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Running title: Duplication of *Hoxd11* causes skeletal changes

Duplication of the *Hoxd11* Gene Causes Alterations in the Axial and Appendicular Skeleton of the Mouse

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ABSTRACT

The Hox genes encode a group of transcription factors essential for proper development of the mouse. Targeted mutation of the *Hoxd11* gene causes reduced male fertility, vertebral transformation, carpal bone fusions, and reductions in digit length. A duplication of the *Hoxd11* gene was created with the expectation that the consequences of restricted over-expression in the appropriate cells would provide further insight into the function of the *Hoxd11* gene product. Genetic assays demonstrated that two tandem copies of *Hoxd11* were functionally indistinguishable from the normal two copies of the gene on separate chromosomes with respect to formation of the axial and appendicular skeleton. Extra copies of *Hoxd11* caused an increase in the lengths of some bones of the forelimb autopod and a decrease in the number of lumbar vertebrae. Further, analysis of the *Hoxd11* duplication demonstrated that the Hoxd11 protein can perform some functions supplied by its paralogue *Hoxa11*. For example, the defects in forelimb bones are corrected when extra copies of *Hoxd11* are present in the *Hoxa11* homozygous mutant background. Thus, it appears that Hoxd11 can quantitatively compensate for the absence of Hoxa11 protein and therefore Hoxa11 and Hoxd11 are functionally equivalent in the zeugopod. However, extra copies of *Hoxd11* did not improve male or female fertility in *Hoxa11* mutants. Interestingly, the insertion of an additional *Hoxd11* locus into the *HoxD* complex does not appear to affect the expression patterns of the neighboring *Hoxd10, d12* or *d13* genes.

Key words: *Hox* genes, gene duplications, limb development, axial skeleton development, gene targeting

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INTRODUCTION

The expansion of Abdominal-B type *Hox* genes from a single *Abd-B* gene in invertebrates to a total of 16 genes in mammals suggest that they have played a significant role in the elaboration of more complex vertebrate morphologies. Mice with mutations in these genes manifest defects in the limbs, vertebral column, urogenital system, and caudal digestive tract (Dolle et al., 1993; Small and Potter, 1993; Davis and Capecchi, 1994; Rijli et al. 1995; Satokata et al. 1995; Suemori et al. 1995; Fromental-Ramain et al. 1996a; Carpenter et al. 1997; Chen and Capecchi 1997). There appears to be a significant degree of functional redundancy among these genes as single mutants often have mild mutant phenotypes while double and triple mutants are very severely affected (Davis et al. 1995; Fromental-Ramain et al. 1996b; Warot et al. 1997; Wahba et al. 2001; Wellik et al., 2002).

Mice with a mutation in the *Hoxa11* gene show transformations in the lumbar/sacral region of the vertebral column, malformations in the ulna, radius and carpal bones of the forelimb and in the tibia and fibula of the hindlimb, and infertility in both males and females (Small and Potter 1993). *Hoxd11* mutants exhibit vertebral transformations similar to those of *Hoxa11* mutants, alterations in the shape of the distal radius and ulna, reductions in the lengths of the phalanges and metacarpals, abnormalities in carpal bones, and male infertility (Davis and Capecchi 1994). The phenotypes of mice lacking three or all four wild type copies of *Hoxa11* and *Hoxd11* reveal the dramatic degree of functional redundancy between these two gene products (Davis et al. 1995). Mice homozygous mutant for one gene while heterozygous for a mutation in the other gene, and mice homozygous mutant for both *Hoxa11* and *Hoxd11,* show more striking

defects in the appendicular and axial skeleton and in the urogenital tract than single homozygous mutants.

Two general methods have been used to gain insight into gene function: creation of loss-of-function mutations and over-expression of gene products. Most over-expression experiments rely on randomly inserted, multi-copy transgenes that elicit high, non-physiological, levels of transcripts or cause ectopic gene expression. Furthermore, *Hox* transgenes can be particularly problematic because they often cause embryonic lethality (Balling et al. 1989; McLain et al. 1992; Wolgemuth et al. 1989). Gene duplications offer an alternative method for providing measured, increased levels of gene product to cells that normally express the gene of interest (Smithies and Kim 1994). For this purpose, we generated a tandem duplication of the *Hoxd11* locus. This allele allowed us to determine the phenotypic consequences resulting from the controlled over-expression of the Hoxd11 protein.

Zakany et al. (1996) showed that a Hoxd11-expressing transgene was able to rescue the axial vertebral phenotypes of *Hoxa11/Hoxd11* or *Hoxa11* mutant mice. However, this study did not address functional redundancy of *Hoxa11* and *Hoxd11* in the limb or in the urogenital tract. Additionally, the level of *Hoxd11* expression driven by the transgenes used in this study was several-fold greater than wild type *Hoxd11* levels (Gérard et al. 1996). In order to determine whether physiological levels of *Hoxd11* expression are able to complement *Hoxa11* and *Hoxa11/Hoxd11* mutant phenotypes, and whether complementation occurs in all tissues showing mutant effects, the *Hoxd11* duplication was crossed into mice mutant for *Hoxa11* and *Hoxd11*.

Duboule and coworkers have proposed that the expression of the 5' *HoxD* genes in the limb is controlled by two *cis*-regulatory elements (Herault et al. 1999). The exact position of each gene within a proposed regulatory interval determines the relative influence that each element has on the promoter and thereby establishes expression patterns within the limb. This model would predict that insertion of 9.5 kb of DNA with an additional promoter into the *HoxD* cluster could cause alterations in the transcription patterns of neighboring 5' *HoxD* genes. An analysis of the expression of *Hoxd10*, *Hoxd12* and *Hoxd13* in embryos homozygous for the *Hoxd11* duplication demonstrated no detectable changes in neighboring gene expression patterns.

METHODS

Generation of mice with a *Hoxd11* duplication

An insertion vector was constructed with 6 kb of sequences 5' and 1.8 kb 3' of the *Hoxd11* gene. These sequences were cloned into the pSSGAP loxP vector (Figure 1B, H. S. Stadler, unpublished). The TK1 negative selectable marker gene was inserted at a SalI site 2.3 kb 5' of the *Hoxd11* coding region, and the vector was linearized with SalI at the 3' end of TK1 prior to electroporation. One targeted cell line was obtained out of 282 cell lines screened, and was used to produce chimeras that transmitted the targeted allele. Progeny of the chimeras were mated with a Cre-deleter mouse to excise the *neo* gene and plasmid sequences that were flanked by loxP sites (Schwenk et al. 1995). Southern blots were initially used to identify mice with the duplicated *Hoxd11* locus (*Dp/+*). Subsequently PCR assays were used for genotyping, except when it was necessary to distinguish *Dp/+* from *Dp/Dp*. Primers used to detect the d11 duplication were 5'-

CAGACAATCAAAGTATTTCACTCAG (sequence 3' of *Hoxd11*) and 5'-TAATACGACTCACTATAGGGCGAA (sequence from the T7 promoter region of the pSSGAPloxP vector).

Mice carrying the *Hoxd11* duplication were crossed with *Hoxa11* and *Hoxd11* mutants to obtain the genotypes used to test genetic complementation. The phenotype of the *Hoxa11* mutant allele used in this study, $Hoxa11^{neo}$, is different from that of the published *Hoxa11* mutant, $Hoxa11^{\Delta+neo}$,(J. Delort, A.P. Davis, and M. Capecchi, unpublished data; Small and Potter, 1993), and is described in greater detail below.

Whole mount *in situ* hybridization

In situ hybridization to whole embryos was carried out as previously described (Boulet and Capecchi 1996) The *Hoxd10* probe, a gift from Dr. Ellen Carpenter, was a 600 bp EcoRV fragment from exon 1. The *Hoxd11* probe was an AccI to BamHI fragment that includes part of the homeobox and the 3' untranslated region. The *Hoxd12* probe was a 900 bp EcoRI-SacI fragment containing part of the homeobox and the 3' untranslated region. The *Hoxd13 probe,* a 1 kb PstI-SacI fragment, contained most of the homeobox plus 3' untranslated region.

Fertility tests

Male $a11^{neo/neo}$ $d11^{+/+}$, $a11^{neo/neo}$ $d11^{Dp/+}$ and $a11^{neo/neo}$ $d11^{Dp/Dp}$ mice were mated to fertile female mice, which were checked daily for vaginal plugs. Each male was tested with at least six females that were subsequently monitored for pregnancy and delivery of

pups. Female mice of the same genotypes were mated with fertile males. Each female was plugged at least three times and checked for pregnancy.

Skeletal analysis and bone measurements

Alizarin red-stained adult skeletons were prepared as described (Mansour et al. 1993). Bones were measured using NIH image software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The right forelimb bones of ten animals of each genotype (+/+, *Dp*/+, and *Dp/Dp*) were measured and significance was determined by t-test using Excel software. Littermates were used as controls and all mice were sacrificed at 8 to 9 weeks of age.

Hoxa11 mutant

The phenotype of the *Hoxa11* mutant mice used in this study, homozygous for the $Hoxa11^{neo}$ allele (Delort, Davis and Capecchi, unpublished data), differs in some respects from that of the published *Hoxa11* mutant, $Hoxa11^{\Delta+neo}$ (Small and Potter 1993). The latter gene disruption introduced a 2.7 kb deletion at the *Hoxa11* locus, while the $Hoxa11^{neo}$ mutation was generated by a simple insertion of the pol2neo cassette (Deng et al. 1993) at an Eco47III site at the 5' end of the homeobox (J. Delort and M. Capecchi, unpublished). While $Hoxa11^{\Delta+neo}$ mice show fusions of the pisiform and triangular carpal bones (P-T fusion), carpal bone fusions were not seen with the $Hoxa11^{neo}$ allele. In addition, axial homeosis was limited to an anterior transformation of the first sacral vertebra to a lumbar identity: the posterior transformation of the 13th thoracic to a lumbar vertebra was only rarely observed. The expressivity of the hindlimb phenotype seen in

Hoxa11$^{\Delta+neo}$ mice, incomplete fusion of the distal tibia and fibula, was also lower in *Hoxa11*^{neo} specimens (data not shown). Nevertheless, the effects on the radius and ulna of both *Hoxa11* alleles were essentially identical, and the forelimb phenotype of mice carrying combinations of the *Hoxa11*^{neo} mutation with the *Hoxd11* mutation was the same as that reported previously (Davis et al. 1995; Figure 3C).

RESULTS

Duplication of the *Hoxd11* locus

The *Hoxd11* locus was duplicated by insertion of a second copy of the coding region and 7.8 kb of flanking sequences, including a region from 3.5 kb upstream of the *Hoxd11* transcription start to 1.8 kb 3' of the polyadenylation signal (Figure 1). Gérard et al. (1993) showed that the latter region was sufficient to direct transgenic ß-galactosidase reporter gene expression in a pattern, along the main body axis, similar to that of the endogenous gene. After mice carrying the duplication were obtained, plasmid sequences and the *neomycin* gene used for positive selection of targeted cell lines were removed by recombination between flanking loxP sites by mating to the *deleter* CRE mouse strain (Schwenk et al. 1995; Figure 1C).

Male and female mice harboring either one or two copies of the duplicated *Hoxd11* allele were viable and fertile. Whole mount *in situ* hybridization analysis of embryos homozygous for the duplication did not reveal any evidence of ectopic expression of *Hoxd11* transcripts, either in the limb buds or in the vertebral column at E11.5 (Figure 2). The most anterior prevertebra previously reported to show expression of *Hoxd11* is prevertebra 27 (pv27) (Gérard et al. 1993). We detected a low level of

Hoxd11 expression in wild type embryos in pv26 by whole mount *in situ* hybridization (Figure 2B). A higher level of expression, especially in the dorsal portion of pv26, was apparent in *Dp/Dp* specimens (Figure 2D), indicating that the level of *Hoxd11* transcripts is increased relative to wild type embryos with only two copies of the *Hoxd11* gene. Similar results were obtained when *Dp/Dp* embryos at E12.5 and E13.5 were compared to wild type (data not shown).

Duplication of *Hoxd11* complements a *Hoxa11* mutation in the forelimb

Whereas mice homozygous for a mutation of the *Hoxd11* gene show only subtle alterations of the distal ends of the radius and ulna (Davis and Capecchi 1994; Favier et al., 1995), the forearm bones of $Hoxa11^{neo/neo}$ mutants are obviously shorter and thicker (Figure 3B) than those of wild type mice (Figure 3A; Small and Potter 1993). The radius and ulna of mice homozygous for the $Hoxa11^{neo/neo}$ mutation and heterozygous for the *Hoxd11* mutation or of the reciprocal genotype, $a11^{neo/+}\ d11^{-/-}$, are much more dramatically affected (Figure 3C). When one copy of the *Hoxd11* duplication is added, i.e. in mice of the genotype $all^{neo/neo}\ d11^{Dp/-}$ (Figure 3D), the radius and ulna appear very similar to those of $a11^{neo/neo}\ d11^{+/+}$ mice. This strongly suggests that, in the context of zeugopod development, Hoxd11 protein activity derived from the tandem duplication is greater than that from one copy of *Hoxd11*, and equivalent to that from two normal copies of *Hoxd11* on separate chromosomes.

In order to determine whether the *Hoxd11* duplication can complement the absence of *Hoxa11* in the forearm, skeletons of $a11^{neo/neo}\ d11^{Dp/+}$ and $a11^{neo/neo}\ d11^{Dp/Dp}$ mice were examined. The radius and ulna of $a11^{neo/neo}\ d11^{Dp/+}$ mice (Figure 3E, F) were

less shortened and thickened than those of *a11*$^{neo/neo}$ *d11*$^{+/+}$ forearms (Figure 3B) while *a11*$^{neo/neo}$ *d11*$^{Dp/Dp}$ mice (Figure 3G, H) appear normal (n=4).

Although the *Hoxa11* mutant used in our experiments does not manifest the carpal phenotype described by Small and Potter (1993; Figure 4B), fusions between the navicular lunate and triangular bones, the triangular and pisiform or all three carpal bones are seen in *Hoxd11* mutants (Davis and Capecchi 1994). In mice of the genotype *a11*$^{neo/neo}$ *d11*$^{+/-}$, the proximal carpal bones, triangular, navicular lunate and pisiform, were fused in all specimens examined (n=3) (Figure 4C). Although fusions of proximal carpals were apparent in four out of five *a11*$^{neo/neo}$ *d11*$^{Dp/-}$ animals (data not shown), they only involved the triangular and navicular lunate, not the pisiform, bones. In the fifth specimen, the navicular lunate and triangular bones were also separate (Figure 4D). This indicates that the *Hoxd11* duplication has some function in development of the wrist region, but, in the context of carpal development, the activity is not quite equivalent to that of two copies of the *Hoxd11* gene on separate chromosomes.

The *Hoxd11* duplication does not complement reproductive defects of *Hoxa11* mutants

Male mice homozygous for the *Hoxa11*$^{\Delta+neo}$ mutation show greatly reduced fertility while females appear to be completely sterile (Small and Potter 1993; Hsieh-Li et al. 1995 and unpublished results). Male *a11*$^{\Delta+neo/\Delta+neo}$ mice show a transformation of vas deferens to epididymis, consisting of increased coiling and decreased lumen diameter, reduced testes size, incomplete descent of testes into the scrotal sac, and evidence of altered spermatogenesis (Hsieh-Li et al. 1995). The uterine environment of *a11*$^{\Delta+neo/\Delta+neo}$ females is unable to support pregnancy (Hsieh-Li et al. 1995; Gendron et al. 1997). We

tested whether the *Hoxa11*^{neo} mutation caused the same degree of reproductive failure. Three of nine *Hoxa11*$^{neo/neo}$ males were fertile. In six matings each, one generated only a single pregnancy, while the others generated two and four pregnancies, respectively. Whereas *Hoxa11*^{neo} homozygous males were more fertile than their *Hoxa11*$^{\Delta+neo}$ counterparts, *Hoxa11*^{neo} females were completely sterile: none of the plugged *a11*$^{neo/neo}$ females became pregnant (n=10).

The effect of one or two copies of the *Hoxd11* duplication on fertility of male and female *Hoxa11* homozygotes was investigated (Table 1). When ten male mice of the genotype *a11*$^{neo/neo}$ *d11*$^{Dp/+}$ were each mated to six wild type females, four produced offspring. On the other hand, none of the females plugged by *a11*$^{neo/neo}$ *d11*$^{Dp/Dp}$ males became pregnant (n=11). Comparison of dissected reproductive tracts of fertile and infertile males did not reveal any aspects of the overall phenotype that correlated with infertility. Abnormal coiling of the vas deferens was observed in every specimen, testis size varied from male to male and from one side to another in some males, testes were never properly descended, and live sperm was found in the epididymis of both fertile and infertile males. The *Hoxd11* duplication had no effect on female fertility, with eleven *a11*$^{neo/neo}$ *d11*$^{Dp/+}$ and nine *a11*$^{neo/neo}$ *d11*$^{Dp/Dp}$ females failing to produce pups or even a visible pregnancy in at least three matings each.

Duplication of *Hoxd11* affects normal skeletal development

Mice mutant for *Hoxa11* or *Hoxd11* or both genes show alterations in the lumbar region of the axial skeleton (Small and Potter 1993; Davis and Capecchi 1994; Davis et al. 1995). Skeletal analysis was repeated using our *Hoxa11*^{neo} allele. When two out of

four total copies of *Hoxa11* plus *Hoxd11* are mutated, seven lumbar vertebrae were often formed instead of the wild type number of six (TABLE 2). When only one wild type copy of *Hoxa11* or *Hoxd11* remained, the number of lumbar vertebrae was always seven. Double mutant homozygotes, with no functional copies of *Hoxa11* or *Hoxd11*, have eight lumbar vertebrae (TABLE 2; Davis et al. 1995). By increasing the number of *hoxd11* transcripts in cells that participate in the formation of the axial skeleton (i.e., in $all^{+/+}$ $d11^{Dp/Dp}$ mice) reduced the number of lumbar vertebrae to five (TABLE 2).

We also carefully examined the forearms of mice with 3 or 4 copies of the *Hoxd11* gene. We did not detect any alterations in overall morphology of the forelimb skeleton (Figure 3; data not shown). Lengths of the radius and ulna of 8-week-old mice were compared to those of wild type littermates, controlling for differences in mouse size by expressing the lengths of the forearm bones as a fraction of humerus length. There was no significant difference in radius/humerus (R/H) or ulna/humerus (U/H) values between wild type and *Dp/+* or *Dp/Dp* mice (TABLE 3). *Hoxd11* mutant homozygous mice show reductions in the lengths of forelimb autopod bones, with the strongest effects on phalange 2 (P2) and the metacarpal of digit II and P2 of digit V (Davis and Capecchi 1994). Favier et al. (1995) reported shortening of metacarpals II, III, and IV and phalange 2 of digit II, but not of P2 of digit V in their *Hoxd11* mutant. When the lengths of *Dp/Dp* digit bones were compared to those of wild type littermates, statistically significant increases in length were observed for some phalanges and metacarpals (TABLE 3, Figure 5). Specifically, the metacarpals of digits II, III, and IV and phalanges 1 and 2 of digit II were longer in *Dp/Dp* than in +/+ mice (TABLE 3). For the metacarpal of digit II, this corresponds to an actual increase from 2.59 ± 0.06 mm in wild type to 2.81 ± 0.07 mm in

Dp/Dp mice (8.5% increase). No significant change in the lengths of phalanges 1 and 2 of digit 5 were observed (TABLE 3). Bone lengths of *Dp/+* mice were intermediate between wild type and *Dp/Dp*, but these values were not statistically significant (TABLE 3).

Insertion of an additional copy of *Hoxd11* into the *HoxD* complex does not affect transcription patterns of neighboring *Hox* genes

Several lines of evidence suggest that transcription of the 5' genes of the *HoxD* cluster is controlled by shared regulatory elements (vander Hoeven et al. 1996). With regard to limb bud expression, it has been proposed that the distance of each promoter from two elements, a zeugopod element and an autopod element, determines transcriptional regulation of the gene (Herault et al. 1998; Herault et al. 1999). Since the *Hoxd11* duplication event inserted 9.5 kb of DNA into the *HoxD* complex, one might expect to see an influence on the timing and/or expression patterns of the neighboring *Hox* genes. Embryos at 11.5 and 12.5 days of gestation were examined by whole mount *in situ* hybridization for patterns of limb bud and prevertebral expression of *Hoxd10, Hoxd12* and *Hoxd13.* No differences could be detected between *Dp/Dp* embryos and wild type littermates with respect to limb bud pattern and anterior limits of expression in the prevertebral column at the embryonic stages examined (Figure 6).

Duplication of *Hoxd11* would not move *Hoxd12* or *Hoxd13* further from a proposed regulating element, but might delay sequential activation initiating at the 3' end of the *HoxD* cluster (Kondo 1999 and references therein). Whole mount *in situ* analysis carried out on 9.5 day embryos using the *Hoxd12* and *Hoxd13* probes detected both

transcripts, suggesting that the *Hoxd11* duplication did not cause a substantial delay in gene activation (*d12* normally appears at E9, *d13* by E9.5; data not shown).

DISCUSSION

The *Hox* gene complex of mammals arose by amplification in cis followed by duplication of the entire unit of thirteen genes to generate four separate clusters. We have generated a cis duplication of the *Hoxd11* gene within the *HoxD* cluster. Though the amount of Hoxd11 protein produced by the duplicated locus has not been quantitated, genetic tests provide strong evidence that it is greater than that supplied by a single copy of *Hoxd11*. Zakany et al. (1996) showed that extra doses of *Hoxd11* were able to rescue the effect of *Hoxa11* loss of function in the vertebral column. Because these experiments were carried out using a randomly inserted multi-copy transgene, the expression level of *Hoxd11* which was able to effect complementation was many fold greater than the physiological level of *Hoxd11* (Gérard et al. 1996). In addition, because the transgene does not recapitulate the normal expression pattern of *Hoxd11* in the limbs (Gérard et al. 1993), the ability of *Hoxd11* to substitute for *Hoxa11* during limb development could not be determined. We have shown that a duplication of the *Hoxd11* locus, causing an increase in *Hoxd11* expression levels of approximately two-fold, was able to rescue zeugopod defects but not the reproductive defects, caused by a mutation in *Hoxa11*.

Paralogous *Hox* genes, those that share the same relative position in each cluster, often share similarities in expression patterns as well as in nucleotide sequences. Numerous studies of *Hox* gene knock-out mice have revealed cases of functional overlap or redundancy between paralogous, as well as non-paralogous, *Hox* genes (Condie and

Capecchi 1994; Davis et al. 1995; Horan et al. 1995; Rancourt et al. 1995; Fromental-Ramain et al. 1996b; Gavalas et al. 1998; Rossel and Capecchi 1999; Wellik et al. 2002). Greer et al. (2000) reported that the Hoxd3 protein, when expressed under control of the *Hoxa3* regulatory elements, is able to rescue a *Hoxa3* mutant. In the experiment reported here, additional copies of the *Hoxd11* gene were able to substitute for *Hoxa11* in the development of the forelimb zeugopod. This provides further support for the proposal that paralogous *Hox* genes are functionally equivalent in spite of only 61% similarity between the Hoxa11 and Hoxd11 proteins.

The results obtained with the *Hoxd11* duplication imply that the spatial, temporal and quantitative aspects of the *Hoxa11* and *Hoxd11* expression patterns corresponded sufficiently in some sites to obtain full rescue. The expression patterns of *Hoxa11* and *Hoxd11* are similar, but not identical, during the course of embryonic development (Dollé et al. 1989; Dollé et al. 1991; Haack and Gruss 1993; Hsieh-Li et al. 1995; A. Boulet, unpublished data). *Hoxa11* and *Hoxd11* expression patterns overlap at very early stages of limb bud outgrowth in the distal and posterior regions of the limb bud. After about E10.75, *Hoxa11* mRNA is no longer found in the most distal forelimb bud (Small and Potter 1993), while *Hoxd11* expression persists in this region through late gestation (Dolle et al., 1989; A. Boulet, unpublished data). After formation of the cartilage condensations for the forelimb skeletal elements (about E12.5 to E16.5), *Hoxa11* and *Hoxd11* are both expressed in a region surrounding the distal ends of the radius and ulna (Favier et al. 1996; Dollé et al. 1989; A. Boulet, unpublished data). Preliminary evidence suggests that defects in the radius and ulna of $Hoxa11^{neo/neo}$, $a11^{neo/neo}\ d11^{+/-}$, and $a11^{neo/+}d11^{-/-}$ mice are due to distal growth plate abnormalities not manifest until later in

gestation (A. Boulet and M. Capecchi, in preparation) which correlates well with this overlap in expression pattern.

In contrast to the limb, *Hoxa11* and *Hoxd11* are expressed in different portions of the female reproductive tract, with *Hoxa11* transcripts in the uterus and *Hoxd11* transcripts reportedly confined to the oviduct (Dollé et al. 1991; Hsieh-Li et al. 1995; Gendron et al. 1997; Taylor et al. 1997). However, although *Hoxa11* and *Hoxd11* are both expressed in the vas deferens of male mice (Dollé et al. 1991; Hsieh-Li et al. 1995), little or no complementation of reduced male fertility in $a11^{neo/neo}$ mice was obtained with the *Hoxd11* duplication. The failure to obtain complementation may indicate that the particular cells expressing *Hoxa11* and *Hoxd11* in the vas deferens are not identical, that there are critical differences in timing or expression level, or even that, in the context of the reproductive tract, the two proteins are not functionally equivalent. Another possibility is that reduced male fertility in $Hoxa11^{neo/neo}$ mice is not due to the defect in vas deferens morphology, which has been interpreted as a transformation of vas deferens to epididymis. Reduced fertility of *Hoxa11* mutant males could instead be due to a combination of the defects seen in the male reproductive tract (Hsieh-Li et al. 1995). Incomplete descent of testes into the scrotal sac, reduced testes size, and altered spermatogenesis are all plausible candidates. The cause of reduced male fertility in *Hoxd11* homozygotes has not been determined: no morphological or histological abnormalities are apparent in the genitourinary tract of $Hoxd11^{-/-}$ males (Davis and Capecchi 1994; Favier et al. 1995).

The effects of the *Hoxd11* gene duplication on the vertebral column and on digit length appear to correspond to two different aspects of *Hox* gene function. In the

vertebral column, increased expression of the Hoxd11 protein causes alterations in vertebral identity, i.e. changes in fate. Thus, the number of lumbar vertebrae is observed to decrease in proportion to the number of functional copies of *Hoxa11* plus *Hoxd11*, requiring a threshold for each transition, from 5 to 6 and from 6 to 7. In contrast, increased *Hoxd11* copy number in the autopod results in increases in bone length without visible changes in digit identity. Though these effects appear different, they may be a consequence of similar roles of *Hox* gene products in the axial and appendicular skeletons. Alterations in vertebral morphology are likely to be due to remodeling of cell condensation patterns, perhaps reflecting effects on cell adhesion properties, and/or to changes in cell proliferation. Similarly, increases in bone length could be due to an expansion in the population of cells condensing to form the cartilage template or to an increase in proliferation either at the time of condensation formation or in the growth plates of the bones occurring later in gestation or after birth. The changes in the length of the phalangeal and metacarpal bones observed in mice with duplicated *Hoxd11* alleles, relative to wild type controls, were not dramatic. However, since numerous *Hox* genes are expressed within the developing autopod during the formation of the precartilaginous condensations, the extent of increase in the lengths of these bones resulting from the *Hox11* duplications is what we should expect if the length of these bones were determined from integration of multiple *Hox* gene signals within the developing autopod. Since many *Hox* genes are used to guide the formation of the tetrapod autopod, during their evolutionary history selection forces have had at their disposal large pools of mutations to draw upon. The effects of such mutations could independently modulate *Hox* gene expression patterns and cumulatively could readily account for the enormous

variations in autopod morphological structures and functions observable among existing tetrapod species.

Duboule and coworkers have proposed models to explain the regulation of 5' *HoxD* gene expression, both for colinear gene activation (Kondo 1999, and references therein) and for precise control of limb patterns by opposite regulatory influences (Herault et al. 1999). When a "neutral" promoter was used to scan regulatory influences across the 5' end of the *HoxD* cluster, the relative levels of transcription in the proximal forearm and distal domains at E11.5 depended upon the position of promoter insertion within this region. While *Hoxd10* is expressed in both proximal forearm and distal autopod domains at this stage, *Hoxd9* expression is not detected in the distal domain (Dollé and Duboule 1989; Dollé et al. 1989). The distance between the *Hoxd9* and *Hoxd10* promoters is approximately 5.5 kb. The 9.5 kb insertion of a second copy of *Hoxd11* moved *Hoxd10* into a position more like that of *Hoxd9*, further from the influence of a distal domain regulatory element. Therefore, we expected that the distal expression domain of *Hoxd10* would be reduced or absent in mice carrying the *Hoxd11* duplication, but *Hoxd10* expression was not observably altered. One explanation would be that local regulatory controls or promoter-specific influences play a role in determining the response to remote regulatory sequences and, consequently, in establishing the precise expression pattern. Analyses of a *Hoxd12* regulatory element and a repressor element located between *Hoxd12* and *Hoxd13* suggest that local regulatory sequences, as well as global influences, play a role in establishment of the final limb pattern (Herault et al. 1998; Kondo 1999).

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In summary, we have shown that increasing the number of copies of *Hoxd11* in the mouse can complement the effects of *Hoxa11* loss-of-function mutations during limb formation. We have also shown that such increases of *Hoxd11* gene copy number result in predictable changes in axial skeleton morphology relative to the effects of *Hoxa11* and *Hoxd11* loss-of-function mutations. However, tandem duplications of *Hoxd11* could not complement male and female sterility phenotypes observed in *Hoxa11* mutant homozygotes. Finally, the fact that an approximate two-fold increase in *Hoxd11* expression was sufficient to bring about morphological changes and increase the lengths of individual bones in the autopod of the mouse has interesting implications for the role of *Hox* genes on the evolution of the tetrapod autopod.

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FIGURE LEGENDS

Figure 1. Targeted duplication of the *Hoxd11* gene. (A) The region of the *HoxD* cluster containing *Hoxd12, Hoxd11* and *Hoxd10*. The direction of transcription is from left to right for each gene. Black boxes designate exons. Regions comprising the 5' and internal probes used to screen cell lines generated by electroporation of the targeting vector are shown. (B) The insertion vector used to generate the *Hoxd11* duplication. A region from the SacI site at the 3' end of *Hoxd12* to an EcoRI site about 1.8 kb downstream of the *Hoxd11* gene was used for gene targeting. The *TK1* gene was inserted at the SalI site about 2 kb upstream from the *Hoxd11* gene for negative selection, and linearization for electroporation was as shown. pUC and pol2neo sequences were flanked by loxP sites to allow subsequent removal by Cre-mediated recombination. Targeted recombinants were identified by hybridization of EcoRV digested DNA with the 5' probe shown in (A). Insertion of the targeting vector reduces the size of the hybridizing EcoRV fragment from 20.6 kb to 14.3 kb. Further analysis using the internal probe shown in (A) confirmed the targeting event. (C) After Cre-mediated recombination, two tandem copies of the *Hoxd11* gene were created (from the SacI site to the EcoRI site), each about 9.5 kb, separated by a single loxP site. RV, EcoRV; X, Xho; Sac, SacI; Nsi, NsiI; S, Sal; RI, EcoRI. (Not all sites for each restriction enzyme are shown.)

Figure 2. Expression of *Hoxd11* in wild type and *Dp/Dp* embryos. The expression of *Hoxd11* in the forelimbs of wild type E11.5 embryos (A) is indistinguishable from that in E11.5 embryos carrying two copies of the *Hoxd11* duplication (*Dp/Dp*) (C). In the

prevertebral column, expression in the most anterior prevertebra, pv26, indicated by arrows, is stronger in the *Dp/Dp* embryo (D) than in the wild type control (B).

Figure 3. Effect of the *Hoxd11* duplication on the forelimb morphology of $Hoxa11^{neo/neo}$ mice. The radius and ulna of $Hoxa11^{neo/neo}$ $d11^{+/+}$ mice (B) are shortened and thickened relative to wild type (A). When one copy of *Hoxd11* is removed from a $Hoxa11^{neo/neo}$ background, these bones are more dramatically affected, with further length reduction and bowing of the radius (C). When the tandem duplication of *Hoxd11* was substituted for the single copy of *Hoxd11*, the radius and ulna resemble those of $Hoxa11^{neo/neo}$ skeletons (D). When the total number of copies of the *Hoxd11* gene was increased to three (*Dp/+*) or four (*Dp/Dp*), radius and ulna morphology approaches that of wild type specimens (E and F, *Dp/+*; G and H, *Dp/Dp*). In particular, the ulna in $a11^{neo/neo}$ $d11^{Dp/Dp}$ skeletons was as thin as in wild type rather than thickened as in $a11^{neo/neo}$ mice (arrows).

Figure 4. Function of the *Hoxd11* duplication in carpal formation. (A) Carpal bones of a wild type adult mouse, viewed from the dorsal side. The navicular lunate (nl) and triangular (t) bones are fused in mice of the genotype $a11^{neo/neo}$ $d11^{+/-}$ (C), but not in mice carrying only the *a11* mutant allele ($a11^{neo}$) used in this study (B). Replacement of the wild type copy of *Hoxd11* with the tandem duplication sometimes prevented the fusion of nl and t, but did not completely restore the wild type morphology of the carpal region (D). Arrows indicate the point where nl and t bones touch in (B) and (D), or the fused bone in (C).

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Figure 5. Forelimb digits of *Dp/Dp* and wild type adult mice. (A) and (B) Distal forelimb of two wild type adult mice. (C) and (D) Distal forelimbs of two *Dp/Dp* animals. mcII, metacarpal of digit II; mcIII, metacarpal of digit III; mcIV, metacarpal of digit IV; mcV, metacarpal of digit V.

Figure 6. Expression patterns of *Hoxd10, Hoxd12* and *Hoxd13* in embryos carrying two copies of the *Hoxd11* duplication. *Hoxd10* expression in *Dp/Dp* embryos at E11.5 (F) and (G) is indistinguishable from the *Hoxd10* pattern in wild type embryos (A) and (B). *Hoxd12* and *Hoxd13* patterns are also unchanged by the insertion of an extra copy of *Hoxd11* into the *HoxD* complex. For *Hoxd12*, compare *Dp/Dp* embryo in (H) and (I) to wild type embryo in (C) and (D). For *Hoxd13*, compare *Dp/Dp* embryo in (J) to wild type embryo in (E).

TABLE 1: Fertility of *Hoxa11* mutants with 0, 1 or 2 copies of the *Hoxd11* duplication

Male genotype	Percent fertile	Female genotype	Percent fertile
$al1^{neo/neo}d11^{+/+}$	33% (n=9)	$al1^{neo/neo}d11^{+/+}$	0% (n=10)
$al1^{neo/neo}d11^{Dp/+}$	40% (n=10)	$al1^{neo/neo}d11^{Dp/+}$	0% (n=11)
$al1^{neo/neo}d11^{Dp/Dp}$	0% (n=11)	$al1^{neo/neo}d11^{Dp/Dp}$	0% (n=9)

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TABLE 2: Effect of *Hoxa11* plus *Hoxd11* copy number on lumbar vertebrae

Hoxa11 copies	*Hoxd11* copies	total copies	# lumbar vertebrae	genotype

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0	0	0	8(n=3)	$a11^{neo/neo}$, $d11^{-/-}$
0	1	1	7(n=7)	$a11^{neo/neo}$, $d11^{+/-}$
1	0	1	7(n=2)	$a11^{neo/+}$, $d11^{-/-}$
1	1	2	7(n=1), 6(n=3)	$a11^{neo/+}$, $d11^{+/-}$
0	2	2	7(n=1), 6(n=5)	$a11^{neo/neo}$, $d11^{+/+}$
2	0	2	7(n=15), 6(n=4)**	$a11^{+/+}$, $d11^{-/-}$
0	2	2	7(n=3), 6(n=2)	$a11^{neo/neo}$, $d11^{Dp/-}$
1	2	3	6(n=20)*	$a11^{neo/+}$, $d11^{+/+}$
0	3	3	6(n=3)	$a11^{neo/neo}$, $d11^{Dp/+}$
2	2	4	6(n=10)	$a11^{+/+}$, $d11^{+/+}$
2	2	4	6(n=1)	$a11^{+/+}$, $d11^{Dp/-}$
0	4	4	6(n=4)	$a11^{neo/neo}$, $d11^{Dp/Dp}$
1	4	5	6(n=1), 5(n=2)	$a11^{neo/+}$, $d11^{Dp/Dp}$
2	3	5	5(n=8), 6(n=2)***	$a11^{+/+}$, $d11^{Dp/+}$
2	4	6	5(n=11)	$a11^{+/+}$, $d11^{Dp/Dp}$

- *unpublished data (A.P. Davis)
- **data from Davis and Capecchi (1994)
- ***specimen with a partial lumbar, partial sacral vertebra after 5 other lumbar vertebrae counted as 6 lumbar

TABLE 3: Relative lengths of forelimb bones in *Dp/Dp* mice

genotype	R/H	U/H	mcII/H	mcIII/H	mcIV/H	mcV/H	dIIp1/H	dIIp2/H	dVp1/H	dVp2/H
+/+	93.1	114.9	22.1	27.8	25.1	16.0	14.6	9.5	13.8	7.6
Dp/+	92.8	114.5	23.3	28.8	25.9	16.3	14.9	9.8	13.8	7.6
Dp/Dp	93.2	114.6	24.2	29.9	26.4	16.7	15.2	9.9	14.1	7.4
% increase			9.5%	7.6%	5.2%	4.4%	4.1%	4.2%	2.2%	
P value			0.0001 *	0.0019 *	0.0064 *	0.0578	0.0431	0.0286	0.1646	

***** statistically significant

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