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(1-10)^* 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. 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,
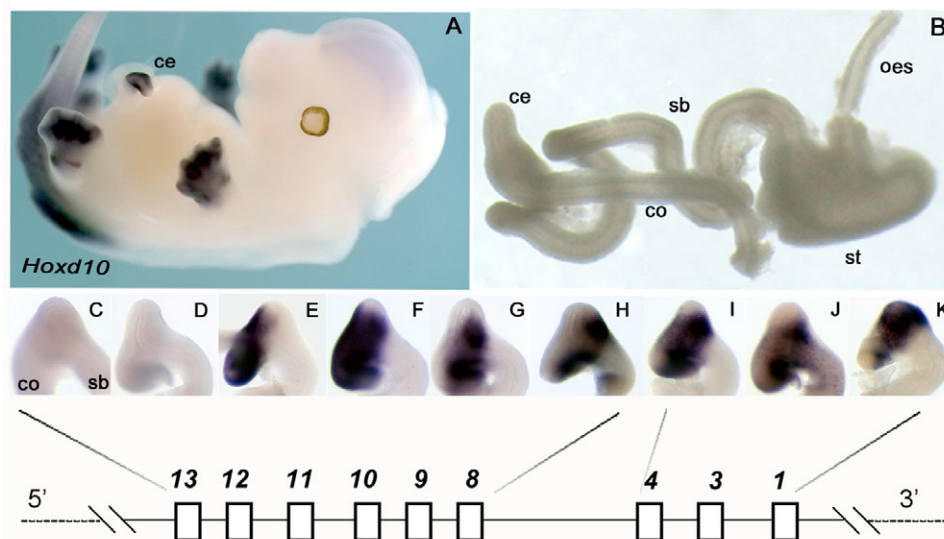

Fig. 1. Co-expression of seven Hoxd genes in posterior midgut. (**A**) Whole-mount RNA in situ hybridization detection of *Hoxd10* transcripts in E13 mouse embryo, showing some sites of expression, including the intestinal hernia. (**B**) Anatomical subdivisions of the mid-gestation murine gastrointestinal 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
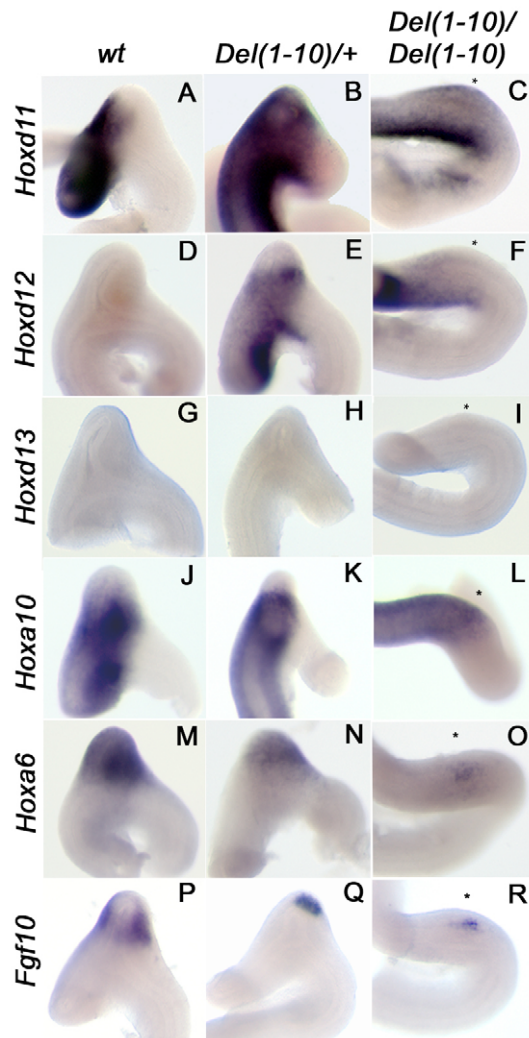

Fig. 2. Defect in the gut of Del(1-10) mutants. Whole-mount RNA in situ hybridization analysis of gene expression in dissected posterior midgut of E12 mouse embryos. *Hoxd11* (**A-C**), *Hoxd12* (**D-F**), *Hoxd13* (**G-I**) *Hoxa10* (**J-L**), *Hoxa6* (**M-O**) and *Fgf10* (**P-R**). Caecum budding 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).

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.

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
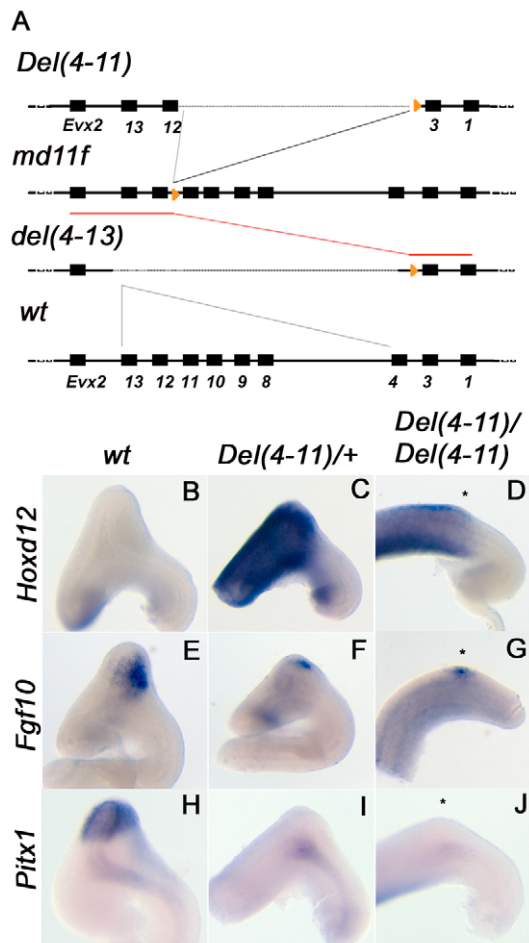
Fig. 3. Role of *Hoxd12* in caecum agenesis. (**A**) Derivation of the *Del(4-11)* allele by TAMERE. (**B-J**) Whole-mount RNA in situ hybridization of *Hoxd12* (B-D), *Fgf10* (E-G) and *Pitx1* (H-J) transcripts in wild-type control (B,E,H), *Del(4-11)*-deficient *HoxD* cluster mutant heterozygous (C,F,I) 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
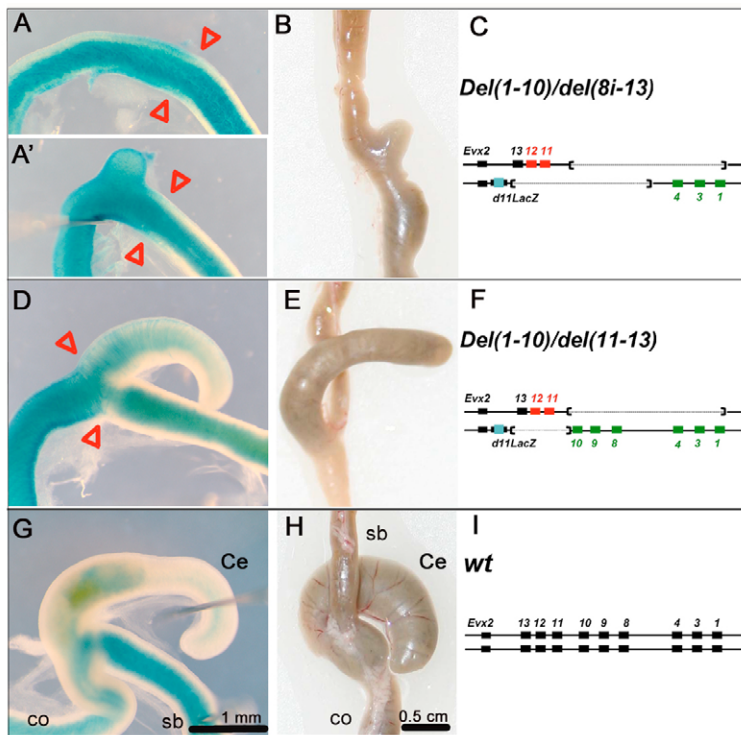
Fig. 4. 'Anterior' Hoxd genes promote adult caecum size in mice. (**A-I**) Dissected ileo-caecal transition zones of three genotypes, illustrating key examples of the phenotypic series presented in Table 1. A,A',D,G depict X-Gal-stained newborn specimens, to confirm the identity of the complementing 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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