xt/Xt* homozygous limb buds showed modifications of these expression patterns not drastically different from those observed in *Del(1–10)* deleted animals. *Hoxd13* early expression extended into the most anterior part of the bud (Zuniga and Zeller, 1999), and a remnant of this pattern was still detected at E10 (Fig. 4K, arrowhead), along with a rather distal domain for the late *Hoxd13* pattern. *Shh* expression was also somewhat extended along the anterior-distal margin (Fig. 4M), although much less extensively than *Hoxd13*, especially as anterior proximal, and also most part of the distal limb domains remained devoid of *Shh* transcripts until later, when an ectopic *Shh* domain is generally scored anteriorly (Buscher et al., 1997). *Fgf10* was slightly down-regulated but again extended into anterior distal and proximal domains (Fig. 4L). *Fgf8* expression was essentially identical to wild-type, indicating the presence of a near normal AER, corresponding to the seemingly normal aspect of these early limb buds, despite their subsequent polydactyly (Fig. 4N).

Xt/Xt;Del(1–10)/Del(1–10) compound animals displayed drastically different expression patterns. *Hoxd13* transcripts accumulated in the anterior limb bud, suggesting a complete shift in the anterior-posterior polarity of the bud. While this was observed for the late *Hoxd13* domain, traces of the early expression suggested a similar inversion of polarity (Fig. 4P; arrow). Accordingly, *Shh*-positive cells were found at an anterior-distal position, corresponding to cells expressing *Hoxd13* Fig. 4R). However, signal intensity was just above detection and only few cells were scored positive. *Shh* expression was not detected in its usual posterior domain. *Fgf10* expression was also severely reduced in quantity, and mostly found in anterior mesenchymal cells, illustrating once again an inversion in the AP polarity (Fig. 4Q; see below). Finally, the pool of *Fgf8*-positive cells was also dramatically reduced. Only a small cluster of positive cells was detected in the anterior limb bud, precisely above the ectopic domains for all three *Hoxd13*, *Fgf10* and *Shh* (Fig. 4S). This virtually non-existing AER coincided with an important reduction in the size of the entire limb bud when compared to all other genotypes.

In *Xt/+;Del(1–10)/Del(1–10)* compound mutants, ectopic expression of *Hoxd13* in anterior regions was clearly detected as well, and a shift of the late distal domain towards the anterior margin was also evident, giving an overall pattern that was intermediate between *Xt/Xt;Del(1–10)/Del(1–10)* and *+/+;Del (1–10)/Del(1–10)*. Accordingly, the intensity of the *Fgf10* signal increased, and the size of the limb bud was also consistently bigger than that of the double homozygous. From this data set, we concluded that expressions of both *Fgf10* in the mesenchyme and *Fgf8* in the newly forming AER were severely altered in the presence of prematurely expressed *Hoxd13* and *Hoxd12*, provided the quantity of GLI3 was either reduced, or completely absent. Because both *Gli3* and *Hoxd* genes are expressed in mesenchyme, we favored an hypothesis whereby these latter gene products would act upon *Fgf10* transcript accumulation. In double mutants, *Fgf10* was massively affected, which prevented formation of a full-grown AER leading to the observed truncations.

Fgf10 in early mutant limb buds

We looked at the expression of *Fgf10* in earlier mutant limb buds, i.e. at a stage where the AER was being established, hence gene expression was unlikely to depend upon AER derived signals (Fig. 5). Two major aspects were immediately scored: firstly, the presence of two copies of the *Del(1–10)* mutant allele drastically reduced the quantity of *Fgf10* transcripts, regardless of the presence or absence of *Gli3* function (Figs. 5B, C). Secondly, the absence of *Gli3* function (*Xt/Xt*) induced a spectacular inversion of AP polarity, independently of the presence or absence of the *Del(1–10)* allele (Figs. 5C, D; see also Fig. 4). Concerning the former aspect, it is likely that the down-regulation of *Fgf10* depended upon the presence of gained posterior *Hox* genes, as two copies of the *Del(1–10)* allele were required to achieve substantial extinction of *Fgf10*, in the absence of *Gli3* function.

The inversion of *Fgf10* polarized expression was mostly dependent upon the absence of *Gli3* function, as it started to occur even with a normal set of *Hox* genes (Fig. 4). In this case, a ‘rotation’ of the *Fgf10* pattern was scored, along with the distalization of the *Shh* expressing domain (Fig. 4L, compare Fig. 4M). While *Del(1–10)* homozygous limb buds had a bilateral expression of *Fgf10*, following that of posterior *Hoxd* genes, further removing *Gli3* function gave the limb a clear, yet not sustainable, inverted polarity (Figs. 4 and 5). Up-regulation of *Fgf10* in the anterior bud may result from the observed gain of posterior *Hoxd11* gene expression there upon loss of *Gli3* function (Zuniga and Zeller, 1999). Likewise, *Del(1–10)* mutant limb buds may induce *Fgf10* expression anteriorly. The

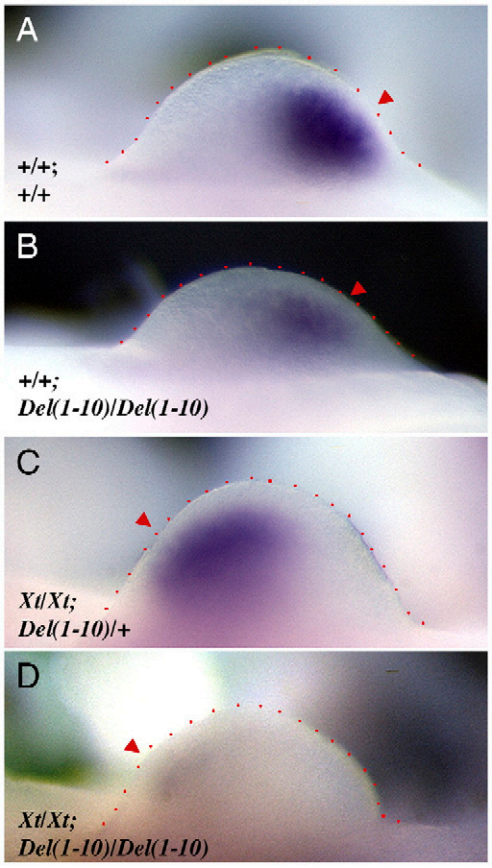


Fig. 5. Suppression of *Fgf10* transcript accumulation in pre-AER stage mutant limb buds and inversion of the anterior to posterior polarity. In normal limb buds (A), *Fgf10* accumulation occurred preferentially in the posterior part (indicated by red arrowhead on the right). (B) In homozygous *Del(1–10)* animals, *Fgf10* transcript accumulation was significantly reduced, yet still detected in the expected posterior domain. (C, D) When the two mutations were combined, an inversion of polarity in the distribution of *Fgf10* was observed (indicated by red arrowhead on the left). In compound homozygous very low level of *Fgf10* signal could be detected (D). When the *HoxD* gain of function was reduced by half, in *Xt/Xt;Del(1–10)/+* mutant (C), transcript accumulation in this anterior domain increased substantially. Therefore, both in the presence and in the absence of *Gli3*, premature posterior *Hoxd* gene expression suppressed *Fgf10* gene transcript level, though this suppression was increased by removing *Gli3* function, leading to more severe truncations (see Figs. 1 and 3).

disappearance of *Fgf10* expression from the posterior margin of the limb in *Del(1–10)/Xt* compound mutants may also reflect the down-regulation of posterior *Hoxd* genes in these cells, yet how the *Xt* mutation stimulates this remains elusive.

Discussion

Genetic analyzes have highlighted the role of the *HoxA* and *HoxD* clusters in tetrapod limb development (Davis et al., 1995; Kmita et al., 2005). Recently, their capacity to regulate the amount and position of *Shh* transcripts, hence to control both proximal to distal growth and the anterior to posterior polarity, was proposed (Tarchini et al., 2006). In this view, the late and posteriorly restricted expression of groups 10 to 13 *Hox* genes is mandatory for further development of the *Shh*-dependent, most distal part of the limb. However, combined *HoxA/HoxD* clusters deficient limbs showed more extensive truncations than those reported for *Shh* mutant mice (Chiang et al., 2001; Kmita et al., 2005), suggesting that *Hox* gene products are required early on, independent of their effect upon *Shh* transcription, likely to control the formation or maintenance of the AER. In this work, we provide evidence that the integrity of the AER depends on the interplay between posterior *Hoxd* genes and *Gli3*, probably mediated through the control of *Fgf10* expression in early limb bud mesenchyme.

Antagonistic role of 5′ HOXD genes and GLI3 in controlling FGF10

When *Gli3* was either half, or fully abrogated, in the presence of prematurely expressed *Hoxd13* and *Hoxd12*, extreme limb truncations occurred. Interestingly, the most affected limbs, in this phenotypic series resembled those observed either after surgical removal of the AER in chick limb buds (Saunders, 1948) or after inactivation of *Fgf* signaling in mice. Genetic analyses of limb development in mice identified *Fgf10* as a major early mesenchymal competence factor (Sekine et al., 1999) and further studies on *Fgf* receptors have associated this early step with the establishment of the AER (Li et al., 2005; Revest et al., 2001; Xu et al., 1998), which will be subsequently the source of *Fgf4* and *Fgf8*. Inactivation of *Fgf8* impacted upon the formation of the stylopod (Lewandoski et al., 2000) and additional inactivation of *Fgf4* prevented the development of all three limb segments. Interestingly, in *Fgf8;Fgf4* compound mutants, *Fgf10* expression was severely reduced, whereas *Shh* transcription was abrogated (Boulet et al., 2004; Sun et al., 2002), pointing to feedback mechanisms in this complex process.

In our experiments, expression of *Fgf10* in the mesenchyme and of *Fgf8* in the forming AER were dramatically reduced, indicating a defect in the *Fgf10* to *Fgf8* arm of the positive circuit maintaining AER function. This important decrease in the amount of *Fgf10* transcripts resulted from prematurely expressed *Hoxd13* and *Hoxd12* genes. Yet this effect was not observed, or at least not to this degree, in the presence of the *Gli3* gene product. Therefore, *Gli3* products were able to mitigate, or protect from, the effects of *Hoxd* gain of function upon *Fgf10* activity, in a dose-dependent manner. In *Del1–10* homozygous mice, initial *Fgf10* expression was indeed readily detectable, whereas only trace amounts of *Fgf10* could still be seen after additional removal of *Gli3* function. In such double mutants, patches of AER were occasionally scored with only dispersed *Fgf8*-positive cells, suggesting that, receiving weak *Fgf10* signal, epidermal cells started responding by activating *Fgf8* transcription, but the pool of responding cells was likely too small and failed to assemble a ridge. In the development of the final phenotype, these most severe constitutions are tantamount to a genetic AER ablation. From previous experiments, in particular those involving simultaneous inactivation of *Fgf8* and *Fgf4,* it is expected that massive apoptosis is involved in bringing about the eventual truncations (Sun et al., 2002). Besides fibroblast growth factors, other signals are involved in AER establishment (Capellini et al., 2006; Hill et al.,

2006; Mariani and Martin, 2003) and we cannot rule out the possibility that other signaling cascades be directly affected by this HOX/GLI3 circuitry.

'Anterior' Hox genes promote AER formation

The deletion of both *HoxA* and *HoxD* clusters lead to severely compromised AER integrity and consequent arrest in the growth of limb buds (Kmita et al., 2005). Here, we show that a similar defect in the AER, associated with severe truncations, can be obtained even in the presence of the complete set of *Hoxa* genes, provided 'posterior' *Hoxd* genes are expressed ectopically and that *Gli3* function be removed. Collectively, these observations can be interpreted in the context of the 'posterior prevalence' rule (Duboule and Morata, 1994). In this view, 'anterior' *Hox* genes are required for proper AER establishment and/or function, whereas 'posterior' genes (e.g. groups 13 to 12) restrict AER longevity; the integration of differential *Hox* inputs being achieved by the regulation of *Fgf10* production. Accordingly, the correct balance, in time and space, between 'anterior' and 'posterior' *Hox* products may thus be central to proper proximal to distal outgrowth, by determining both the onset and the regression of the AER. The fact that transgene-driven expression of either *Hoxb8* (Charite et al., 1994) or *Hoxa9* (Williams et al., 2006) during early mouse limb development induced polydactyly and enlarged limb buds is compatible with an increase of AER output as a consequence of exaggerated 'anterior' *Hox* gene function.

In *Del(1–10)* homozygous animals, the gain of function of *Hoxd12* and *Hoxd13* in the anterior part of the early limb bud did not drastically impair AER formation. Instead, an ectopic *Shh* domain was observed anteriorly, leading to the formation of a bilateral symmetric limb, slightly reduced in length. When further removing the *Gli3* function, a drastic effect was seen on AER formation, which prevented the assessment of the effect upon *Shh* transcription, even though a weak domain was sometimes observed at a distal and anterior position. Therefore, we conclude that GLI3 does not interfere with the capacity of *Hoxd13*, *Hoxd12* or *Hoxd11* to trigger *Shh* expression. In contrast, *Gli3* products appear to protect anterior *Hoxd* gene products against the prevalent effect of posterior products and concurrent impairment of AER formation. While the underlying molecular mechanism is elusive, the fact that GLI3 and posterior HOX products directly bind to each other (Chen et al., 2004) suggest that GLI3 may sequester HOX products, thus preventing their deleterious effects. Whether or not this protective effect of GLI3 occurs in wild-type physiological conditions is more difficult to assess. The facility of obtaining proximal limb truncation upon ectopic expression of *Hoxd13* in transgenic mice, even in presence of a full complement of Gli3 (Williams et al., 2006) may reflect excessive amounts of ectopic products. Also, posterior HOX products must be expressed early enough to elicit *Shh* induction (from groups 10 to 13; Tarchini et al., 2006), at a time when the AER is being fully established. It is thus conceivable that GLI3 products would prevent this latter structure to be aborted, in the case where these prevalent HOX proteins would be present.

Inversion of the AP polarity

In limb buds with both posterior *Hoxd* gene gain of function, and loss of *Gli3* function, a striking inversion of polarity was observed before the growth of the limb aborted due to the absence of AER. This inversion of polarity involves two separate changes: the gain of expression of several genes in the anterior part of the bud, and the concurrent loss of expression of the same genes in the posterior part. We believe this inversion follows posterior *Hoxd* gene ectopic expression, which is itself due the combined effect of the *Del(1–10)* gain of function (Zakany et al., 2004) and the absence of *Gli3*-mediated *Hox* repression in the anterior bud (Zuniga and Zeller, 1999). In the *Del(1–10)* deletion, *Hoxd11*, *Hoxd12* and *Hoxd13* are placed near the telomeric end of the cluster, where they fall under the control of regulatory elements that normally act on 'anterior' *Hox* genes. Therefore, these remaining three genes acquired a more anterior type of expression pattern in the early bud (Zakany et al., 2004), corresponding to the anterior specificity of those genes located in 3′ of the cluster (Tarchini et al., 2006). This anteriorization was nevertheless not complete, likely due to the property of *Gli3* to antagonize posterior *Hoxd* gene in the anterior limb bud, leading to the symmetrical distribution of e.g. *Hoxd13*.

However, whenever the dose of *Gli3* was reduced, a full anteriorization of the patterns was observed, as a result of the reduction in (or absence of) *Gli3*-mediated repression. The full anterior pattern of posterior *Hoxd* genes, as illustrated by *Hoxd13* (Fig. 4) was expectedly able to elicit *Shh* transcription in an anterior spot (Tarchini et al., 2006), underlying the few *Fgf8*-positive AER cells. A fully inverted limb was nonetheless impossible to produce since the protective effect of *Gli3* against posterior prevalence had been removed, leading to the concurrent growth arrest (see above).

Hox genes; a link between RA and Fgf signaling in limb buds?

The drastic defects observed in *Del(1–10);Xt* compound mutants limbs are remarkably similar to those described in animals with altered levels of retinoic acid, suggesting that related developmental pathways were affected in both cases. In the absence of endogenous RA synthesis, i.e. in animals lacking the function of *Raldh2*, the modifications in the expression of *Fgf10*, *Shh* and *Fgf8* were related to those reported in this paper. In particular, *Shh* was lost in the normal posterior limb bud of *Raldh2* mutants, whereas appearing delocalized in the distal region (Mic et al., 2004; Niederreither et al., 2002). Furthermore, as a consequence of RA depletion, posterior *Hoxd* genes became prematurely and ectopically expressed in early limb buds (Niederreither et al., 2002). These analogies suggest that an important role for RA signaling is to prevent posterior *Hox* genes to be transcribed in the early bud. In the absence of RA, *Hoxd12* and *Hoxd13* transcription was activated

prematurely and altered *Fgf10* gene expression, thus precluding bud growth.

On the other hand, in embryos mutated for the *Cyp261b* gene, i.e. with an increased level of RA, *Hoxd12* and *Hoxd13* transcription in limb buds was delayed (Yashiro et al., 2004) leading to severe defects in the stylopod, zeugopod and autopod. Interestingly, the eventual phenotypes of *Raldh2* and *Cyp261b* mutant limbs are quite alike, involving massive alterations of all limb regions, including the humerus. These phenotypes nevertheless arise through distinct mechanisms, as witnessed by the expression pattern of *Fgf8* in the AER: while reduced in *Raldh2* mutant, it was increased in *Cyp261b* mutants, in good correlation with either increased, or decreased *Hoxd12* and *Hoxd13* expression, respectively. The most severe genetic constitutions we report in this work are reminiscent to early vitamin A deficiencies. We tested the expression of *Meis1*, a gene that is under the control of RA signaling (Fig. S1) and observed normal expression patterns of this gene in our five key mutant combinations. We take this as an evidence that RA signal was initially received from the flank and that *Fgf*-dependent suppression of *Meis1* transcript accumulation in proximal bud was effective (Mercader et al., 2000).

The function of RA in the activation of *Hox* gene transcription has been abundantly documented, both on particular *Hox* genes, via their RAREs (Serpente et al., 2005) and at the level of entire clusters. In this latter case, RA was able to trigger collinear *Hox* genes activation in cultured EC cells (Simeone et al., 1990). It is thus possible that RA plays a role in the sequential activation of *Hox* genes during limb bud development by favoring expression of anterior *Hox* genes first, while delaying expression of the posterior members. This would allow for the AER to be established and functional, via *Fgf10* regulation, before the massive expression of posterior genes in the autopods would abrogate it. In this task of maintaining posterior *Hox* genes silent or harmless, RA would be helped by *Gli3*, first by its repressive effect on posterior *Hox* gene transcription in anterior cells (Zuniga and Zeller, 1999) then by the protective effect against potential posterior HOX products, which we describe in this work.

However, this explanatory framework fails to account for the fact that no major proximal defect occurs in *Gli*3 minus animals, i.e. in the presence of detectable ectopic posterior *Hox* genes products in the early anterior limb bud (Zuniga and Zeller, 1999). We think that this may result from a dose effect, the amount and sustainability of these ectopic products being much below those observed in the *Del(1–10)* gain of function (Zakany et al., 2004). Also, in the *Gli3* mutant limb buds, ectopic posterior products must 'compete' against (or abrogate) the full complement of anterior *Hox* genes, whereas the complete set of anterior *Hoxd* genes is removed in the *Del(1–10)* mutants, in addition to a strong gain of expression. Such a dosage effect is well supported by the trans-heterozygous phenotypes described above. The fact that limbs develop well in the absence of *Gli3* function, despite the strong expression of posterior *Hoxd* genes in the distal autopod (late phase in Tarchini et al., 2006), suggests that the dramatic phenotypes observed in this work derive from perturbations occurring at a very early stage of limb bud development, a stage critical for the formation of the AER, in agreement with the described kinetics of the posterior *Hoxd* gene gain of function in *Del(1–10)* animals (Zakany et al., 2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.03.517.

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Paper 2:

Hox gene function in vertebrate gut morphogenesis: the case of the caecum

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Hox gene function in vertebrate gut morphogenesis: the case of the caecum

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The digestive tract is made of different subdivisions with various functions. During embryonic development, the developing intestine expresses combinations of Hox genes along its anterior to posterior axis, suggesting a role for these genes in this regionalization process. In particular, the transition from small to large intestine is labelled by the transcription of all Hoxd genes except *Hoxd12* and *Hoxd13*, the latter two genes being transcribed only near the anus. Here, we describe two lines of mice that express *Hoxd12* ectopically within this morphological transition. As a consequence, budding of the caecum is impeded, leading to complete agenesis in homozygous individuals. This effect is concurrent with a dramatic reduction of both *Fgf10* and *Pitx1* expression. Furthermore, the interactions between 'anterior' Hox genes and ectopic *Hoxd12* suggest a model whereby anterior and posterior Hox products compete in controlling *Fgf10* signalling, which is required for the growth of this organ in mice. These results illuminate components of the genetic cascade necessary for the emergence of this gut segment, crucial for many vertebrates.

KEY WORDS: Hox target genes, Budding morphogenesis, Genetic analysis, Gut regionalization, Mouse organogenesis

INTRODUCTION

The caecum is a pouch of the digestive tube, located at the junction between the small and the large intestine, which is essential for many vertebrate species to digest dietary cellulose. In herbivorous species, where it represents a crucial gastrointestinal (GI) organ, the relative size of the adult caecum is much larger than that of carnivores. In addition, there is variation in the presence or absence of the caecum even among mammals (Langer, 2001), hence discovering genetic determinants of caecum growth may contribute to diverse types of investigations into both ontogenesis and phylogenesis of the gastrointestinal system.

The role of Hox genes in patterning the mammalian GI tract in addition to the skeleton, the nervous system and the genitals has been documented for some time, in particular with respect to the differentiation of both the muscular layer and the epithelium. While systematic analyses of expression patterns in mice have revealed a coordinated expression strategy (Sekimoto et al., 1998; Pitera et al., 1999; Kawazoe et al., 2002), more recent studies employing various methodologies of gene expression profiling have also supported the involvement of many Hox genes, including those in the *HoxD* cluster, in regionalization (Bates et al., 2002; Choi et al., 2006).

Furthermore, examination of mice with modified Hox gene expression levels has provided decisive evidence for their function during development, as ranges of anatomical defects were discovered along the anteroposterior axis of the GI tract. Hox deficiencies due to the inactivation of single genes such as *Hoxc4* and *Hoxa5*, as well as overexpression of either *Hoxc8* or *Hoxa4*, were shown to affect the oesophagus, stomach or intestine, respectively (Boulet and Capecchi, 1996; Aubin et al., 2002; Pollock et al., 1992; Wolgemuth et al., 1989). We had previously shown that, in the absence either of all Hoxd genes, or of the *Hoxd4* to *Hoxd13* genomic interval, the genesis of both the ileo-caecal and anal sphincters was severely impaired, even though the gross anatomy was normal (Zakany and Duboule, 1999; Zakany et al., 2001). Furthermore, targeted inactivation of *Hoxd12* or *Hoxd13* affected the proper morphology of the anal sphincter selectively (Kondo et al., 1996).

The caecum forms at the limit between the ileum and the colon; in mice, it begins to grow at day 10 of embryonic development, and one day later it protrudes out of the abdominal cavity and is included in the intestinal hernia. A large number of Hox genes are co-expressed in posterior midgut, in a region that coincides with the future budding of the caecum (Dolle et al., 1991; Kawazoe et al., 2002; Levin et al., 1997; Pitera et al., 1999; Roberts et al., 1995; Sekimoto et al., 1998). By contrast, the expression of the most 'posterior' Hox genes, such as *Hoxd12* and *Hoxd13* is excluded from this precise region (Dolle et al., 1991; Kmita et al., 2000). Interestingly, the expression of the *HoxD* cluster genes in this particular region, the transition from the ileum to the colon, did not appear to follow the rule of collinearity, unlike that seen for the expression of these genes in other axial structures. Indeed, several genes belonging to the *HoxD* cluster were reported to be co-expressed at around the position of the future caecum, probably in response to a global regulatory mechanism located in 3′ of (telomeric to-) the cluster (Kmita et al., 2000; Spitz et al., 2005), suggesting that these transcription factors may be instrumental in the development of this organ.

In this report, we further investigate the importance of the embryonic Hox expression domains for the proper formation of the ileo-caecal transition. First, we confirm that *HoxD* cluster genes are excluded from the anterior small bowel and we show that all Hoxd genes, with the exception of *Hoxd12* and *Hoxd13*, are heavily co-expressed in a limited segment of the posterior midgut. Next, by investigating novel mutant lines involving partial deficiencies of the *HoxD* cluster, we show that a robust gain of expression of *Hoxd12* in the posterior midgut correlates, in time and place, with the absence of caecum budding originating from this region. In these foetuses, however, specific expression of Hoxa genes was maintained. We also show that *Hoxd12* gain of function inhibits the outgrowth of the caecum, probably by interfering with fibroblast

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growth factor signalling, in particular *Fgf10*, which normally depends upon the activity of anterior Hox gene products. These results strongly suggest that several Hox gene(s) are required for the proper formation of the ileum-to-colon transition and concurrent budding of the caecum.

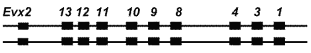
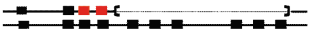
MATERIALS AND METHODS

Mouse stocks, TAMERE, crosses and genotyping

In order to obtain the various genotypes shown in Table 1, mice heterozygous for the $HoxD^{Del(1\text{-}10)}$ allele (Zakany et al., 2004) [referred to as '*Del*(*1-10*)'] were crossed with either *del*(*1-13*) (Zakany et al., 2001), *del*(*4-13*) (Zakany and Duboule, 1999), *del*(*8i-13*) (Tarchini et al., 2005) or *del*(*11-13*) (Zakany and Duboule, 1996). To produce the novel *Del*(*4-11*) allele, we used targeted meiotic recombination (TAMERE) (Hérault et al., 1998) after a cross between the *del*(*4-13*) allele and the *md11f* allele (Beckers and Duboule, 1998). Heterozygous *Hoxd1/lac* mice (Zakany et al., 2001) were crossed together in order to monitor the expression of *Hoxd1/lac* reporter gene by X-Gal assay or to detect *lacZ* transcript accumulation. For the production of the novel *Del*(*4-11*) allele, 'transloxer' males were produced containing the two *HoxD* alleles *del*(*4-13*) and *md11f*, along with the *Sycp1*CRE transgene. Three recombinant pups were obtained after genotyping 171 progeny (1.7%), one of which carried the intended allele, and the two others the predicted reciprocal allele (see Fig. 3).

PCR primers used for genotyping were as follows: Inv1 (5′-CCAC-CCTGCTAAATAAACGCT-3′) and 5′d10b (5′-GGTTGCCTCTTTT-CCTCTGTCTC-3′) to detect the wild-type *HoxD* allele; Inv1 and 3′d1b1 (5′-CTATTCAAAGGTGGGGAGCAGTC-3′) to detect the *Del*(*1-10*)

Table 1. Range of caecum defects scored in newborn mice of *HoxD* mutant stocks

HoxD genotype		Phenotypic classes A	H	D	N	Total
wt				2	71	73
Del(1-10)/+			4	63	3	70
Del(1-10)/Del(1-10)		8				8
Del(1-10)/del(1-13)		3	10	2		15
Del(1-10)/del(4-13)		7	11	3		21
Del(1-10)/del(8i-13)		4	4			8
Del(1-10)/del(11-13)			2	16		18
del(1-13)/del(1-13)				2	6	8
del(1-13)/+					31	31
del(4-13)/+					24	24
del(8i-13)/+					16	16
del(11-13)/+				1	21	22

Genotypes are listed on the left and graphically represented in the middle; the incidence of the respective phenotypic classes is indicated on the right. Different colours signal the differential influence of 'posterior' and 'anterior' Hox genes on caecum morphogenesis. *Del(1-10)*-associated *Hoxd12* and *Hoxd11* gain-of-functions are in red, referring to their ectopic expression in caecum bud. In the other alleles, *Hoxd1*, *3*, *4*, *8*, *9* and *10* genes are in green, referring to their buffering activity against the activity of more-posterior genes. The *Hoxd11/lac* reporter transgene (whose expression in caecum is documented in Fig. 4A,A′,D) is in blue. The four phenotypic classes were defined as follows. Class A: absent (agenesis; see Fig. 4A for illustration) or very short caecum bud without epithelial invasion (atresia; see Fig. 4A′,B for illustration). Class H: short caecum, of less than half the normal length (hypoplasia). Class D: thin caecum, not overtly shorter than normal (dysplasia; see Fig. 4D,E for illustration of this mildly abnormal morphology). Class N: caecum having normal proportions and substantial lumen (normal; see Fig. 4G,H for illustration). The vast majority of wild-type (wt) specimens belonged to class N or occasionally to class D. For this reason, data from classes A and H were combined as 'abnormal' and those from classes N and D were combined as 'normal' for the purposes of statistical hypothesis testing (see below). *Del(1-10)/+* heterozygous mice fitted mostly into class D, rarely into the more severe class H. In a few cases, specimens were normal and thus assigned to class N. *Del(1-10)/Del(1-10)* homozygous mice represented the most abnormal group. The caecum was absent with 100% penetrance, assigning these mutants to class A. Although not included in this table, *Del(4-11)/Del(4-11)* homozygotes were also completely caecum-less and would thus qualify as class A. *Del(1-10)/del(1-13)* compound mutants never showed normal caecum. Instead, caecum agenesis, atresia and severe hypoplasia were most often represented. *Del(1-10)/del(4-13)* and *Del(1-10)/del(8i-13)* showed the same abnormal distribution, with minor repartition between the phenotypic classes. *Del(1-10)/del(8i-13)* compound mutants were able to survive to adulthood, allowing recording of the postnatal caecum morphology (see Fig. 4A, agenesis; Fig. 4A′,B, atresia), the equivalent to the phenotypic class A. *Del(1-10)/del(11-13)* compound mutants mostly fell into the moderate abnormal class D and occasionally into class H. Complete agenesis or atresia was not seen in any of the 18 compound mutants observed. These compound mutants survived well, and the morphology of their adult caeca is shown in Fig. 4D,E, mirroring the phenotype of class D. Homozygous *HoxD*-deficient animals, *del(1-13)/del(1-13),* showed a caecum size close to normal, although they were lacking the ileo-caecal sphincter as compared with wt mice. Remarkably, caecum agenesis or atresia never occurred in these animals. A comparison of *del(1-13)/del(1-13)* with *Del(1-10)/del(1-13)* clearly indicated that caecum agenesis was induced by a gain-of-function effect, as it required the presence of the *Hoxd11* and *Hoxd12* loci. Indeed, both these genes, when associated with the *Del(1-10)* allele, were ectopically expressed up to the conjunction between small and large intestine at the time when caecum budding normally occurred. The rest of the heterozygous genotypes were observed in the respective crosses aimed at isolating the compound mutants discussed above. The vast majority fell under class N and were only occasionally classified as mildly abnormal under class D. The data presented in the table have been subjected to statistical analysis: incidence in two pools, classes A+H representing 'abnormal' outcomes, versus classes D+N, representing 'normal' or close to normal outcomes, were compared by Fischer's exact test and the χ^2 test. Differences that were not statistically significant are: *Del(1-10)/Del(1-10)* versus *Del(1-10)/del(1-13)* (Fischer's test, *P*=1.4E+00); *Del(1-10)/del(1-13)* versus *Del(1-10)/del(4-13)* (Fischer's test, *P*=4.1E-01); *Del(1-10)/del(1-13)* versus *Del(1-10)/del(8i-13)* (Fischer's test, *P*=1.4E+00). The first comparison indicates that in the presence of a single dose of *Del(1-10)* allele-associated 'posterior' *HoxD* gain-of-function, the caecum was affected in a similar way as in homozygous *Del(1-10)*, provided the other chromosome was completely *HoxD*-deficient. This made possible the assessment of the phenotypic correction effect of the nested cluster deficiencies. The latter two comparisons indicate that in spite of the simultaneous presence of 'anterior' genes like *Hoxd1* and *Hoxd3*, or *Hoxd1*, *Hoxd3* and *Hoxd4*, respectively, the ectopic 'posterior' *HoxD*-induced gut dysmorphology was still predominant. Statistically significant differences are: wt versus *Del(1-10)/Del(1-10)* (χ^2 test, *P*<0.001); *Del(1-10)/del(1-13)* versus *+/Del(1-10)* (χ^2 test, *P*<0.001); *Del(1-10)/del(1-13)* versus *Del(1-10)/del(11-13)* (χ^2 test, *P*<0.001). The latter two comparisons, in particular, indicate that the presence of the wild-type allele or the *Hoxd1-Hoxd10* loci at the *HoxD* cluster neutralized in trans the effect of ectopic posterior Hoxd gene expression on caecum morphogenesis.

or *del*(*1-13*) alleles; Inv1 and 5′d3b (5′-GGGATGTCAAATCTT-CTTGGAGTG-3′) to detect the *del*(*4-13*) allele; Inv1 and 5′d4b (5′-TGGCAACCAACCGTTTCTTTC-3′) to detect the *del*(*8i-13*) allele; Xfwd (5′-TACCCTGCTGTTCACTCCGTTG-3′) and Xrev (5′-TGTGTCCT-TGTCCTTGCTTATTCG-3′) to detect the *md11A* allele and Xfwd and 5′d3b to detect the *Del*(*4-11*) allele.

Gastrointestinal tract dissection and whole-mount in situ hybridization

The morning of the recovery of vaginal plug was counted as day 0 of embryonic development. Foetuses were collected at gestational days 11, 12 or 13 and full-length gastrointestinal tracts were dissected in PBS then fixed and processed according to standard procedures. The RNA probes used to detect *HoxD* expression were the following: *Hoxd1* (Zakany et al., 2001), *Hoxd3* (Condie and Capecchi, 1993), *Hoxd4* (Featherstone et al., 1988), *Hoxd8* (Izpisua-Belmonte et al., 1990), *Hoxd9* (Zappavigna et al., 1991), *Hoxd10* and *Hoxd11* (Gerard et al., 1996), *Hoxd12* (Izpisua-Belmonte et al., 1991), *Hoxd13* (Dolle et al., 1991). The remaining RNA probes were *Hoxa6* (Sekimoto et al., 1998), *Hoxa10* (Favier et al., 1996), *Fgf10* (Bellusci et al., 1997) and *Pitx1* (Logan et al., 1998). For the complementation assay, newborns were recovered, their full GI was dissected, documented and genotyped.

RESULTS AND DISCUSSION

Posterior specificity of the *HoxD* cluster

We first established the expression pattern of all nine gene members of the *HoxD* cluster in wild-type embryos at mid-gestation (E12), at a time when the caecum is located in the intestinal hernia (Fig. 1A,B). Consistent with earlier observations, we found that *Hoxd4*, *Hoxd8* and *Hoxd9* are co-expressed in the developing caecum, in addition to *Hoxd1*, *Hoxd3*, *Hoxd10* and *Hoxd11*. From *Hoxd1* to *Hoxd10*, expression was detected up to the ileo-caecal transition. By contrast, *Hoxd11* transcripts were restricted to the posterior half of the caecum bud (Fig. 1E), whereas the more ‘posterior’ genes *Hoxd12* and *Hoxd13* (Fig. 1C,D) were transcribed only in the most caudal part of the GI tract (Dolle et al., 1991; Kondo et al., 1996).

Despite the expression of most Hoxd and other Hox genes in the developing caecum, foregut derivatives appeared to be devoid of Hoxd transcripts. For instance, while expression of all three *Hox4* paralogous genes was scored in E12 stomach mesenchyme (Kawazoe et al., 2002; Pitera et al., 1999), we were unable to detect either *Hoxd1*, or *Hoxd4*, transcripts in embryonic stomach. Because of the rapid degradation of *Hoxd1* mRNA (Zakany et al., 2001), we performed in situ hybridization with a *Hoxd1* probe on wild-type foetuses, and explored *lacZ*-specific transcript accumulation in embryos carrying the *Hoxd1/lacZ* knock-in allele. In contrast to the robust *lacZ* expression in the caecum (Fig. 1C), staining was not seen in stomach. Similarly, *Hoxd3* was weakly expressed in stomach, compared with midgut, indicating a relative restriction of Hoxd gene expression to the posterior gut.

Ectopic expression of Hoxd genes

Over recent years, a collection of mouse lines carrying rearrangements at the *HoxD* locus were produced by targeted meiotic recombination (TAMERE) (Hérault et al., 1998), in order to study gene regulation at this locus. In several lines harbouring deletions of one or multiple Hoxd genes, the remaining genes usually changed their expression patterns, in agreement with their new respective position within the Hox cluster. Accordingly, mice carrying such deletions usually showed both loss-of-function and gain-of-function phenotypes. In particular, severe alterations were obtained when ‘posterior’ Hoxd genes such as *Hoxd12* or *Hoxd13* were expressed in more anterior territories, either in the trunk (Kmita et al., 2000), or in the limbs (Zakany et al., 2004), due to the antagonizing effect of the most posterior HOX products over anterior ones, a property referred to as ‘posterior prevalence’ (Duboule, 1991; Duboule and Morata, 1994).

Mice homozygous for a deletion of the anterior part of the cluster, from *Hoxd1* to *Hoxd10* including [the *Del*(*1-10*) allele], were born in mendelian proportions and newborns appeared overtly normal,

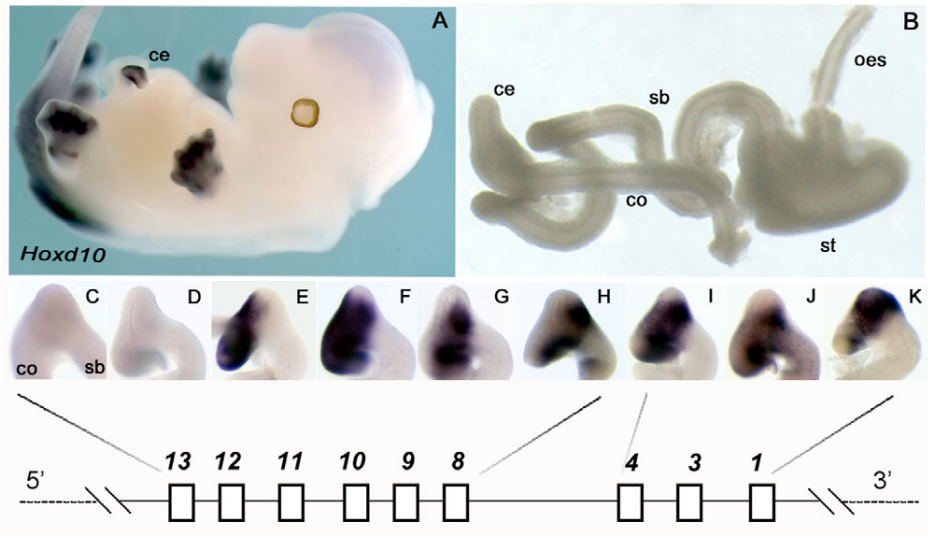


Fig. 1. Co-expression of seven Hoxd genes in posterior midgut. (**A**) Whole-mount RNA in situ hybridization detection of *Hoxd10* transcripts in E13 mouse embryo, showing some sites of expression, including the intestinal hernia. (**B**) Anatomical subdivisions of the mid-gestation murine gastrointestinal system at E12. (**C-K**) Detection of *Hoxd13* (C), *Hoxd12* (D), *Hoxd11* (E), *Hoxd10* (F), *Hoxd9* (G), *Hoxd8* (H), *Hoxd4* (I), *Hoxd3* (J) and *Hoxd1* (K) transcripts in dissected gut of E12.5 mouse embryos. The contiguous loci *Hoxd1* to *Hoxd10* are all co-expressed in the posterior midgut, in the region that involves the incipient caecum bud (F-K). *Hoxd11* is excluded from the anterior (ileal) part, but is expressed in the posterior (colonic) part of the caecum bud (E). *Hoxd12* (D) and *Hoxd13* (C) expression is not detected in this region. ce, caecum; co, colon; oes, oesophagus; sb, small bowel; st, stomach.

yet none of them survived due to acute respiratory failure. Interestingly, all homozygous animals showed a severe agenesis of the caecum. We investigated whether this defect was due to the combined loss of function of several Hoxd genes in *cis* by analysing mice homozygous for a complete deficiency of the *HoxD* cluster [the *del*(*1-13*) allele]. In *del*(*1-13*) homozygous mice, however, the caecum was never absent. This observation indicated that the absence of caecum in *Del*(*1-10*) homozygous individuals was caused by a gain-of-function mechanism involving either *Hoxd11*, *Hoxd12* or *Hoxd13*, rather than by a combined loss of function.

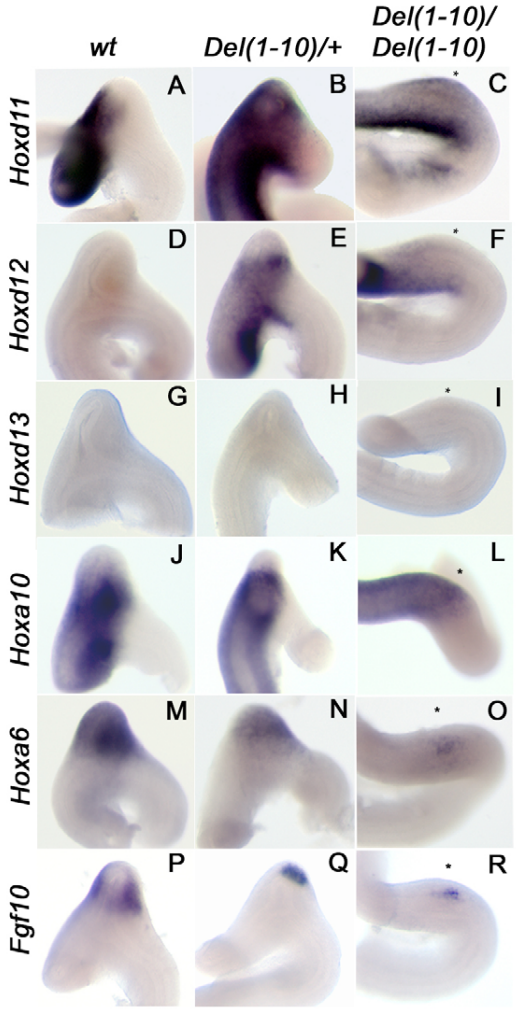


Fig. 2. Defect in the gut of Del(1-10) mutants. Whole-mount RNA in situ hybridization analysis of gene expression in dissected posterior midgut of E12 mouse embryos. *Hoxd11* (**A-C**), *Hoxd12* (**D-F**), *Hoxd13* (**G-I**) *Hoxa10* (**J-L**), *Hoxa6* (**M-O**) and *Fgf10* (**P-R**). Caecum budding is well underway in wild-type controls (A,D,G,J,M,P), perceptibly delayed in heterozygotes (B,E,H,K,N,Q) and is absent from homozygous specimens (C,F,I,L,O,R). In heterozygous specimens, ectopic *Hoxd11* and *Hoxd12* expression is detected in the anterior part and in the entire caecum bud, respectively (B,E). In homozygotes, *Hoxd11* and *Hoxd12* expression is detectable in the presumptive area for caecum budding (asterisks in C,F). In all three genotypes, including homozygous embryos, the demarcation between *Hoxa10*-negative and *Hoxa10*-positive regions seems to be rather faithfully maintained (J-L). Retarded bud growth in heterozygous specimens is correlated with reduced hybridisation signals for *Hoxa6* (N) and *Fgf10* (Q). Absence of bud growth correlates with the massive reduction of the expression domain of both these genes, leaving only of a tiny expression domain for both *Hoxa6* (O) and *Fgf10* (R).

To determine which one(s) of these three genes could be causative of this phenotype, we performed RNA whole-mount in situ hybridization on dissected GI tracts of E12 embryos, from the lower oesophagus to the rectum (Fig. 1B). Strikingly, the expression patterns of both *Hoxd11* (Fig. 2B) and *Hoxd12* (Fig. 2E) changed, in heterozygous mutants, to become similar to that of *Hoxd10* (Fig. 1F). Both *Hoxd11* and *Hoxd12* transcripts were readily detected in the most posterior part of the ileum as well as in the caecum. In such heterozygous animals, a marked delay in the progression of caecum budding was clearly scored (Fig. 2B,E,H), whereas no budding at all was visible in homozygous littermates (Fig. 2C,F,I). Therefore, both *Hoxd11* and *Hoxd12* became ectopically expressed in the mesenchyme of the whole caecum up to the ileo-caecal transition, and this gain-of-function condition correlated with the suppression of caecum budding in homozygous *Del*(*1-10*) embryos. By contrast, no ectopic *Hoxd13* expression could be seen in the digestive tract (Fig. 2H,I).

We then investigated whether the absence of caecum was due to a deficit in budding or to a more global problem of gut (mis-)specification, due to aberrant regulation of those Hox genes labelling the ileum-to-colon transition. To this aim, we used the *Hoxa10* and *Hoxa6* probes. In wild-type animals, *Hoxa10* is expressed in the anterior colon up to the ileo-caecal valve, including the budding caecum. In *Del*(*1-10*) heterozygous animals, *Hoxa10* expression was not importantly modified and still labelled the ileum-to-colon transition, reminiscent of the ectopic patterns of both *Hoxd11* and *Hoxd12* (Fig. 2J-L), suggesting that caecum agenesis was not due to a transcriptional effect of the gained genes over other Hox genes transcription. In contrast to *Hoxa10*, *Hoxa6* signal is normally restricted to the budding caecum. Whereas in heterozygous *Del*(*1-10*) embryos, the signal was expectedly reduced, homozygous mutant GI tracts still showed a *Hoxa6* signal, but only in a small group of cells located at the expected position for the caecum bud (Fig. 2M-O). From these observations we conclude that the overall molecular GI tract specification, as indicated by the *HoxA*-cluster-specific probes, was maintained even in homozygous mutants that did not develop a caecum. Consequently we searched for other genetic constitutions that result in caecum agenesis or hypoplasia, but without known involvement of general regionalization.

The development of the caecum is strongly impaired in mice, where either fibroblast growth factor genes (Fairbanks et al., 2004; Zhang et al., 2006) or receptors (Burns et al., 2004) are inactivated, in particular *Fgf10*, which is selectively expressed in the mesenchyme of the wild-type budding caecum (Fairbanks et al., 2004) (Fig. 2P). In the *Del*(*1-10*) mutant embryos, we found that *Fgf10* transcript accumulation was reduced in heterozygotes and almost completely absent in homozygotes, leaving a small cluster of *Fgf10*-expressing cells in the ileo-colonic loop (Fig. 2Q-R). These observations suggest that caecum outgrowth is under the control of *Fgf10*, the expression of which may require the activity of several Hox genes in a defined region of the developing intestinal tract. In the absence of all Hoxd genes [*del*(*1-13*)], Hox genes from other clusters can still instruct presumptive cells to activate *Fgf10* signalling, thus leading to the budding of a caecum. By contrast, the presence of ectopic *Hoxd12* in this precise intestinal segment will abrogate the functions of more ‘anterior’ gene products from all clusters, via posterior prevalence. Accordingly, *Fgf10* will fail to be produced and caecum budding will be suppressed. Interestingly, this situation is analogous to that recently reported to happen during

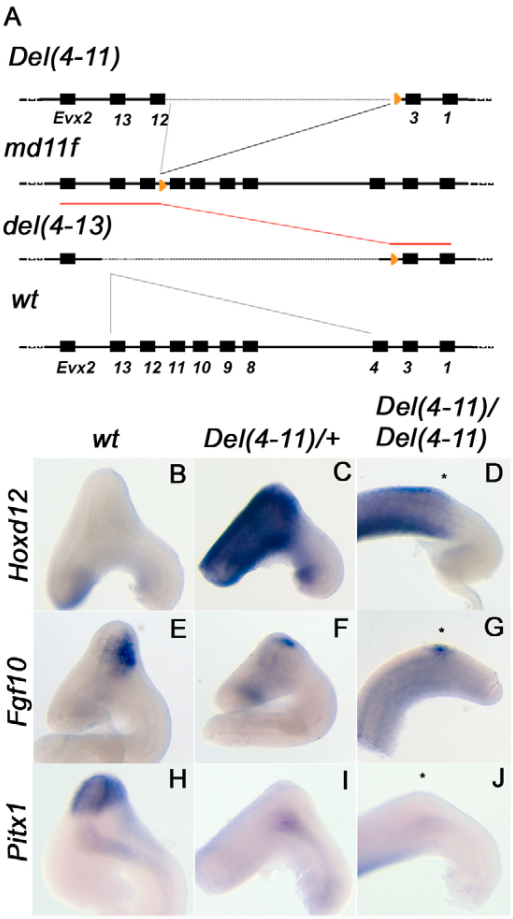


Fig. 3. Role of *Hoxd12* in caecum agenesis. (**A**) Derivation of the *Del*(*4-11*) allele by TAMERE. (**B-J**) Whole-mount RNA in situ hybridization of *Hoxd12* (B-D), *Fgf10* (E-G) and *Pitx1* (H-J) transcripts in wild-type control (B,E,H), *Del*(*4-11*)-deficient *HoxD* cluster mutant heterozygous (C,F,I) and homozygous (D,G,J) posterior midguts at E12.5. In wild-type controls, the *Hoxd12* transcript is always absent from the caecum bud (B), whereas localized expression of both *Fgf10* (E) and *Pitx1* (H) are always detectable. Caecum bud growth is conspicuously retarded in *Del*(*4-11*) heterozygous specimens and is correlated with robust *Hoxd12* transcript accumulation (C), reduced hybridization signals for *Fgf10* (F) and undetectable signal for *Pitx1* (I) in bud mesenchyme. Absence of bud growth in homozygous specimens is correlated with extremely reduced of *Fgf10* (asterisk in G) and complete absence of localized *Pitx1* (J).

early limb budding, where ectopic expression of both *Hoxd12* and *Hoxd13* could abrogate the *Fgf10*-dependent growth of forelimb buds (Zakany et al., 2007).

In this scenario, the ectopic expression of *Hoxd12* plays the key role via its concurrent deleterious effect upon the functions of other Hox genes. We challenged this hypothesis by producing and analysing yet another *HoxD* cluster deletion allele; *Del*(*4-11*) (Fig. 3A). In *Del*(*4-11*) F2 newborn progeny, all three genotypes were present in mendelian proportions. Strikingly, all homozygous embryos completely lacked the caecum. In situ hybridization analysis revealed a massive ectopic expression of *Hoxd12* in posterior midgut mesenchyme (Fig. 3B-D), mimicking the normal expression pattern of *Hoxd10* (Fig. 1F). The presumptive caecum of homozygous mice was consistently reduced to a small deformation of the gut, right inside the ectopic *Hoxd12* expression domain. As for the case of *Del*(*1-10*), *Hoxd13* was not gained in this presumptive caecum area in *Del*(*4-10*) mutant intestines at E12 (not shown), but the level of *Fgf10* transcript was reduced (Fig. 3E-G). We also looked at the expression of the *Pitx1* gene, whose transcripts are found both in the epithelium and mesenchyme of the developing gut (Lanctôt et al., 1997) and, as shown here, accumulate selectively in the mesenchyme of the growing caecum (Fig. 3H). Here again, this specific expression of *Pitx1* was severely reduced in caecum mesenchyme of heterozygotes, whereas it was absent from homozygous specimens (Fig. 3I-J). Posterior midgut development was thus similarly compromised in both *Del*(*1-10*) and *Del*(*4-11*) homozygous animals. There was a robust correlation between all aspects of the caecum defect, on the one hand, and the ectopic expression of *Hoxd12* and concurrent dose-dependent suppression of *Fgf10* and *Pitx1* transcripts in prospective caecum bud mesenchyme, on the other hand. We thus concluded that the induction and/or growth of the caecum are affected by ectopic expression of *Hoxd12*. Whether the loss of *Fgf10*, *Hoxa6* and *Pitx1* expression reflects the loss of the corresponding 'presumptive caecal cells' or, alternatively, the downregulation of these genes in these cells remains to be addressed. We did not fully assess the genetic cascade underlying the suppressive effect of HOX proteins on *Pitx1* and *Fgf10* transcription in developing caecum mesenchyme. However, *Pitx1* expression was gained in the second branchial arch of mice lacking *Hoxa2* following Hox interference with *Fgf* signalling (Bobola et al., 2003). Also, the data from the genetic and molecular embryological analysis presented here, together with those concerning early limb budding (Zakany et al., 2007), suggest that the Hox genes *Fgf10* and possibly *Pitx1* are components of a mesenchyme-specific genetic hierarchy that controls caecum budding.

These observations support an instructive role for 'anterior' Hox genes in the definition of a restricted territory from where the caecum will emerge. This precise area corresponds to an important morphological transition in the intestine, the position of which is probably also dependent upon the coherent expression of these same Hox genes. Induction of caecum budding and its elongation require a localized source of growth factors, as provided by *Fgf10* signalling, downstream of Hox gene expression. We interpret our results in the context of posterior prevalence, according to which the function of a given Hox gene may be impeded by the presence of more posterior Hox product in the same cells (Duboule and Morata, 1994), in particular from the most posterior *Hox12* and *Hox13* groups. Because the absence of the whole *HoxD* cluster induced only a relatively mild posterior midgut malformation, we think that the expression of Hox genes left in the other clusters is equally capable of promoting posterior midgut development. However, in the case of internal *HoxD* cluster deletions, the ectopic expression of *Hoxd12* abrogates the functions of several co-expressed 'anterior' Hox genes, leading to the inability to transcribe *Fgf10* and consequent absence of budding.

Hoxd genes and the posterior midgut

In order to further document this conclusion, we produced a set of genetic configurations to fine-tune the doses of various Hox gene products. Because the *Del*(*1-10*) allele arguably delivers less ectopic activity of *Hoxd12* than the *Del*(*4-11*) allele, we used the former together with selected *HoxD* cluster deficiencies, which by themselves do not induce ectopic gene expression. The rationale of these crosses was to manipulate doses of 'anterior' genes on the top of a fixed, standard level of ectopic *Hoxd12* in the presumptive region for caecum budding (Table 1). First, we produced compound

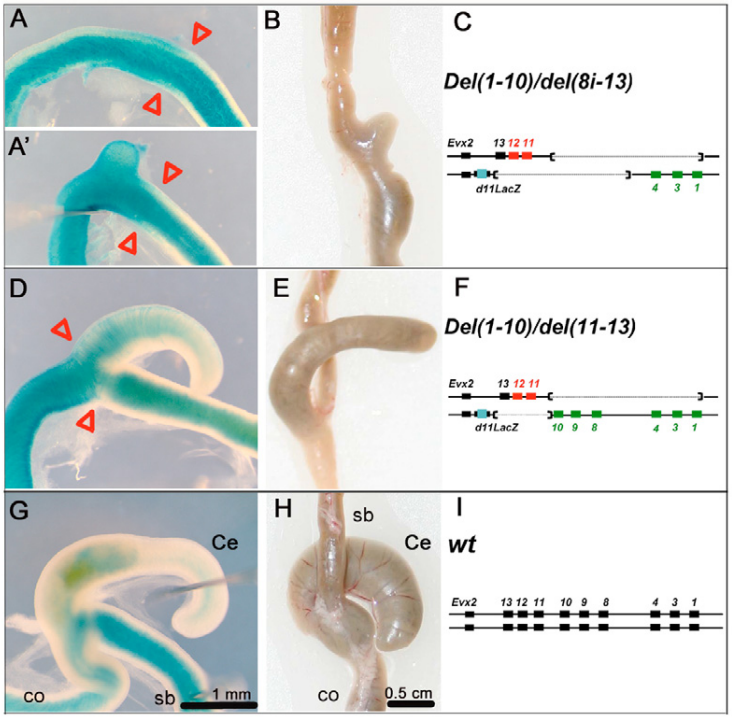


Fig. 4. 'Anterior' Hoxd genes promote adult caecum size in mice. (**A-I**) Dissected ileo-caecal transition zones of three genotypes, illustrating key examples of the phenotypic series presented in Table 1. A,A',D,G depict X-Gal-stained newborn specimens, to confirm the identity of the complementing alleles. Arrowheads in A,A',D point to the anterior limit of *Hoxd11/lac* marker gene expression in mesenchyme, associated with the respective alleles, depicted in the line diagrams on the right. In gut mesenchyme, *del(8i-13)* shows a more anterior *Hoxd11/lac* expression involving the posterior ileum (A,A'), while the reporter gene associated with the *del(11-13)* allele is limited at the ileo-caecal junction, reminiscent of endogenous *Hoxd11*. Blue staining in G is due to endogenous activity in enterocytes, which does not involve the gut mesenchyme. The *lac* fusion protein does not have *Hoxd11* function, consistent with lack of caecum growth promotion by the *del(8i-13)* allele (A,A',B) in the presence of ectopic *Hoxd12*. By contrast, in the case of *del(11-13)*, the presence of the caecum (D,E) indicates growth promotion by *Hoxd8*, *Hoxd9* and *Hoxd10* present in addition to the *Hoxd1*, *Hoxd3 and Hoxd4* loci, which alone were not sufficient to neutralize ectopic *Hoxd12*. Control guts are shown in G and H for comparison.

mutants with the *del(1-13)* allele, i.e. a full deletion of the *HoxD* cluster. Interestingly, a proportion of *Del(1-10)/del(1-13)* *trans*-heterozygous individuals showed a phenocopy of the *Del(1-10)* homozygous phenotype, pointing to a strong influence of gene dose balance: in the absence of one haplotype of the *HoxD* cluster, half the dose of ectopic *Hoxd12* gene product was sufficient to induce caecum agenesis. The occurrence of caecum agenesis in *Del(1-10)/del(1-13)* mice, compared with *Del(1-10)/+* heterozygous mice, demonstrated that caecum development depends on the presence of 'anterior' Hoxd genes, capable of counterbalancing the deleterious effect of ectopic *Hoxd12*. In other words, higher doses of anterior HOXD gene products make a full posterior prevalence by HOXD12 difficult to achieve.

We next used a set of partial deletions to assess the importance of particular 'anterior' Hoxd genes in protecting against the deleterious effect of *Hoxd12* product. Three compound mutants were analysed, which combined gain of *Hoxd12* with various partial *HoxD* deletions as the other allele; *Del(1-10)/del(4-13)*, *Del(1-10)/del(8i-13)* and *Del(1-10)/del(11-13)* (Fig. 4A-I). Out of these combinations, *Del(1-10)/del(11-13)* embryos were the only ones to develop a normal caecum (Fig. 4D-F), similarly to *Del(1-10)/+* heterozygous individuals. This genetic analysis, through a quantitative measurement, revealed the equivalence of one dose of *Del(1-10)*-associated ectopic *Hoxd12*, with one haplotype of *Hoxd1*, *Hoxd3*, *Hoxd4*, *Hoxd8*, *Hoxd9* and *Hoxd10*.

Two doses of ectopic *Hoxd12*, in *Del(1-10)* homozygous, were capable of inactivating all the non-*HoxD*-derived caecum-promoting Hox activity, in all homozygous animals tested. Furthermore, even a single dose of gained *Hoxd12* was capable of abrogating the *HoxA*, *HoxB* and *HoxC* gene function in a number of *Del(1-10)/del(8i-13)* individuals, in addition to an activity possibly provided by *Hoxd1*, *Hoxd3* and *Hoxd4* (Fig. 4A-C). From this, we conclude that in posterior midgut, the *HoxA*, *HoxB* and *HoxC* clusters together provide no more function than a single haplotype of the *HoxD* cluster does.

Hox function and postnatal growth

Quantitative modulation in the balance between 'anterior' Hox genes and ectopic *Hoxd12* led to a phenotypic series involving more or less affected individuals, some of which survived for several weeks. In particular, most *Del(4-11)* heterozygous and some *Del(1-10)/del(8i-13)* compound mutants survived postnatally having either no, or reduced, caeca (Fig. 4B). In the *Del(4-11)* pedigree, we noticed a marked variation, and heterozygous mice proved lighter than their wild-type littermates. We took individual body mass readings of four litters sired by the same third generation backcross male with wild-type C57Bl6 females, and two litters of heterozygous parents. Of a total of 39 typed progeny, 17 were wild type and 22 were of *Del(4-11)* heterozygous genotype. At 4 weeks of age, the average body mass was 10.9 and 9.2 g, respectively, indicating approximately 20% deficit in *Del(4-11)* heterozygotes. This statistically significant body mass deficit persisted into adulthood.

Similar observations carried out on mice heterozygous for a full *HoxD* deficiency showed less than 10% body mass reduction, a figure statistically non-significant. In conclusion, *Del(4-11)* heterozygous mice do not thrive as well as wild-type littermates, which may indicate reduced digestion efficacy due to a shorter gut. We believe that this effect would be even more substantial on a less complete and mostly vegetal chow, as caecum and upper colon are sites of bacterial cellulose decomposition of nutritional importance. Therefore, these *HoxD* cluster mutants represent a valuable genetic resource to investigate gut patterning in general, and postnatal adaptive responses to environmental factors in particular (Wostmann and Bruckner-Kardoss, 1959), as well as the concurrent effects on body mass control (Backhed et al., 2004; Samuel and Gordon, 2006).

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Discussion first paper

The Tetrapods limb is organized along proximo-distal axis in three domains, stylopod, zeugopod and autopod. In forelimbs these bony segments are represented by the humerus, the radius and ulna, the carpals, metacarpals and digits. The equivalent units in hindlimbs are the femur the tibia and fibula, the tarsals, metatarsals and toes. The diverse morphology of the zeugopodal and autopodal bones shows that limbs are polarized according the anterior versus posterior and the proximo versus distal limb axis.

During embryogenesis limb outgrowth along the proximo-distal axis is promoted by the AER whose excision results in limb truncation following the loss of the distal domain. The implantation of beads soaked with FGF in the ectoderm mimics the function of the AER and limb outgrowth (Fallon, et al., 1994; Niswander, et al., 1993), suggesting that the activity of the AER on limb mesenchyme is mediated by fibroblast growth factors. Conversely the determination of the anterior-posterior limb polarity depends on the ZPA, a group of mesenchymal cells situated at the posterior margin of the limb bud and identified by the synthesis of *Shh*. Signals exchanged between the AER and ZPA generate a loop mechanism that triggers both limb patterning and elongation (Niswander, et al., 1994). *Hoxd* and *Hoxa* genes are also fundamental to promote limbs development along the proximo-distal axis as the paralogous -9, -11 and -13 sustain the growth respectively of the stylopod, zeugopod and autopod (Davis et al., 1995; Fromental-Ramain, et al., 1996; Wellik and Capecchi, 2003). In *wt* limbs *Hoxd* genes are induced in two temporally distinct waves defined as early and late phase, each believed to depend on the activity of specific global regulatory elements, external to the cluster. These regions are referred to respectively as ELCR (early locus control region) and GCR (global control region), located telomeric and centromeric to the *HoxD* cluster (Spitz, et al., 2003; Tarchini, and Duboule, 2006; Zakany, et al., 2004). During the early phase of induction *Hoxd10* to *Hoxd13* are transcribed in nested domains in the posterior mesenchyme of the limb bud, prior of *Shh* expression (Tarchini, et al., 2006). By the "early" phase *5' Hoxd* genes establish positional identity inducing *Shh* expression in the ZPA. During the "late" phase this posterior restriction disappears and 5'end *Hox* gene expression domains extend to the anterior handplate within the digit area (Kmita, et al., 2002).

Mice lacking *Shh* had distally truncated limbs characterized by the under-development of stylopodal and zeugopodal structures and by the failure to form the autopod. Such phenotype suggested that *Shh* is crucial to promote limb growth along its proximo-distal axis and is fundamental for the formation of hands and feet (Chiang, et al., 2001; Kraus, et al., 2001). In similar way in simultaneous absence of *HoxA* and *HoxD* clusters severe limb truncations arise, evidenced by a short humerus fused to a distal cartilage element (Kmita, et al., 2005). Further analysis showed that these limb buds were devoid of *Shh* and that their AER was partially degenerated. In conclusion the loss-of-function of *HoxD* and *HoxA* gives rise to distally truncated limbs owing the lack of *Shh* synthesis and the maintenance of the AER. Likewise, in absence of the entire *HoxD* cluster the selective removal of *Hoxd8* to *Hoxd13* on *HoxA* null background suppressed the formation of the radius and ulna and shortened the humerus (Tarchini, et al., 2006). Indeed the morphogenesis of the radius and ulna occurs along with the induction of *Shh* by the last four genes of the cluster (*Hoxd10*, *Hoxd11*, *Hoxd12* and *Hoxd13*). Conversely the length of the humerus relies on *Hoxd8* and *Hoxd9* which, unlike *Hoxd10* to *Hoxd13,* can not elicit *Shh* expression (Tarchini, et al., 2006).

These results showed that in limb buds the most *5'Hox* induce *Shh* in the ZPA and therefore *Shh* is downstream *Hox* activity. After induction, *Shh* in the ZPA is maintained by a positive feed-back loop with both FGF8 and FGF4 produced in the AER (Boulet, et al., 2004; Laufer, et al., 1994; Lewandoski, et al., 2000; Sun, et al., 2000; Sun, et al., 2002). The expression of *Fgf8* is fundamental for the development of the zeugopod (Boulet et al., 2004); indeed its depletion as well as that of *Fgf4* in the AER affects the proximo-distal growth of the limb and cause severe truncations (Boulet et al., 2004; Sun, et al., 2002). Notably *Fgf10* expression in limb mesenchyme induces *Fgf8* expression in the AER (Min, et al., 1998; Sekine, et al., 1999) and is in turn maintained by the latter in growing limb bud. The way by which the loss *Fgf8* hampers limb growth is by blocking the amplification of *Fgf10* signaling in limb bud (Boulet, et al., 2004).

With the present work, we continued the study of *Hoxd* function in limb development by means of analyzing partial deficiencies of the *HoxD* cluster. We obtained a number of limb truncations along the proximo-distal axis dependent on the simultaneous gain-of-function of *5'Hoxd* genes and loss-of-function of *Gli3* transcription factor. Furthermore new experimental evidence shifted our initial focus from *Shh* to *Fgf10* and we demonstrated that the 5'*Hoxd* interference with FGF signaling damages the AER and ultimately blocks limb elongation. Our story started with the observation that the ectopic *5'Hox* gene expression induces *Shh* at the anterior margin of the limb bud, giving rise to digits with posterior identity (Zakany et al., 2004). This suggested that in a normal situation, in order to avoid the growth of a dysmorphic limb, the distribution of *Hox* transcripts in limb bud needs to be carefully regulated. One of the molecules thought to be involved in this mechanism was the *Gli3* transcription factor whose activity was shown to control *Hox* transcription in limbs. The outline of the hypothesis was the following. The spatial distribution of *Gli3* transcripts in early limb bud is rather anterior, complementary to the areas where *5' Hoxd* genes and *Shh* are expressed (Zuniga and Zeller, 1999; Buscher, et al., 1997). Once *5' Hoxd* genes are activated by the ELCR, *Gli3* maintains them repressed within the anterior limb mesenchyme. The *Extra-toes* mutation (*Xt*) generated a *Gli3* loss of function and gives rise to polydactylous limbs (Shimmang, 1992). In these mice *5' Hoxd* genes are induced ectopically in the anterior margin of the limb bud (Buscher, et al., 1997; Zuniga and Zeller, 1999), suggesting that *Gli3* might restrain the number of digits to five through *5' Hoxd* gene repression. In attempt to further challenge this hypothesis we generated mice homozygous both for *Xt* and the deletion of the full *HoxD* cluster *(Del1-13)*. These mice still displayed polydactyly characteristic of *Xt* homozygous mice. Like in the case of ectopic expression of *5'Hoxd* genes, *Shh* is induced ectopically in the anterior margin of *Xt* limbs (Buscher et al., 1997). However, the polydactylous phenotype of these mice was shown to be *Shh* independent (Litingtung, et al., 2002; Te Welcher et al., 2002-b). The presence of severe polydactyly in our *Xt/Xt;Del(1-13)/Del(1-13)* mutants suggests that the polydactyly in *Xt* mice is independent of the ectopic *Hoxd* gain in the anterior limb. A recent work published by Sheth, et al., 2007; is in agreement with the above conclusion. The authors generated double mutants lacking the function of *Gli3* and carrying a partial deficiency for the posterior *Hoxd* genes, removing from *Hoxd11* to *Hoxd13*. In double homozygous mutants *Xt/Xt; Del (11-13)/Del(11-13)* mice the specific lack of posterior *Hoxd* genes -in absence of *Gli3*- did not rescue the pentadactyly, but gave rise to a different kind of polydactylous phenotype, suggesting that posterior *Hox* likely interact with *Gli3* to restrain the number of digits.

Despite these first series of results, we decided to verify in another manner the possible genetic interaction between *Hox* and Gli3. We then changed our approach moving from the loss-of-function of *Del(1-13)* mice to the gain-of-function of posterior *Hox* genes in mice lacking the genomic region from *Hoxd1* to *Hoxd10*.

Del(1-10) homozygous mice develop forelimbs with a double posterior symmetry (Zakany, et al., 2004). While in *wt* mice 5'*Hoxd* genes are expressed at around E9.5-E10 restricted to the posterior limb bud mesenchyme, in *Del(1-10*) *Hoxd11, Hoxd12* and *Hoxd13* are distributed throughout the whole limb bud from the onset of budding through the early stage. At later stages these genes remain expressed at both anterior and posterior domains, providing a molecular explanation for the symmetrical limb phenotype (Zakany, et al., 2004).

During the late phase of induction both *Hoxd12* and *Hoxd13* are restricted to the limb digit domain, whereas *Hoxd11* and *Hoxd10* are represented both in digits and in the proximal limb domain. The *Hoxd11/Hoxa11* loss-of-function gives rise to abnormal limbs lacking the radius and ulna (Davis, et al., 1995). The *Hoxd13* and *Hoxd12* gain-of-function along the proximo-distal axis might represent an alternative way to induce a truncated limb. Experiment performed in chicken showed that *Hoxd13* counteracts the activity of *Hoxd11* in limbs, generating a phenotype consisting in the shortening of stylopodal and zeugopodal bones (Goff and Tabin, 1997). Similar results (short zeugopod) were obtained by the mis-expression of *Hoxa13* in chicken limbs (Yokouchi, et al., 1995). In mouse the uniform expression of *Hoxd12* throughout the hindlimb mesenchyme affected the elongation of the anterior zeugopod (Knezevic, et al., 1997). The reported observations supported the posterior prevalence model, by which the product of posterior genes interferes with that of anterior ones once they are coexpressed in the same domain. The best example of posterior prevalence is given by the spontaneous mutant *Ulnaless*, which consists in the absence of ulna and fibula from the limbs of this mutant (Peichel, et al., 1997). *Hoxd12* and *Hoxd13* expression was gained in the anterior-proximal domain of the limb bud and simultaneously decrease from the digit domain (Herault, et al., 1997; Peichel, et al., 1997; Spitz, et al., 2003). Therefore the expression of *Hoxd12* and *Hoxd13* in a region where they are normally absent, affect the zeugopodal development.

To assess the impact *Hoxd* gain-of-function in *Xt* background we crossed *Xt* with *Del(1-10)* mice, obtaining all the possible genetic combinations. We analyzed the forelimbs of end of term fetuses carrying both *Xt* and *Del1-10* mutations counting the number of digits and we found an unexpected result. Double homozygous for *Xt* and *Del(1-10)* had a rudimentary limb characterized by a single bony element in place of the humerus, radius and ulna, while often only one finger was present distally. Surprisingly the malformation affecting these mutants involves the entire limb along its proximo-distal axis, abolishing the features of all the three segments stylopod, zeugopod and autopod. This extensive defect involving the totality of the limb was never observed in *Del(1-10)* mice. The limb bilateral symmetry of these latter affected the polarity of digits and carpal bones (Zakany, et al., 2004) and the overall length of the limb was only slightly shorter owing the reduction in length of the humerus. The limb shortening observed in *Del(1-10)/Del(1-10)* mice can not be explained by *Hoxd* loss-of-function alone, indeed *Del(1-13/Del(1-13)* mice develop a normal zeugopod. Thus the phenotype of *Xt/Xt*; *Del(1-10)/Del(1-10)* mutants is the outcome of the simultaneous lack of functional *Gli3* and the gain of posterior *Hoxd* in limb buds, as the *Hoxd* mis-expression alone is not sufficient to fully abrogate the development of the limb of *Del(1-10/Del(1-10)*.

In order to evaluate the relative impact of the *Hoxd* gain-of-function over the loss of *Gli3*, we measured carefully the limb bones for each genotype. We grouped the animals in phenotypic classes based on the length of their humeri. In this way we found that the size of the humerus in half of the *Xt*+/-; *Del(1-10)*+/- (double heterozygous) mice was the same of *Del(1-10*)/*Del1-10* homozygous. This particular feature might derive from the decrease of *Gli3* in the anterior limb bud, consequent to the gain of *Hoxd11* and *Hoxd13*. However in situ hybridization analysis showed that *Gli3* was still consistently present in the anterior limb bud of *Del(1-10)* homozygous. In other words the *Gli3* dose in *Del(1-10)* forelimbs was not reduced to half like in *Xt; Del(1-10)* double heterozygous. This is consistent with observation that the enforced expression of *Hoxd12* does not affect the level of *Gli3* transcripts (Chen, et al., 2004). Therefore the reduction of the humerus length in *Del(1-10)* mice was imputable to the *Hoxd* gain-of-function, but the presence of functional *Gli3* still protected limb elongation from the negative effect of the latter genes. Indeed in compounds for both the mutations the limb shortening was dependent on the amount of *Gli3* left rather than by the gain of *Hoxd.* A strong decrease of the humerus length was observed in homozygous for the *Del(1-10)* deficiency where a copy of the *Gli3* gene was missing (*Xt/+;Del(1-10)/Del(1-10)*). Remarkably *Xt/Xt*; *Del(1-10)*/+ almost lacked the humerus and displayed a fusion of radius and ulna, whereas *Xt/Xt*; *Del(1-10)/Del(1-10)* limbs exhibited the most severe phenotype.

The strong limb phenotype observed likely reflects the perturbation of the molecular network regulating the interactions between limb mesenchyme and AER. We analyzed the expression of *Fgf10*, *Shh* and *Fgf8* in limbs of *Xt* and *Del(1-10)* compound mutants along with *Hoxd13*, being all these molecules crucial for limb morphogenesis. We focused on E10 stage of development when the AER is just formed and *Hoxd13* shifts from the early to the late phase of induction. In contrast with all the other genotypes analyzed *Xt/Xt*; *Del(1-10)/(Del1-10)* double homozygous limbs almost lack *Fgf10* expression. Only few cells are *Fgf10* positive in the anterior limb bud and no signal is detected in the remaining regions. The downregulation of *Fgf10* is associated to the accumulation of *Hoxd12* and *Hoxd13* transcripts in the anterior part of the limb bud. The *Shh* signal is restricted to an anterior small region underlying a short AER, which is labeled by the *Fgf8* expression. This *Shh* positive domain is no longer maintained owing the disruption of the AER.

As mentioned above in the text the outgrowth of the limb is promoted by the reciprocal signaling between bud mesenchyme and AER mediated by FGF. The *Fgf10* expression in lateral plate mesoderm of limb field induces *Fgf8* expression in the AER and the latter maintains *Fgf10* in mesenchyme (Ohuchi, et al., 1997). The selective inactivation of *Fgf8* or *Fgf10* receptor (*Fgfr2-IIIb*) in the ectoderm of the prospective AER at early developmental stage impairs FGF signaling and causes severe limb truncations (Lewandoski, et al., 2000; Moon, et al, 2000; Revest, et al., 2001, Sun, et al., 2002; Xu, et al., 1998). In absence of *Fgf8*, the expression of *Fgf10* is not longer maintained as well as the inactivation of *Fgf10* receptor affects both *Fgf10* and *Fgf8* in the AER (Moon, et al., 2000; Revest, et al., 2001). Thus one or more segments of the limbs are lost whenever the signal between *Fgf10* in the mesenchyme and *Fgf8* in the ectoderm is interrupted. These defects are the consequence of the degeneration of the AER and apoptosis or diminished proliferation of the precursor cells.

Despite the *5' Hoxd* gain-of-function in *Del(1-10)/Del(1-10)* mice *Fgf10* is still represented in two groups of cells situated at the anterior and posterior margin of in limb bud. This suggests that the deleterious effect of posterior *Hoxd* gain-of-function on *Fgf10* is buffered by the activity of *Gli3,* consequently the AER is viable and sufficient

to promote limb growth. The limb truncations of our *Xt/Xt*;*Del(1-10)/(Del1-10)* mutants correlate with the downregulation of *Fgf10* expression in limb bud mesenchyme, and with the premature degeneration of the AER. This provides evidence that in our mice the loop between *Fgf10* and *Fgf8* is interrupted. Remarkably, we noticed that in *Xt/Xt*;*Del(1-10)/(Del1-10)* limbs *Hoxd13* is gained in the same domain were *Fgf10* expression is lost. This supports the concept that in absence of functional *Gli3, 5'Hoxd* gene products block the synthesis of *Fgf10* in mesenchyme and indirectly affect *Fgf8* in the AER, whose disruption leads to the suppression of limb elongation.

We can imagine that the formation of the AER depends on the balance between anterior *Hox* genes and posterior ones that respectively promote or counteract the formation of the latter. As already mentioned the lack of both the *HoxD* and *HoxA* cluster affects the integrity of the AER and cause limb truncations (Kmita, et al., 2005). In our case the absence of *Gli3* and the gain of *5' Hoxd* gene expression in the limb anterior domain are sufficient to generate truncation in presence of the full *HoxA* cluster. The way by which *Gli3* interacts with *Hoxd* in wt limbs might be both at transcriptional and post-transcriptional levels (Chen, et al., 2004; Zuniga and Zeller, 1999). The mis-expression of *Hoxd12* in *Xt/+* background modifies the identity of digital pattern, compared to *Xt/Xt* mice where all digits are identical. It was found that *Hoxd12* and *Gli3* products bind each other and the digital phenotype was attributed to the interaction of these two proteins (Chen, et al., 2004). In this view *Gli3* might be a cofactor of *5' Hoxd* gene products, and protect the limb bud from the posterior prevalence exerted from the latter over the activity of more anterior *Hox* genes. The ratio between proteins might explain why if the dose of gained genes is very high *Gli3* activity is not sufficient to protect the limb from the effect of *Hoxd13* (Williams, et al., 2006). Conversely if the dose of gained genes is not elevated does not have impact on zeugopod morphogenesis of mice lacking *Gli3*.

Additional observations of the expression pattern of *Hoxd13*, *Shh*, *Fgf10* and *Fgf8* in double mutants indicated, that the polarity of the limb is completely inverted. For instance the posterior *Shh* domain is absent from double mutants limb buds. Nevertheless *Fgf10* expression is shifted from the posterior to the anterior-medial half of the limb bud in *Xt* mice, while is almost erased in *Xt/Xt;Del(1-10)/(Del1-10)*.

To circumvent the bias exerted from the AER on *Fgf10*/*Fgf8* loop we looked at the pattern of the latter at earlier stages (E9), when the AER is not yet formed. We found that *Fgf10* transcripts are restricted to the posterior half of *wt* limb bud, whereas this pattern is completely inversed in Xt/Xt limbs. This rotation depends on the lack of *Gli3* function, since is not observed in *Del(1-10)/Del(1-10)* mutants. In *Xt/Xt;Del(1-10)/Del(1-10)* limbs we observed both the inversion and the suppression of *Fgf10* signal. We then concluded that the rotation of *Fgf10* pattern is due to the lack of *Gli3*, whereas its suppression is consequent to the *Hoxd* gain of function. At present we don't have further clues that could elucidate the molecular mechanisms involved in this process.

The limb truncation observed in *Xt/Xt;Del(1-10)/Del(1-10)* mice is reminiscent of the consequences of aberrant retinoic acid signaling. The depletion of RA in rats at different gestational stages gives rise to hypoplastic limbs, associated with mis-regulation of *Hoxd13* and *Hoxd12* in limb mesenchyme, in addition to and *Fgf4/Fgf8* in the AER (Power., et al., 1999). The implantation of beads releasing RA showed that the latter mimics the activity of the ZPA and is involved in limb anterior-posterior specification (Tickle, et al., 2002). In addition RA induces the expression of *Meis1* genes in the proximal domain of the limb bud (Mercader, et al., 2000). The latter is a marker of the proximal part of the limb, and its over-expression is associated to the truncation of distal

limb (Capdevila, et al., 1999; Mercader, et al., 1999). The role of RA on proximo-distal limb development was further elucidated following the functional inactivation of the enzyme *Raldh2*, involved in the biosynthesis of RA from retinol. The lack of this gene caused severe limb truncations whose extent was finely artificially modified by the supplementation with RA (Niederreither, et al., 2002). In the worst case observed the phenotype consisted in a limb where the scapula was the only recognizable bone reminiscent of our *Xt/Xt;Del(1-10)/Del(1-10)* mice. Interestingly each considered category of *Raldh2-/-* mutants displayed deviations of *Shh*, *Fgf8* and *Fgf10* from *wt* pattern. *Shh* signal was absent or localized in distal position of the limb bud. *Fgf8* mirrored the formation of a short, or malformed, AER, while *Fgf10* expression was often biased trough a more anterior domain. On the other hand either *Gli3* or *Hoxd11,* and *Hoxd12* transcripts were abnormal, the former being less abundant in the anterior limb bud and the latter gained all over the anterior-posterior limb axis, and in a more proximal domain (Niederreither, et al., 2002).

In contraposition with *Raldh2* deficiency, it is possible to modify RA signaling affecting its degradation, or else inactivating the isoform 1B of the enzyme CYP26 and causing an excess of RA signaling in limb buds (Yashiro, et al., 2004). The limbs of these mice were often hypodactylous and carried fusions between the zeugopod and stylopod, making difficult to discriminate the identity of bones. The analysis of markers in mutant limb buds showed that the expression of *Hoxd12* and *Hoxd13* was downregulated at the expense of the distal expansion of *Meis1* and *Meis2* expression domains. Therefore the absence of *Cyp261b*, as well as the excess of RA caused limb proximalization. One of the common outcomes of the works reported above was that RA affects limb development along the PD axis by mis-regulation of *5' Ho*x gene expression, along with the ZPA and AER defects. However the mechanism by which the two phenotypes were originated is different. One of the key molecules beside *Fgf* and *Hox* was represented by *Meis1*. While *Cyp261b-/-* mice showed a more distal restriction of *Meis1* mRNA in limb bud (Yashiro, et al., 2004) the expression boundary of the latter in *Raldh2-/-* mice was not displaced to ectopic position. To show that RA signalling was received from limb bud in mutants we analyzed *Meis1* expression in all our genetic combinations, including *Xt/Xt;Del(1-10)/Del(1-10)* mice and we did not find difference between these and wt. Thus proximal identity identified by *Meis1* expression was maintained in mutants, suggesting that proximal limb truncation were not due to the mis-positioning of the anatomic boundary between stylopod and autopod.

RA acts upstream *Hox* transcription and induces their collinear expression in cultured cells (Simeone, et al., 1990). On the other hand the phenotype we observe in our mutants is similar to that originated by the Vitamin A deficiency as shown by the gain of *5' Hox* gene function and the alteration of *Shh*, *Fgf8* and *Fgf10* expression pattern. Therefore we can speculate that during limb morphogenesis both RA and *Gli3* counteract the deleterious effect of a premature or ectopic 5'*Hox* expression.

Discussion second paper

Prior to the wave of epithelial differentiation, the GI tube is constituted by an inner lining of epithelium, and an outer lining of mesenchyme. The area represented by the esophagus and stomach is called foregut whereas midgut and hindgut contribute to the small and large intestine. The most anterior part of large intestine is situated posterior to the caecum, the latter of which emerges from GI at E11.5 stage and elongates until E18 (reference Burns, et al., 2004). At early stage of development the caecum is located inside the embryo, later owing the coiling of the gut inside the abdomen it protrudes out of the abdominal wall together with the intestinal hernia. However this situation is not definitive and the intestinal hernia returns inside the body by the end of the gestation (Kaufman and Bard, 1999).

We became interested in determining the *Hox* influence on embryonic caecum development as these genes were shown to be involved in GI specification along the anterior posterior axis.

Former studies focused on the *Hox* expression profile in the developing gut have shown that members of each cluster are induced along the anterior-posterior axis of the gut primordium at fetal stages (Deschamps and Wijerde, 1993; Dollé, et al., 1991; Gaunt, et al., 1988; Gaunt et al., 1989; Geada, et al., 1992; Izpisua-belmonte, et al., 1990, Le Mouellic, et al., 1988). In this respect, a substantial amount of data is provided by a few works (Kawazoe et al., 2002; Pitera, et al., 1999; Sekimoto et al., 1998) that carried out the systematic analysis of *Hox* expression by in situ hybridization at crucial stages of gut morphogenesis. These papers show that the *Hox* anterior expression boundaries in GI are established from E12.5 stage onwards. However the *Hox* profile along the AP axis of the GI is discontinuous as a number of genes are represented in stomach and in posterior midgut, whereas are excluded from the anterior intestinal segment. The common conclusion drown by these studies was that the *Hox* activity sustains the specification of the gut, like in vertebral column and neural tube. This assumption was supported by the description of mice carrying gastric or intestinal malformations following *Hox* inactivation (see introduction of this thesis for a detailed description).

The caecum is a functionally and anatomically peculiar component of the posterior midgut. It is amazing how this organ can reach a complex structure and huge size in mammals like rabbits (Snipes, et al., 1982). The caecum has a microbial flora that helps to degrade the fibers contained in the food completing the digestive process started in stomach (Backhed et al., 2005; Eckburg, et al., 2005). Comparative studies in mammals showed that herbivores develop a more voluminous caecum than carnivores, reflecting their need to process large amounts of cellulose. Albeit mice are omnivores, the caecum in adults is bigger than stomach. Here the food is retained, further metabolized then expulsed into the large intestine. The passage of chow from small intestine to the caecum is regulated by the activity of a muscular ring represented by the ileo-caecal sphincter. Previous studies showed that *Hoxd* loss-of-function hinder the formation of this sphincter (Zakany and Duboule 1999), illustrating a role of the *HoxD* cluster in the posterior midgut.

With the present work we first established the *Hoxd* transcription profile in GI at E12.5 stage of development, when the gut is still undifferentiated but the principal subdivisions along the AP axis are already delineated. To this purpose we performed in situ hybridization analysis on whole mount GI dissected from wt embryos by using RNA probes specific for each *Hoxd* gene. We found that at this stage of development all the members of the *HoxD* cluster are transcribed in wt GI. Interestingly transcripts of all

genes from *Hoxd1* up to *Hoxd11* are well detected in the caecum, in contrast to *Hoxd12* and *Hoxd13* that are excluded from this region. The signal from *Hoxd1* to *Hoxd10* stops just posterior to the caecum bud. Curiously, *Hoxd11* is transcribed exclusively in the posterior half of the bud through the anterior part of the large intestine. Previous experiments had suggested the presence of a "hernia gut enhancer" positioned at the same 3' end side to the *HoxD* cluster (Kmita, et al., 2000-b). While the genes from *Hoxd1* to *Hoxd10* are expressed in early caecum bud under the influence of this enhancer, *Hoxd12* and *Hoxd13* escape from its activity. The detection of such great number of *Hoxd* genes in embryonic caecum suggested that the simultaneous requirement of their products is probably needed for the morphogenesis of this organ. Conversely the expression of *Hoxd12* and *Hoxd13* may affect the growth of caecum in negative way. Indeed their mRNA is restricted to the most terminal GI segment represented by the rectum. Thus focusing on the ileo-colonic transition we remarked that *Hoxd* genes fall into two groups of 3' end and 5' end genes. While the 3' end, or "anterior" genes are expressed in caecum bud the 5' end or "posterior" ones are limited to the terminal part of the GI.

In foregut and midgut *Hoxd* function seem not to be relevant. Indeed *Hoxd3* is the only gene transcribed in stomach and none of *Hoxd* genes is expressed in small intestine. These findings suggested that the induction of *Hoxd* genes in developing GI does not follow the collinearity rule, at least not as in trunk. The work of Sekimoto analyzed the pattern of three consecutive genes from different paralogous groups and showed collinearity in intestine but not in stomach (Sekimoto, et al., 1998). Kawazoe (et al., 2002) has built a *Hox* expression profile grouping results from the two works. A number of genes are expressed in stomach, like *Hoxb4* and *Hoxb8* despite their position within the *HoxB* cluster. Conversely none of the *Hoxd* gene analyzed (*Hoxd4-d8-d9*) was transcribed in stomach mesenchyme, in agreement with our observations. In E12.5 caecum *Hoxa5-a6-a7-a9*; *Hoxb5-b7-b8*; *Hoxc5-6-8-9*; *Hoxd4-d8-d9* are all represented (Sekimoto, et al., 1998; Kawazoe, et al., 2002). Among them genes like *Hoxb5* to *Hoxb8* are transcribed in stomach, absent from the anterior midgut and rising in posterior midgut and caecum. This appearance is clearly not collinear. This complex expression pattern is intriguing; it suggests that a combination of diverse *Hox* proteins is needed in functionally distinct GI elements and let us suppose the existence of a special mechanism of regulation of *Hox* transcription.

As the fetal stomach, small and large intestine, the caecum bud is composed of endoderm surrounded by mesoderm. Caecum elongation occurs by the simultaneous growth of the epithelial bud and mesenchymal anlagen around it, likely relying on reciprocal interactions between these two layers. The histological analysis of labeled caecum bud at E12.5 showed that the expression of anterior *Hoxd* genes, including *Hoxd11,* is restricted to the mesenchymal layer. By contrast the epithelium of the bud is negative. *Hoxd12* transcripts are also restricted to the mesenchyme of the terminal GI tract, whereas only *Hoxd13* mRNA is found both in rectal mesenchyme and epithelium. Similarly the mRNA profile of the murine *HoxA*, *HoxC* and *HoxB* clusters in GI is limited to the mesenchyme (except for *Hoxa13* that is transcribed in rectal epithelium). Presumably the mRNA and its product coexist within the same embryonic layer however the lack of specific antibodies made impossible the immunocytochemical detection of *Hox* proteins in caecum bud. To circumvent this problem we took advantage of an engineered mouse line where the *egfp* coding sequence was inserted in the *Hoxd11* gene (Cobb, J., data not shown). In these mice the 5' end of *Hoxd11* gene and *egfp* are transcribed on the same mRNA. The analysis of dissected GI at stages corresponding to caecal elongation suggested that likely the distribution of *Hoxd11*

protein matches that of *Hoxd11* mRNA. Indeed the fluorescence was limited to the mesenchyme of caecum and colon, confirming the results obtained by in situ hybridization. Because of these results we concluded that the specification of the caecum involves a number of *Hox* products acting in mesenchymal cells, through inductive interactions with the epithelium.

Despite the abundance of descriptive data showing *Hox* expression in GI, to our knowledge none of the precedent studies assessed the *Hox* function in caecum budding. However the interference of a "non-*Hox*" homeodomain protein with caecum growth has been observed. In this context a group showed the absence of the caecum in transgenic mice expressing *Pdx1*, under the control of *Hoxa4* promoter (Heller, et al., 1998). Under normal circumstances *Pdx1* is transcribed in the endoderm of the pancreatic primordium, however in these transgenics it was misexpressed in mesenchyme of the transition between small and large intestine, mirroring *Hoxa4* pattern. Unexpectedly transgenic mice showed dysmorphogenesis of the proximal colon and lacked the caecum. This work suggested that *Pdx1* might compete with other homeobox proteins for the control of caecum specification.

To assess the contribution of *HoxD* cluster in caecum morphogenesis we took advantage of a number of strains in which the *HoxD* cluster was modified by internal deletion of genes. In particular newborn mice carrying a deficiency spanning from *Hoxd1* up to *Hoxd10 (Del1-10)* (described in Zakany, et al., 2004) displayed the complete agenesis of the caecum when in homozygous configuration. Curiously heterozygous developed a caecum of reduced size compared to wt. This defect was also observed at E12.5 stage of development, as the caecum bud was smaller. Since the *Del(1-10)* deletion removes six out of nine *Hoxd* genes, it might be argued, that the agenesis of the caecum was caused by the loss-of-function of the anterior genes. However mice lacking the *HoxD* cluster develop a caecum of correct size, suggesting that is not the gene deficiency that blocks the caecum bud outgrowth.

The *Del(1-10)* deletion "displaces" the most posterior genes left in the cluster nearby to the early limb control region ELCR located 3'end to the cluster (Zakany, et al., 2004). This region was discovered because in these mice *Hoxd11* and *Hoxd13* are gained in the anterior limb bud mesenchyme at early stage of development, like anterior genes in *wt*. In similar way the *Del(1-10)* deficiency may position the *Hox* posterior genes in proximity to the hernia gut enhancer likely affecting their expression in GI. To assess this prediction we performed *in situ* hybridization on E12.5 *Del(1-10)* homozygous and heterozygous embryos to detect the expression of *Hoxd11*, *Hoxd12* and *Hoxd13* in GI. The pattern of these three genes in rectum remained unchanged. However in all *Del(1-10)* homozygous and heterozygous specimens both *Hoxd11* and *Hoxd12* were gained all over the caecal region. *Hoxd13,* was still absent from caecum bud even in homozygous. The accurate inspection of embryonic layers revealed that both *Hoxd11* and *Hoxd12* were gained in mesenchyme up to the limit represented by the ileo-caecal junction whereas the epithelium remained negative. We assumed that *Hox* gain-of-function in *Del(1-10)* posterior midgut suppresses the caecum bud formation. The more recently established *Del(4-11)* line lacking from *Hoxd3* up to *Hoxd11* strongly supported this analysis. As in *Del(1-10)* mice, *Del(4-11)* homozygous lack the caecum and heterozygous develop a caecum of intermediate proportions. In *Del(4-11)* mice only *Hoxd12* was gained in the intestinal region between the ileum and colon, as *Hoxd13* transcripts remained limited to the rectum. On the basis of these results we concluded that ectopic *Hoxd12* is sufficient alone to abolish caecum budding in *Del(4-11)* line. Concerning *Del(1-10)* mice we can imagine two alternative scenarios: either the product of *Hoxd11* and *Hoxd12* are both contribute to inhibit caecal morphogenesis,

or else *Hoxd12* is dominant over *Hoxd11* activity and is the only responsible for the phenotype.

In summary, since *Del(1-13)* homozygous have a caecum of normal proportions we concluded that the agenesis of the caecum in *Del(4-11)* and *Del(1-10)* lines is related to the gain of function of posterior *Hox* genes, *Hoxd12* in particular, over anterior ones (from the same and/or other complexes), and not to the loss-of-function of the first six genes of the *HoxD* cluster. These results agree with the posterior prevalence model, according to which 5' end *Hox* genes compete with 3' end at post-transcriptional level for the control of a subset of common target genes. Partial deletion of the *HoxD* cluster led to gain-of-function of posterior genes at the ileo-colonic transition hampering the execution of a predetermined developmental program that sustains the caecum growth.

Since the *Hox* gain-of-function in trunk confers a posterior fate to anterior vertebrae, one might argue by analogy that the agenesis of the caecum might represent a from a homeotic transformation. We did not observe any misplaced structure morphologically similar to an ectopic caecum along the AP axis of GI of mutants. Nevertheless the agenesis of this organ might be a consequence of mis-specification of the posterior midgut, following the transcriptional deregulation of *Hox* genes normally expressed in this region. We proved that this is not the case as *Hoxa10* and *Hox6* expression in GI of deleted mice was maintained in the appropriate region. Curiously *Hoxa6* expression in *Del(1-10)* was observed in fewer mesenchymal cells compared to *wt* caeca. It might be possible that the *Hox* gain-of-function influences *Hoxa6* regional expression indirectly, affecting an intermediate factor involved in *Hoxa6* induction or maintenance.

The caecum of homozygous *Del(4-11)* and *Del(1-10)* newborns is reduced to a thin protuberance without lumen, and the epithelium is surrounded by a poor amount of mesenchyme. *Fgf10* is expressed in caecum mesenchyme, whereas its receptor is expressed in overlying epithelium (Burns, et al., 2004; Fairbanks, et al., 2004-a). In lung primordia *Fgf10* functions as chemoattractant and mitogen interacting with its receptor *(*FGFR2b*)* localized in the epithelium (Bellusci, et al., 1997; Park, et al., 1998), suggesting a similar activity in early caecum. Mice deficient for *Fgf10* gene, as well as mice lacking FGFR2b, display a number of gastro-intestinal defects, one of which is the atresia of the caecum (Burns, et al., 2004). In our homozygous *Del(4-11)* and *Del(1-10)* mice the caecum is not atretic but absent: since a bud does not form the epithelium cannot invade the mesenchyme. We focused on *Fgf10* as factor likely involved in caecum outgrowth. Indeed the *Fgf10* expression in homozygous caeca was strongly downregulated, in heterozygous was proportionally more abundant but never reached the *wt* pattern.

In *wt* the expression profile of *Hoxd1* up to *Hoxd10* in caecum mesenchyme overlaps with *Fgf10*. In *Del(1-10)* and *Del(4-11)* mice *Hoxd12* and/or *Hoxd11* are ectopically expressed in the ileo-caecal loop and *Fgf10* is downregulated in the same area. In our view *Hox* anterior genes support caecum development reinforcing the *Fgf10* pathway, but they are antagonized by the gain of posterior *Hox* genes, *Hoxd12* in particular. The latter suppress the formation of the caecum bud through *Fgf10* inhibition. So far a correlation between *Hox* gain-of-function and *Fgf10* expression has been found only in limbs of *Del(1-10)* mice (paper 1 of this thesis).

Despite the absence of epithelial bud the ileo-colonic transition of *Del(1-10)* and *Del(4-11)* mice is marked by a small and flat mesenchymal protuberance, which is not devoid of *Fgf10* mRNA. Similarly *Fgf10* null mice have a more pronounced mesenchymal bud that mainly degenerates at late stages of development (Burns, et al., 2004). These two observations suggest that *Fgf10* is not essential for the initiation of

caecum bud, but for the formation of epithelial sprout. Thus *Hoxd12* and/or *Hoxd11* affect the caecum outgrowth inhibiting its elongation.

Since a direct interaction between *Hox* gene products and the and *Fgf10* gene has not been assessed, we searched for molecules that might relay the two categories of genes in a functional pathway.

We considered *Pitx1* as a homeobox gene expressed all along the developing GI, both in mesenchyme and epithelium (Lanctot, et al., 1997; and data not shown). *Pitx1*, *Pitx2* and *Pitx3* represent a family of transcription factors produced in different anatomical parts of the embryo (reviewed in Gage, et al., 1999). *Pitx1* is expressed in stomodeum (ectodermic origin), and mesenchyme of the first branchial arch. In the posterior part of the body this factor is well known because of its expression in mesenchyme of embryonic hindlimbs (Lanctot, et al., 1997; Marcil et al., 2003). In contrast to *Pitx2* that is involved in the development of asymmetric organs like heart and gut (Logan, et al., 1998-b; Campione, et al., 1999), *Pitx1* function in GI has not been established yet. We found that the gain-of-function of posterior genes in *Del(1-10)* mice represses *Pitx1* expression in hindlimb mesenchyme (data not shown), suggesting that *Hox* genes and *Pitx1* genetically interact within this region.

Thus by analogy with the limb model we sought to verify if *Pitx1* is expressed in wt caecum. Our results showed *Pitx1* mRNA in caecum bud epithelium and in the overlying mesenchyme. Conversely *Del(1-10)* and *Del(4-11)* homozygous mice lack *Pitx1* expression in mesenchyme, while heterozygous displayed a reduced intensity of the signal. Still the *Pitx1* expression profile in embryonic caecum mirrors that of *Fgf10* with a relevant difference. While *Pitx1* expression in homozygous ileo-caecal transition is abolished, *Fgf10* is still induced in a small group of mesenchymal cells. We concluded that *Pitx1* and *Fgf10* activity in caecum bud are related and likely the amplification of *Fgf10* signal in caecum bud depends on *Pitx1* activity. The group of Bobola et al., (2003) showed that *Hoxa2* represses *Pitx1* transcription in the second branchial arch, acting on *Fgf* signaling. In this specific case the mis-expression of *Hoxa2* in first branchial arch inhibited the local Fgf expression and blocked the synthesis of *Pitx1* in *Fgf* responsive cells. By analogy we reasoned that in early caecum *Pitx1* might be readout of mesenchymal *Fgf10*. The *Hox* misexpression in *Del(1-10)* and *Del(4-11)* would render the cells insensitive to *Fgf10*, inhibiting the synthesis of *Pitx1*. The absence of the latter product would affect *Fgf10* expression in the same cells, blocking the expansion of *Fgf* signal in paracrine way. In order to validate our assumptions it would be useful to assess whether *Pitx1* null mice develop the caecum in their intestine.

In attempt to better elucidate the mechanism by which *Hox* genes affect caecal elongation, we analyzed other markers like *Shh*, which is normally transcribed in the epithelium of developing GI, and its receptor *Patched* induced in the adjacent mesenchyme. In chick GI the mis-expression of *Shh* induces ectopically *Hoxd11* and *Hoxd13* at the site of injection (Roberts et al., 1995). Conversely the *Hoxd11* gain-of-function in the anterior forelimbs mesenchyme of *Del(1-10)* mice induces the expression of *Shh* in the same region (Zakany et al., 2004). These works showed that even in different anatomic context, the activity of *Shh* and *Hox* genes are related. In accordance with the limb model, the *Hox* gain-of-function in GI of *Del(1-10)* animals may activate the *Shh* pathway in intestinal mesenchyme. Thus we controlled the expression of both *Shh* and *Patched* in homozygous *Del(1-10)* mice (data not shown). However we did not observe any divergence from the wt pattern. Thus we concluded that caecum agenesis in these mutants is not associated to the alteration of *Shh* pathway. An ulterior confirmation derives from the analysis of GI of *Xt* mice. We know that *Gli3*

is upstream *Hox* genes, and regulate negatively their transcription (Zuniga and Zeller, 1999). However and despite the presence of gastric anomalies described in these mice (Kim, et al., 2005), their caecum developed a normally (data not shown), suggesting that the function of *Gli3* is not crucial in this context. Analogously the *Xt* mutation on *Del(1-10)* heterozygous background did not show defect in caecal morphogenesis (data not shown).

Qualitatively one of the co-expressed *Hoxd* genes may be more relevant than others in promoting caecum morphogenesis. On the other hand we can assume that *Hoxd* anterior genes are functionally redundant in caecum bud and that all of them are necessary to specify this region. In contrast the size of the caecum in *Del(1-10)* GI is inverse proportional to the dose of posterior genes gained ectopically. This is consistent with the caecum agenesis in homozygous and dysplastic caecum in heterozygous animals (in the latter one dose of anterior and one of posterior genes coexist in bud mesenchyme). To assess *in vivo* the opposite effect of 3' end and 5' end *Hox* genes on caecum growth we established a complementation assay. This test allowed us to manipulate the gene dose of anterior genes over a constant amount of *Hoxd11* and *Hoxd12* in ileo-caecal transition of *Del(1-10)* mice. Basically we imagined that the sequential addition of *Hox* genes at the 3'end of the cluster might restore the bud elongation in *Del(1-10)* background. Therefore we crossed *Del(1-10)* mice both with *Del(1-13)* and *Del(4-13)*. Then we dissected the GI of double recombinant and evaluate the size of caecum in newborns compared to controls. According their size and aspect we distinguished dissected caeca in phenotypic classes: agenesis, hypoplasia, and dysplasia. The hypoplastic caecum (or atretic) is usually very short and does not have epithelium; dysplastic caecum is represented by a longer and thin bud of epithelium surrounded by a reduced amount of mesenchyme. We reasoned that *Del(1-10)/Del(1-13)* mice would show a small caecum compared to *Del(1-10)/Del(4-13)*, which coexpress *Hoxd1*, *Hoxd3*, *Hoxd11* and *Hoxd12* in the same GI segment. However the gross analysis revealed that the size of caecum was pretty similar in both the categories. This is consistent with the absence of caecum observed in *Del(4-11)* mice, where *Hoxd1*, *Hoxd3*, *Hoxd12* and *Hoxd13* are left in the cluster. Thus one haplotype of *Hoxd1* and *Hoxd3* is not sufficient to balance the suppressive activity of ectopic posterior genes on caecum growth. Pups obtained from breeding of mice carrying from *Hoxd1* up to *Hoxd4* (*Del(8i-13)*) with *Del(1-10)* never showed more than a hypoplastic caecum bud. The situation changed dramatically when *Del(1-10)* mice were crossed with a strain lacking *Hoxd11*, *Hoxd12* and *Hoxd13* (*Del(11-13)*). All compound mutants had a caecum with a size comparable to that of *Del(1-10)* heterozygous mice. In conclusion the addition of *Hoxd8*, *Hoxd9* and *Hoxd10* in *trans* is decisive to restore the development of the caecum. Reasoning in terms of genetic dose of gained genes we concluded that one haplotype of *Hoxd* anterior genes is necessary to counteract the gain of posterior *Hox* genes. At this genetic resolution we were not able to discriminate between the influence of the *Hoxd8*, *Hoxd9* and *Hoxd10* addition to the cluster. To assess the impact of *Hoxd8* and *Hoxd9* over *Hoxd11* and *Hoxd12* on caecum morphogenesis it would be interesting to breed *Del(1-10)* with *Del(10-11)* mice (lacking only *Hoxd10* and *Hoxd11*).

Unfortunately *Del(1-10)* and *Del(4-11)* homozygous mice die soon after birth, and don not allow us to assess the impact of the absence of caecum on their metabolism. What we know is that in young litters the development of a hypoplastic caecum may have consequence on survival of the animal. We observed that before weaning *Del(1-10)/Del(4-13)* and *Del(1-10)*/*Del(1-13)* litters do not increase their body weight as *wt*, a great majority of these compounds mice died within this period. *Del(4-11)* heterozygous

mice displayed a body mass consistently inferior compared to *wt*. All these animals had a short gut phenotype, but we were not able to discriminate if this phenomenon is the cause of the death rather than the effect of the "stunted" growth. According to our observations of *Del(4-11)* heterozygous in particular, the presence or absence of caecum in mice is determinant for the accumulation of body mass in the first three-four weeks of life.

In humans the caecum ends with a specialized structure called appendix that corresponds to the true apex of caecum. Several medical reports describe as common the absence of the appendix however there is not a recent documentation about the congenital absence of caecum. Collins (1951) classified the abnormal caecal development in humans in groups from one to five. In particular the group one includes individuals without caecum and appendix, the group two is represented by a rudimentary caecum without appendix, whereas in group three the caecum is normal but lacks appendix. The class one anomalies are very rare (10 per cent of cases examined) and likely are not the cause of death of the analyzed individuals, some of which had quite long survival. From these results we deduce that despite its relatively complex organization the caecum in humans is not fundamental for life.

Résumé en français.

Durant le développement de vertébrés, les gènes *Hox* sont exprimés de manière colinéaire au niveau de l'axe principal du corps et de membres. Cela signifie que leurs domaines d'expression reflètent l'ordre qui lie les gènes dans le complexe. La coordination, spatiale et temporelle, de l'expression des gènes qui font partie de complexes *HoxA*, *HoxB*, *HoxC* et *HoxD* contribue de manière décisive à la spécification des régions du corps, suite à l'activité différentielle de facteurs de transcription homeotiques produits par chacun des 39 gènes *Hox*.

Jusqu'à présent la fonction des gènes *HoxA* et *HoxD* a été particulièrement étudiée dans le tronc aussi que dans les membres des tétrapodes. Plus récemment des nouvelles études montrent un rôle joué par les *Hox* dans le développement de l'appareil gastro-intestinal.

Avec cette dernière étude qui fait l'objet de cette thèse, nous avons concentré nos efforts sur la fonction des gènes *Hox* dans le développement des membres, ainsi que de l'appareil digestif. Ce travail résume les résultats des expériences conduites sur des lignées des souris où plusieurs gènes *HoxD* à la fois ont été éliminés de façon ciblée. Ces lignées ont été nommées respectivement *Del(1-10)* et *Del(4-11)* pour indiquer précisément le groupe de gènes qui ont été supprimés par la délétion.

Du point de vue anatomique, les quatre membres des tétrapodes sont caractérisés par un segment proximal près du corps, ou stylopode, un segment intermédiaire ou zeugopode et un segment distal, l'autopode. Ce dernier correspond à la main au niveau des membres supérieurs et au pied, dans les membres inférieurs. Les membres croissent selon les trois axes de polarité proximo-distal, antéro-postérieur et dorso-ventral. Pendant le développement embryonnaire des membres, les gènes *HoxD* sont caractérisés par deux phases d'expression. Durant la première et plus précoce, tous les gènes du groupe (de *Hoxd1* à *Hoxd13*) sont transcrits dans le bourgeon. L'ARNm des gènes antérieurs (*Hoxd1* à *Hoxd9*, qui sont localisés à l'extrémité 3' du complexe) est distribué dans le bourgeon de façon uniforme, en revanche les transcrits des gènes postérieurs (*Hoxd10* à *Hoxd13*, qui sont localisés à l'extrémité plus 5') sont exprimés dans le domaine le plus postérieur. Durant la deuxième phase (tardive) seulement les gènes *Hoxd9* à *Hoxd13* sont activés, et parmi ces derniers *Hoxd11* à *Hoxd13* sont transcrits dans le domaine le plus distal correspondant aux doigts (et aux orteils). Cette organisation spatiale et temporelle est responsable du développement des membres dans ses segments selon l'axe proximo-distal.

L'analyse génétique des souris a montré que l'anatomie de chaque partie des membres est déterminée par la fonction du gène *Hoxd* qui est le plus postérieur parmi les gènes qui sont transcrits dans la même région. Par exemple, la fonction de *Hoxd9* est limitée à l'humérus, qui est l'élément osseux le plus proximal, alors que *Hoxd12* et *Hoxd13* sont prédominants dans le développement de la main et des doigts. Cette propreté caractéristique des Hox prend le nom de « prévalence postérieure ». Selon les principes de cette règle, les gènes Hox qui sont exprimés dans la même cellule sont soumis à une hiérarchie fonctionnelle entre gènes antérieurs et postérieurs, qui se manifeste au niveau des protéines.

Dans notre laboratoire, plusieurs sites *loxP* ont été placés dans des positions stratégiques au sein du groupe *HoxD*, afin d'éliminer de façon ciblée un certain nombre de gènes. L'utilisation d'une technique (TAMERE) basée sur des événements de recombinaison méiotique, qui ont lieu dans la ligne germinal des souris mâles entre *loxP*

situés sur chromosomes différents, a permis de créer un nombre élevé de réarrangements génomiques.

Deux des lignées obtenues sont nommées respectivement *Del(1-10)* et *Del(4-11)* et présentent des délétions partielles du groupe *HoxD* qui éliminent respectivement les gènes *Hoxd1* à *Hoxd10*, ou *Hoxd4* à *Hoxd11*.

La délétion *Del(1-10)* provoque l'expression prématurée de *Hoxd12* et *Hoxd13* en position ectopique dans le bourgeon des membres. Cette dernière est suivie par la transcription de *Shh* dans le même domaine, qui est la cause de la formation d'une symétrie double postérieure des os de la main et des doigts. Le nombre des doigts et leur morphologie sont contrôlés par la fonction du facteur de transcription Gli3. De fait, une lignée de souris caractérisée par une mutation qui inactive le gène *Gli3* (*Xt*) développe des membres polydactyles. Dans cette lignée, l'expression des gènes Hox postérieurs est activée ectopiquement dans la partie antérieure du bourgeon. Donc il a été supposé que Gli3 contrôle le nombre de doigts en supprimant la fonction des gènes *Hox* postérieurs dans le bourgeon antérieur. Afin de découvrir ultérieurement la relation existant entre *Gli3* et les gènes *HoxD* dans le développement des membres antérieurs nous avons croisé des souris avec une complète déficience du complexe (cette lignée est nommée *Del(1-13)*) avec des souris *Xt*. Ensuite nous avons analysé la morphologie des membres des nouveau-nés. De façon inattendue, le nombre et la morphologie des doigts étaient les mêmes que chez les souris *Xt*, ce qui suggère que le gain de fonction des gènes *Hox* postérieurs n'est pas la cause de la polydactylie. Néanmoins, le gain de fonction des gènes *Hoxd11*, *Hoxd12* et *Hoxd13* dans la lignée *Del(1-10)* est souvent la cause de la polydactylie. Pour mettre au clair cette très complexe situation nous avons croisé des souris *Del(1-10)* avec des souris *Xt*, afin de pouvoir déterminer l'influence de Gli3 sur le gain de fonction des gènes *HoxD* postérieurs. Nous avons remarqué que les souris homozygotes pour la délétion *Del(1-10)* en plus d'une symétrie de l'autopode avaient un humérus plus court. Le défaut du membre proximal était renforcé chez les souris doubles homozygotes *Xt* et *Del(1-10)* avec une sévère troncation des membres supérieurs où l'humérus avait disparu alors que le reste du membre montrait seulement des restes de zeugopode et d'autopode. Une analyse détaillée a montré que l'humérus se raccourcissait progressivement, proportionnellement avec la réduction de la dose de *Gli3* et l'augmentation de l'expression ectopique de *Hoxd12* et de *Hoxd13*. La progression de la croissance des membres supérieurs dépend des facteurs FGF10 et SHH sécrétés par le mesechyme et de FGF8 qui est secrété par la « crête épidermique apicale » (CEA en français, ou AER en anglais, qui signifie « apical ectodermal ridge »). La sévère troncation des membres supérieurs a suggéré que chez les souris *Xt* et *Del(1-10)* double mutants, l'établissement ou le maintien de la CEA sont fortement compromis, probablement par interférance avec les facteurs FGF10 et SHH sécrétés par le mésenchyme. Les hybridations in situ effectuées sur les doubles mutants à un stade précoce, quand la CEA vient juste de se former, ont montré une perturbation de l'expression normale de tous ces gènes. Dans les membres supérieurs de souris *Xt* et *Del(1-10)* l'expression de *Hoxd13* était augmentée dans le mésenchyme antérieur, alors que l'expression de *Fgf10* était diminuée dans le domaine le plus proximal et déplacée dans une zone complémentaire à la zone d'expression de *Hoxd13*. Dans le bourgeon de membres des souris double homozygotes, *Hoxd13* était transcrit dans l'extrémité antérieure du mésenchyme, alors que Fgf10 n'était quasiment plus exprimé. Dans le même contexte *Shh* n'était plus exprimé dans la partie postérieure du bourgeon alors qu'il apparaissait dans un groupe de cellules localisées dans la marge antérieur-mediane. La CEA était maintenue seulement dans cette région particulière, tandis qu'elle était absente dans le reste du bourgeon, comme le montrait l'importante perte du signal

Fgf8. Donc l'expression prématurée ou ectopique de *Hoxd12* et *Hoxd13* en absence de la fonction de *Gli3* arrête la croissance du bourgeon en interférant avec les facteurs secrétés par le mésenchyme et dont la fonction est de préserver l'intégrité de la CEA. En conséquence la croissance proximo-distale des membres supérieurs est sévèrement perturbée.

De la même manière que dans les axes principaux du corps, les gènes *Hox* sont fonctionnels dans le système gastro-intestinal du fœtus. De nombreuses études ont présenté une analyse systématique de l'expression des gènes *Hox* dans le système gastro-intestinal embryonnaire, mais jusqu'à présent leur profil complet n'est pas connu. Étant donné que des mutations dans les gènes *HoxA* et *HoxC* sont la cause des malformations de l'estomac ou de l'intestin, il a été suggéré que les gènes *Hox* sont impliqués dans la spécification de l'identité régionale de l'intestin. Il a été aussi montré que *Hoxd12* et *Hoxd13* sont nécessaires pour la morphogènese des sphincters anal et iléo-cæcal. Pour ce que concerne leur mécanisme d'action, les gènes Hox participent aux échanges moléculaires entre épithélium et mésenchyme dirigeant la spécification du système gastro-intestinal au niveau de l'axe antérieur-posterieur. Le développement du caecum dans la souris représente une situation favorable pour l'étude de la fonction des gènes Hox dans la morphogènese de l'intestin. Le caecum est un organe situé entre l'intestin grêle et le colon chez les mammifères. Sa taille est particulièrement importante chez les animaux avec un régime alimentaire riche en fibres. La morphogènese du caecum chez la souris commence au jour 11 du développement embryonnaire, à partir du moment où le segment d'intestin d'où il émerge sort de la paroi abdominale en formant l'hernie intestinale. Au début de notre étude nous avons effectué une analyse systématique de l'expression des gènes du groupe *HoxD* dans l'intestin embryonnaire de la souris. Nous avons observé que la majeure partie des gènes *HoxD* est transcrite dans le mésenchyme du caecum et qu'en particulier tous les gènes de *Hoxd1* à *Hoxd11* sont exprimés dans le bourgeon du caecum alors que *Hoxd12* et *Hoxd13* sont exclus de la même région. Ces observations ont suggéré que la présence ou l'absence du produit des gènes *Hox* dans le bourgeon du caecum pouvait jouer un rôle clé dans sa croissance. Effectivement, les souris homozygotes pour la délétion *Del(1-10)* présentent une complète absence du caecum, alors que les souris hétérozygotes développent un caecum de taille réduite. Pour découvrir les facteurs moléculaires impliqués dans la suppression de la croissance du caecum nous avons effectué une hybridation in situ sur la totalité de l'appareil digestif embryonnaire en utilisant des sondes capables de détecter l'activité des gènes *HoxD* restés intacts dans le complexe. Dans le caecum de souris *Del(1-10)*, *Hoxd11* et *Hoxd12* sont transcrits avec une limite antérieure atteignant la jonction entre l'iléon et le caecum. Ensuite, nous avons poursuivi nos recherches sur une autre souche de souris, appelée *Del(4-11)*. Dans ces dernières, les gènes *Hoxd1*, *Hoxd3* et *Hoxd12*, *Hoxd13* étaient intacts, respectivement aux extrémités *3'* et *5'* du groupe *HoxD*. Les souris *Del(4-11)* n'ont pas de caecum. Les souris homozygotes et hétérozygotes pour cette délétion montrent une expression ectopique de *Hoxd12* dans la région à partir de laquelle le caecum se développe. Dans ce cas, le produit du gène *Hoxd12* est capable de supprimer l'activité morphogénétique de HOXD1 et HOXD3 en bloquant la formation du caecum. En cherchant d'autres facteurs capables de promouvoir la morphogènese du caecum nous avons trouvé que les souris dans lesquelles la fonction de *Fgf10* est compromise présentent une atrésie du caecum. Dans nos souris *Del(1-10)* et *Del(4-11)*, l'expression de *Fgf10* était presque absente des cellules qui expriment *Hoxd12* de façon ectopique. Très probablement, le gain de fonction de *Hoxd12* supprime la croissance du caecum en bloquant l'activité des gènes *HoxD* antérieurs, cela à travers l'inhibition de *Fgf10*. En quête d'autres molécules qui pourraient être déterminantes pour le

développement du caecum, nous nous sommes concentrés sur le facteur de transcription Pitx1. Nous avons observé que l'ARNm de *Pitx1* est accumulé dans le mésenchyme du bourgeon du caecum alors que dans les souris homozygotes et hétérozygotes pour *Del(1-10)* et-ou *Del(4-11)* il est absent de la même région. En conséquence, nous affirmons que dans ces souris, l'expression ectopique des gènes *Hox* postérieurs au niveau de la jonction iléo-caecale réprime l'expression de *Fgf10* et *Pitx1* dans les cellules du mésenchyme. De cette manière, l'amplification du signal FGF10 est interrompue en donnant un impact négatif à la croissance du caecum. Pour confirmer l'hypothèse que pendant la formation du bourgeon, des membres ou du caecum, le défaut de croissance était provoqué par l'inactivation de la fonction des gènes *Hox* antérieurs, nous avons établi une analyse génétique de complémentation. Nous avons croisé des souris *Del(1-10)* avec des allèles *HoxD* où les gènes *Hoxd1*, *Hoxd3*, *Hoxd4*, *Hoxd8*, *Hoxd9* et *Hoxd10* était ajoutés progressivement. De cette façon, le gain de fonction des gènes postérieurs est titré avec des doses additionnelles de produits antérieurs qui, les unes par rapport aux autres, ont en apparence une fonction équivalente. Dans les souris qui portent les gènes de *Hoxd1* à *Hoxd10* sur un chromosome et de *Hoxd11* à *Hoxd13* sur l'autre, le développement du caecum était pleinement récupéré. En conclusion, les gènes *HoxD* postérieurs bloquent la croissance du caecum en neutralisant l'activité des gènes *Hox* antérieurs. Ainsi, cette étude met en évidence l'incidence morphogénétique du mécanisme de prévalence postérieure.

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