Polyalanine expansion in HOXA13: three new affected families and the molecular consequences in a mouse model

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Polyalanine expansions in two of three large imperfect trinucleotide repeats encoded by the first exon of \textit{HOXA13} have been reported in hand–foot–genital syndrome (HFGS). Here we report additional families with expansions in the third repeat of 11 and 12 alanine residues, the latter being the largest expansion reported. We also report a patient with a novel, \textit{de novo} 8-alanine expansion in the first large repeat. Thus, expansions in all three large \textit{HOXA13} polyalanine repeats can cause HFGS. To determine the molecular basis for impaired HOXA13 function, we performed homologous recombination in ES cells in mice to expand the size of the third largest polyalanine tract by 10 residues (HOXA13\textsubscript{ALA28}). Mutant mice were indistinguishable from \textit{Hoxa13} null mice. Mutant limb buds had normal steady-state \textit{Hoxa13} RNA expression, normal mRNA splicing and reduced levels of steady-state protein. \textit{In vitro} translation efficiency of the HOXA13\textsubscript{ALA28} protein was normal. Thus, loss of function is secondary to a reduction in the \textit{in vivo} abundance of the expanded protein likely due to degradation.

\section*{INTRODUCTION}

In-frame polyalanine expansions encoded by imperfect triplet repeats are an emerging cause of human genetic diseases. Polyalanine expansions in \textit{HOXD13}, \textit{HOXA13}, \textit{RUNX2}, \textit{ARX}, \textit{PABPN1}, \textit{FOXL2}, \textit{ZIC2}, \textit{SOX3} and \textit{PHOX2B} have been reported in human genetic diseases (1,2). The mutations exert their effects through different mechanisms.

Hand–foot–genital syndrome (HFGS) is an autosomal dominant condition caused by mutations in \textit{HOXA13} (3–9). Most mutations, including polyalanine tract expansions, encoded by imperfect triplet repeats in \textit{HOXA13} cause haplo-insufficiency and lead to a common phenotype with bilaterally symmetrical shortening of the thumbs and halluces and clinobrachydactyly of the second and fifth fingers. Genitourinary anomalies, including hypospadias, ureteral reflux and varying degrees of incomplete Mullerian fusion, may also occur (reviewed in 9). Missense mutations may cause variable phenotypes (4,10). Further, chromosomal translocation involving the 5’ end of \textit{NUP98} and the 3’ end of numerous \textit{HOX} genes has been associated with hematologic malignancies. \textit{NUP98/HOXA13} fusion is associated with acute myeloid leukemia or myelodysplastic syndrome (19,20).

We have shown previously that polyalanine expansions arose in the \textit{HOXA13} gene after the reptilian/proto-mammalian divergence occurred approximately 310 million years ago (11). For purposes of clarity, the largest \textit{HOXA13} polyalanine tracts are labeled I–III in this paper, representing lengths of

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14, 12 and 18 residues, respectively, and are encoded by imperfect trinucleotide repeats (11; Fig. 9). The length and homopolymeric nature of these repeated domains are strongly conserved in extant mammals.

Length polymorphism for the largest HOXA13 polyalanine tract has been reported by Lavoie et al. (1) using PCR on 84 unrelated chromosomes. Tracts of 18 (98%) or 12 (~2%) alanines were observed, and no phenotypic manifestations of individuals with the shorter tract were described. In their study of the polyalanine tracts of proteins, larger polyalanine tracts (>13) are more likely to be polymorphic. Unequal crossing-over has been hypothesized to be the molecular mechanism by which pathogenic polyalanine expansions occur (12). Unequal crossing-over generates two meiotic products, thus it would be expected that shorter polyalanine tracts for HOXA13 must be produced, yet no tract III shorter than 12 has ever been described.

Patients with HOXA13 in-frame polyalanine expansions exhibit a phenotype similar to those with presumed null alleles (similar hand–foot phenotype to a deletion of most of the cluster). In contrast, synpolydactyly, a disorder associated with HOXD13 polyalanine expansions in mice and humans, is distinct in that the mechanism of the disease is dominant-negative and severity increases as the length of the tract increases (13–17). For these, a HOXD13 endogenous polyalanine tract of 15, also encoded by an imperfect trinucleotide repeat, expands by 7–14 alanines. Thus, the effects of polyalanine expansions of these two highly related, paralogous HOX transcription factors appear to be different. Moreover, the mechanism of HOXA13 loss of function in polyalanine tract expansions is unknown.

In this paper, we report additional families with HOXA13 polyalanine expansions. We also explore the molecular basis of apparent loss of function in HOXA13 polyalanine expansions by presenting phenotypic and molecular data of a mouse model engineered to have 10 additional alanines in the largest tract.

RESULTS

Clinical descriptions and molecular analysis
Pedigrees are shown in Figure 1.

Family A: Expansion III, +11A. The proband is a German Caucasian woman (III-1) who had shortened first digits and clinodactyly of digit V on both hands (Fig. 2). Radiologically, the first metacarpal was shortened; there were pointed distal phalanges, shortened medial phalanges V, left clinodactyly V, carpal fusion and a misshaped scaphoid. She had small feet (size 32–34 European), broadly shaped halluces, extension deficits of digits II–V, short phalanges and typical distal hallucal appearance (Fig. 2). She had many urinary tract infections as a child, including pyelonephritis; ultrasound of the urinary tract was unremarkable, except that in the past few years she has developed renal stones. All other family members were unaffected. PCR amplification and sequencing revealed an alanine expansion of 11 residues in the third large repeat (normal 18 residues) and is shown in Figure 3.

Family B: Expansion III, +12A. This large pedigree shows 24 affected individuals. Typical hand and foot abnormalities were observed (Fig. 2). Importantly, GU anomalies were very prevalent particularly in females (Table 1). For those affected males for whom we had clinical information, there was hypospadias or other GU anomalies. DNA sequencing of the expanded allele is shown in Figure 3.

Family C: Expansion I, +8A. The proband, IV-1, is a German Caucasian male who had a normally appearing thumb and unremarkable phalanges on examination, except perhaps for shortened metacarpal 1 and mild fifth finger clinodactyly (Fig. 2). Radiologically, his hands were nearly normal. His feet showed mild hallux varus and shortened distal great toes. He had no history of urinary tract infections, but had penoscrotal hypospadias. His father, paternal grandmother and grand paternal aunt were also affected. The paternal grandmother and her sister reside in Russia and nothing is known except that they have hand and foot abnormalities. His sister, IV-2 was normal and had an ultrasound of the abdomen, which was normal. Father, III-1, was born in Kazakhstan, and there were no clinical records. He had a surgical procedure to correct glandular hypospadias. Gel electrophoresis and sequencing of PCR products from IV-1 and III-1 revealed both to carry a heterozygous eight-residue
expansion of the first large polyalanine repeat from 14 to 22 alanines (Fig. 3). This expansion was absent in the unaffected daughter, IV-2. This is the first expansion to be reported in this repeat, and it is stable through at least one generation.

**Generation of engineered mouse Hoxa13 polyalanine expansion**

HOXA13 in-frame polyalanine expansions appear to produce a loss of function allele. In contrast, HOXD13 polyalanine expansions lead to a dominant phenotype that is more severe as the size of the expansion increases. A mouse Hoxd13 polyalanine expansion allele arose spontaneously and mimics the human phenotype; this allele has been useful for exploring the mechanism of action of this mutation (16,17). To explore the molecular mechanisms for the effects of HOXA13 polyalanine expansions, we constructed a HOXA13 alanine expansion (10 additional alanine residues in repeat III) in a targeting vector and performed homologous recombination in ES cells to replace one Hoxa13 allele of the mouse genome. The strategy is shown in Figure 4A. As described in Materials and Methods, we used PCR and Southern blotting on ES colonies surviving selection in G418 to confirm the structure of the replaced allele (Fig. 4B and C). Transient Cre expression was used to remove the PGK-Neo cassette, and independent ES cell clones were then selected by PCR across the remaining loxP site in the intron for blastocyst injection. Three different injections resulted in two chimeric males, each having shortening of the nails and terminal phalanges of the first digits on the hindfeet. One male passed the expanded alanine repeat to offspring. Heterozygous mutants showed shortening of the first digit or nail of the hindfoot and occasional cutaneous syndactyly between digits two and three (data not shown), consistent with heterozygous loss of function alleles (21,22,24,25). The phenotype of embryonic mutant fore- and hindlimbs at E13.5 is shown in Figure 5, which are representative of numerous mutant embryos examined at several stages. No discernible morphologic difference at this level of analysis could be found between polyalanine expansion heterozygous or homozygous mice and mice with an engineered deletion of the Hoxa13 gene (Fig. 5) (21,22). No polyalanine expansion homozygotes survived to birth, which is consistent with prenatal death associated with Hoxa13 null alleles (23,25). These results suggest that the polyalanine expansion leads to a loss of function consistent with predictions from human mutations.

**Molecular characterization of HOXA13 polyalanine expression**

To determine the mechanism for the apparent null phenotype of the polyalanine expansion, we examined in vivo limb bud RNA and protein expression from the expanded allele. To examine HOXA13 splicing and steady-state mRNA abundance, we performed RT–PCR on wild-type and mutant limb bud RNA. As shown in Figure 6A, no apparent difference in the abundance of Hoxa13 RNA was observed in mutant limb buds, using RT–PCR. In addition, no abnormal splicing products were observed, suggesting that the remaining loxP site and additional sequences in the intron did not interfere with splicing.

To determine whether Hoxa13 mRNA was expressed appropriately in developing limb buds, we performed whole-mount RNA in situ hybridization on wild-type and mutant limb buds (Fig. 6B, top two panels). Hoxa13Ala28 RNA was found in the expected spatial domains and abundance in distal limb buds at E11.5, suggesting that the mRNA is normally expressed.

To determine whether the steady-state abundance of polyalanine expansion HOXA13 protein (HOXA13Ala28) is normal, whole-mount immunohistochemistry was performed using an affinity-purified, anti-HOXA13 antibody (Fig. 6B, lower three panels). A reduction in the steady-state abundance of the protein in mutant limb buds was observed, although the pattern of expression was normal. Western blotting using protein lysates from wild-type and mutant limb buds confirmed the reduced abundance of the protein in vivo (Fig. 7). Reduced protein abundance could be secondary to a reduction in efficiency of translation or to degradation or combined effects.

To determine whether the translation efficiency of the RNA encoding the expanded protein, we performed in vitro transcription/translation (Fig. 8). When compared with the wild-type Hoxa13 allele, the translation efficiency of HOXA13Ala28 is significantly reduced, consistent with the in vivo results. This suggests that the reduced protein abundance is due to a decrease in translation efficiency rather than degradation.
was equal to wild-type HOXA13. Thus, the apparent loss of function exerted by the in-frame alanine expansion is most likely related to degradation of the protein in vivo.

**DISCUSSION**

Polyalanine expansions within all three of the largest (14, 12 and 18 alanine residues) endogenous polyalanine tracts in HOXA13 have now been shown to give rise to HFGS (Fig. 9). The range of expansion so far described is 6–12 residues. The phenotype among patients with expansions is similar and the skeletal and GU effects appear to be secondary to loss of function. Polyalanine expansions are stable through many generations, unlike the expansions observed in typical trinucleotide repeat disorders. We have tested, in a mouse model, the molecular consequences on RNA and protein expression of expansion in polyalanine tract III.

Polyalanine expansions appear to have different pathogenic mechanisms of action (2). In our study, mice harboring a 10-residue polyalanine expansion of Hoxa13 exhibit typical phenotypic findings of Hoxa13 loss of function. The loss of function is not secondary to transcriptional shutdown, interrupted splicing or unstable mRNA as revealed by RT–PCR across the exon–intron boundaries and by RNA whole-mount in situ hybridization. Thus, in contrast to fragile X expansions, which lead to FMR1 hypermethylation and loss of transcription, HOXA13 polyalanine expansions affect post-transcriptional processes. Furthermore, protein abundance in developing limb buds and transfected cells was lower for the expanded protein, yet in vitro translation efficiency appears to be normal. Thus, significant degradation of the protein in vivo must be occurring. Albrecht et al. (34) show that HOXD13, HOXA13, RUNX2 and SOX3 polyalanine expanded proteins form cytoplasmic aggregates. These aggregates are not localized to the aggresome, yet are degraded in vivo and colocalize with chaperones Hsp40 and Hsp70. The ratio of nuclear/cytoplasmic protein is related to the length of the HOXD13 polyalanine repeat. Thus, consequences of polyalanine expansion mutations may be explained by a common mechanism involving misfolding, degradation and cytoplasmic aggregation of polyalanine expanded proteins.

![Figure 3. DNA sequence chromatograms of affected family members. Tracings A and C were obtained on gel-purified PCR products. Family A sequence shown (III-1) was confirmed by opposite strand sequencing (data not shown). The Family B sequence chromatogram was obtained by sequencing cloned PCR products.](image-url)
Table 1. Genitourinary and reproductive tract problems in affected family members of Family B

<table>
<thead>
<tr>
<th>Female pedigree member</th>
<th>GU/reproductive tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-5</td>
<td>Bladder incontinence</td>
</tr>
<tr>
<td>III-2</td>
<td>None</td>
</tr>
<tr>
<td>III-3</td>
<td>Five miscarriages, late term; surgery to unify uterus; many urinary tract infections</td>
</tr>
<tr>
<td>IV-3</td>
<td>Enlarged urethra at birth; multiple urinary tract infections; surgery at 16 years to reduce urethral size</td>
</tr>
<tr>
<td>IV-4</td>
<td>Ureteral reflux; longitudinal vaginal septum; two miscarriages; numerous urinary infections</td>
</tr>
<tr>
<td>V-3</td>
<td>Multiple urinary tract infections as infant; scarring left kidney; multiple IVU, no ureteral reflux; urethra misplaced; urinary frequency; double vagina with surgical correction at 20 years; imperforate hymen requiring hysteroscopy at 14 years; three miscarriages</td>
</tr>
<tr>
<td>VI-7</td>
<td>Vaginal septum requiring surgical correction; no urinary infection</td>
</tr>
<tr>
<td>VI-12</td>
<td>Longitudinal vaginal septum surgery corrected at 16 years; ureteral reflux with surgical correction at 3 years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Male pedigree member</th>
<th>Genitourinary tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1, 4, 6</td>
<td>?</td>
</tr>
<tr>
<td>III-1</td>
<td>?</td>
</tr>
<tr>
<td>III-4</td>
<td>?</td>
</tr>
<tr>
<td>IV-1</td>
<td>+Hypospadias</td>
</tr>
<tr>
<td>IV-2</td>
<td>+Hypospadias</td>
</tr>
<tr>
<td>IV-6</td>
<td>?</td>
</tr>
<tr>
<td>IV-8</td>
<td>?</td>
</tr>
<tr>
<td>IV-9</td>
<td>?</td>
</tr>
<tr>
<td>IV-10</td>
<td>+Hypospadias</td>
</tr>
<tr>
<td>V-5</td>
<td>?</td>
</tr>
<tr>
<td>V-12</td>
<td>?</td>
</tr>
<tr>
<td>VI-6</td>
<td>Small genitalia; club feet; bilateral inguinal hernia; undescended testicles; hip dislocation requiring 11 body casts; one-sided weakness</td>
</tr>
<tr>
<td>VI-8</td>
<td>?</td>
</tr>
</tbody>
</table>

What is not clear at this stage is why polyalanine expansions for HOXA13 appear to act mostly as loss of function mutations in the limbs, whereas HOXD13 polyalanine expansions act in a dominant-negative way. Further work will be required to determine the basis for this difference.

An intriguing clinico-pathologic issue is the seemingly greater severity of GU anomalies and problems within Family B with the largest expansion reported so far (+12 in repeat III) by comparison with other families with HOXA13 mutations. The statistical power is not strong enough to make genotype/phenotype conclusions relative to GU tract defect severity or penetrance and polyalanine expansions. A seemingly high rate of hypospadias and other GU problems was reported by Frisén et al. (6) in a family in whom a tract II polyalanine expansion of eight residues (to a total of 18) was observed. Hypospadias in HOXA13 deficiency may be caused by loss of Bmp7 and Fgf8 signaling in the urethral plate epithelium, as shown in Hoxa13 mutant mice (24). The downstream molecular consequences of HOXA13 deficiency in the defects in the upper urinary system or for incomplete Mullerian fusion in humans are not understood; however, Fgf and BMP signaling warrant close examination. Despite the uncertainty about whether a polyalanine expansion in HOXA13 induces greater risk for genitourinary defects, it is conceivable that the nature and extent of the protein sequestration and/or degradation in the cytoplasm may be unique in different tissues of expression. Thus, a potential increase in GU anomalies in families with HOXA13 polyalanine expansions may represent dominant-negative effects of this allele, whereas dominant-negative effects of HOXD13 expansions are seen in the limbs.

It is unclear whether all large repeats have the same limit for non-pathogenic expansion. At least for the third repeat, once the repeat length exceeds 24, HOXA13 function appears to decline. The 6-alanine expansion in large repeat II (12, +6) reported by Frisén et al. (6) was associated with typical features of HFGS, and some members had a large gap between the first and second toes and more frequent urogenital problems. Surprisingly, the total length of the mutated tract is only 18 alanine residues, which is the normal endogenous length for polyalanine tract III. Polyalanine expansion of eight residues in the first large repeat in Family C is also not tolerated; this increases the total number of alanine residues to 22, which is less than the minimum expanded pathogenic repeat III (+6 alanine residues to a total of 24) reported so far (4,9). Polyalanine expansions in different domains of the protein may be tolerated variably.

Unequal crossing-over in meiosis has been hypothesized as the molecular mechanism for expansions, and presumably contractions, as these are the two products of this process (12). Formal proof of this mechanism has not been presented in HOXA13 alanine variation. Proof would require accurate delineation of the haplotypes of each parent and the affected offspring around the expanded gene, and expansions resulting from unequal crossing-over between sister chromatids cannot resolve the question. Errors during DNA replication could also generate polyalanine length variation. The murine Hypodactyly mutation is a deletion of 50 bp in the first exon of Hoxa13, which we hypothesized was catalyzed by unequal recombination or misalignment during replication of two identical 10 bp repeats (27). In yeast, replication slippage events resulting from mispairing of repeated sequences on opposite strands can lead to extended deletions/duplications (28–30). These extended deletions/duplications, defined as changes of 5–100 bp (18), are generally catalyzed by short direct repeats of 3–12 bp in length. The direction of replication can influence the mutational spectrum, further implicating DNA replication as a repair checkpoint for extended deletions/duplications (28).

Our lab has recently identified a family with an in-frame HOXA13 contraction of repeat III from 18 to 8 alanine residues, which is stable over at least three generations (data not shown). Such a short allele size was not observed in the studies of Lavoie et al. (1) or in our preliminary evaluation of 60 additional human chromosomes (data not shown).
This family will be described elsewhere, but it suggests that very short polyalanine tracts can be generated that might be useful for assessing mechanism(s) of HOXA13 polyalanine length variation and also to ascertain whether a minimum length is necessary for normal mammalian HOXA13 function.

**MATERIALS AND METHODS**

**Probands and families**

Clinical diagnoses were made by J. W. Innis, K. Lehmann and D. Mikulic. Families were enrolled and were consented with Institutional IRB-approved consent documents. Blood or...
cheek scrapings were used to prepare genomic DNA. PCR analysis across the first exon of HOXA13 was accomplished according to prior protocols (4), and bi-directional sequencing was performed. Results presented were based on at least two independent PCR amplifications followed by DNA sequencing of gel-purified PCR products (Families A and C) or individual cloned fragments (Family B).

**Generation of targeting construct for Hoxa13 alanine expansion**

Isogenic (129/Sv) genomic clones of the mouse Hoxa13 gene were isolated from a phage P1 clone (31). ES cell line (AB-1), a neomycin-resistant feeder layer cell line expressing LIF (SNL 76/7 STO cells), neomycin-resistance and diphtheria toxin selection cassettes, and Cre-expression vector (pCAGGS-Cre) (32) were used. The targeting vector was created as follows (restriction map, Fig. 4). The HindIII site within the polylinker of pTZ18R was deleted and an MluI site was created in place of it. The MluI site cleavage was followed by insertion of an adapter to create an NsiI site, which is compatible for ligation with PstI cleaved ends. This vector was cleaved with NsiI and PstI and two large isogenic PstI fragments spanning the Hoxa13 gene were inserted. The 5' segment spans ~4.5 kb and ends ~0.5 kb 5' of the Hoxa13 transcription initiation site (26). The 3' PstI segment spans all of exon 1, the intron and ends 100 bp 5' of the first polyadenylation site (27). The assembly of this clone created pTZ-8.5 kb PstI. This clone has a single HindIII restriction site within the native intron into which a neomycin resistance cassette (Neo) flanked by loxP sites driven by the PGK promoter was inserted to create p8.5 + Neo. [The neomycin and PGK-diphtheria toxin (DTA) cassettes were obtained from pLxNDTA. pLxNDTA was first cleaved with ClaI and religated to remove the single SalI site, creating pLxNDTASal. pLxNDTASal was cleaved with NorI and this site was ligated to a NorI/HindIII adapter. The resulting clone was cleaved with HindIII to remove the neomycin resistance cassette, which was then placed within the HindIII site in the first intron of Hoxa13.] p8.5 + Neo was linearized with SalI and the PGK-DTA cassette, obtained by SalI/XhoI digestion from pLxNDTA, was inserted creating p8.5ND-WT (WT refers to wild-type), a plasmid of ~15 kb size. In this clone, the XhoI/SalI fusion on one side of the insert is not receleavable with either enzyme. Restriction enzyme digestion was used to verify the structure of the plasmid clone.

**Construction of alanine expansion III (Hoxa13Ala28)**

Alanine expansion was created within p8.5ND-WT by cleavage with SfiI (‘s’ sites in Fig. 4), which removes a 216 bp fragment leaving cohesive ends, and replacement with a reconstructed double-stranded (ds) oligonucleotide with 30 extra codons for alanine residues. Two pairs (four separate oligonucleotides) of complementary long oligonucleotides were synthesized and HPLC-purified at the University of Michigan oligonucleotide synthesis core lab. Complementary For-1 (8775H; 121 nt) and Rev-1 (8776H; 116 nt) oligonucleotides comprised the 5' side of the reconstructed SfiI insert. For-1 sequence was 5'-C

![Figure 5. Phenotype of mutant forelimb and hindlimb buds is similar to engineered loss of function alleles (21,22). E13.5 embryos were obtained and genotyped. Embryos were stained with Alcian Blue as described before (22). E12.5 limb buds were also analyzed (data not shown). Note loss of digit one in mutant forelimbs and hindlimbs. Digits are numbered I–V, with I being the thumb or hallux. Ala28 refers to the expanded polyalanine tract allele. No homozygote was ever recovered at birth.](資源)
Complementary For-2-ALA (8777H; 112 nt) and Rev-2-ALA (8778H; 117 nt) oligonucleotides comprised the 3' side of the reconstructed SfiI insert. For-2-ALA sequence was 5'-CGGCCGCCGCTGCCGCCGCCGCGGCTGCGCGGCGAGCAGCAGCTGCGCGCG-3'; the PstI restriction site included within this set is underlined and was used to verify insertion. Rev-2-ALA sequence was 5'-CGGCCGCCGCTGCCGCCGCCGCGGCTGCGCGGCGAGCAGCAGCTGCGCGCGCG-3'; the PstI restriction site included within this set is underlined and was used to verify insertion. The dried oligonucleotides were rehydrated and annealed by boiling for 5 min followed by overnight cooling. The two newly created ds oligonucleotides each had one specific cohesive SfiI end that could not anneal with the other oligonucleotide. Each ds oligonucleotide also had a cohesive end that could be annealed and ligated to the other long ds oligonucleotide. The size of the ds oligonucleotides was confirmed using 2% agarose gel electrophoresis. Ten micrograms of each annealed ds oligonucleotide was precipitated separately, resuspended and phosphorylated using T4 polynucleotide kinase. The two ds oligonucleotides were mixed and ligated to each other unidirectionally. The success of the ligation was confirmed by agarose gel electrophoresis, and then the ~230 bp product was gel purified by 6% polyacrylamide gel electrophoresis and then ligated into SfiI cut vector p8.5ND-WT. Coding region modifications were verified by restriction digestion with PstI, Sau3A, SfiI and SmaI followed by gel electrophoresis and DNA sequencing with 35S-labeled dATP and an Excel II kit (Epicentre).

**Generation of Hoxa13 targeted ES cell lines**

The targeting construct was linearized with MluI and introduced into AB1 ES cells, passage 9, by electroporation.
Twenty-four hours after transfection, the medium was changed to include G418 at 240 mg/ml to select for recombinants. Individual G418-resistant colonies were propagated in multiwell tissue culture plates. Genomic DNA was prepared from ES cells using ES cell digestion buffer (1/C2 Valdes NK50 without beta-mercaptoethanol, 0.5% NP-40, 0.5% Tween 20, 1 mg/ml proteinase K) (25). Proteinase K was inactivated at 85°C for 15 min, and PCR was performed on 10-fold dilutions of pooled preparations of 10 individual colonies to screen for colonies with homologous recombination events. This screening PCR employed a primer from the neomycin resistance cassette and a second primer designed from 3' Hoxa13 genomic sequences outside of the targeting vector sequences, which generated a PCR product of 2.3 kb. PCR was performed successfully with forward primer Neo-A (5'-GGTGGGCTCTATGGCTTCTG AGG-3' ; primer P1 in Fig. 4) and reverse primer BglII-3' (5'-GTTCCGCGTAG AAAAGTAACTCGG-3' ; primer P2 in Fig. 4). BglII-3' is downstream of the 3' end of the targeting vector genomic sequence. Individual colonies within positive pools were then identified. Of 256 ES colonies surviving selection in G418, 19 (7.4%) had undergone homologous recombination. Subsequently, PCR across the expanded alanine repeat of the first exon was performed to verify the presence of one wild-type allele and one expanded allele in those cells. PCR utilized forward primer (9764D; 5'-ATCGAGCCCGTCATGTTTCTCTACGACAAC-3' ; primer P3 in Fig. 4) and reverse primer (391I; 5'-GCTCTGTGCCGCCGCCGAGTGCCGCCGCCGAGCAGGGACT-3' ; primer P4 in Fig. 4). Of the 19 positive clones, 10 had both a wild-type length PCR product and the larger product characteristic of the modified allele. Therefore, recombination of the exon one modification had occurred only 50% of the time even though this segment was six times longer than the distance between exon one and the neomycin resistance cassette in the first intron. Genomic DNA from these 10 clones was digested with restriction enzymes and evaluated by Southern blotting to assess the overall integrity of the targeted locus. DNA was digested with HindIII, BstXI or PstI, separated by agarose gel electrophoresis, blotted and probed with a SfiI–HindIII fragment (Probe A, Fig. 4) of the Hoxa13 gene corresponding to the end of exon one and the 5' half of the intron. HindIII was used as one restriction enzyme for the analysis because a fragment of 7 kb should be detected with this probe by cleavage within intron 1 and a site outside of the targeting construct. BstXI sites flank the intron and do not cleave the neomycin cassette, thus BstXI was used to verify that the inserted cassette increased the size of the intron. PstI was used because the alanine insertion was engineered screen for colonies with homologous recombination events.
to harbor a PstI site and cleavage within the neomycin cassette and within the exon one modification should produce an 800 bp product, demonstrating physical linkage of the two modifications. Two of the 10 clones had a non-stoichiometric ratio between the BsrXI fragments suggesting the potential for wild-type ES cell contamination, and these clones were not studied further. The remaining eight clones fulfilled all expectations for restriction digestion.

The neomycin resistance cassette was removed from the first intron of the targeted Hoxa13 locus in ES cells by transient transfection of Cre recombinase-expressing plasmid (pCAGGS-CRE) (32). Two of the eight clones described earlier were chosen for transient Cre expression, C3 and F6. Passage 12 C3 and F6 ES cells were thawed, grown and transfected. Cells were seeded and 96 independent clones that had good morphology were picked (passage 15) for each line. Detection of the PGK-Neo cassette between the loxP sites was verified by PCR using primers H3-intron-for (392I; 5'-CTTGCCTTAGCATTCTGCAC-3'); primer P5 in Fig. 4) and H3-intron-rev (393I; 5'-TTCCCATCTTGAGAATCGA TGTC-3'; primer P6 in Fig. 4). Successful Cre recombination generates an allele that produces a PCR product 130 bp larger than the native intronic product and includes construct DNA along with a single loxP site. Successful Cre recombination was demonstrated for 43.4% (10/23 screened C3) and 40.9% (9/22 screened F6) of the clones.

**Generation of chimeric mice**

Alanine expansion ES clones C3-2C and F6-2E at passage 18 and C3-2A at passage 19, each lacking the neomycin resistance cassette, were injected separately into C57Bl6/J blastocysts to create chimeric mice. Two chimeric males were generated from each cell line and both males showed shorted nails and first digits of their hindfeet. C3-2A failed to generate any viable offspring. C3 failed to develop normally and within the exon one modification should produce a 442 bp product. An RT–PCR control spanning introns of the peptidyl-prolyl isomerase C gene was used. No abnormal splicing of the PGK-Neo cassette between the loxP sites was observed. Successful Cre recombination was demonstrated for 43.4% (10/23 screened C3) and 40.9% (9/22 screened F6) of the clones.

**RT–PCR of mouse limb bud RNA**

Primer TW5 (reverse primer for RT–PCR: 5'-CTTGTTGGAG CTTATACGTCGTTGAGGATGGAGACGC-3') was used for reverse transcription of total RNA from limb buds of wild-type, heterozygous or homozygous mutants. Primers TW45 (forward 5'-TCTGGGATACATCTGAGCAGTGGAGAC-3') and TW5 (3' end of primer is 3 bp downstream of splice acceptor in second exon) were subsequently used on the RT product to amplify a 442 bp product. An RT–PCR control spanning introns of the peptidyl-prolyl isomerase C gene was used. No abnormality of the abundance or size was found. No abnormal splicing products were identified.

**Whole-mount in situ hybridization**

Embryos were collected from matings of C57Bl/6J mice at E11.5. Embryos were staged by assigning noon of the day of vaginal plug identification as E0.5. Antisense mRNA probes were transcribed in vitro as previously described for Hoxa13 (22). Whole-mount in situ hybridization with a single digoxigenin-labeled RNA probe was performed as previously described (22), except that BM purple (Roche) was used as substrate for the alkaline phosphatase.

**Western blotting**

Mouse limb buds from E11.5–E12.5 were harvested and each set of four was prepared as described (26). Genomic DNA samples from each embryo were genotyped twice by PCR to identify wild-type, heterozygous and homozygous mutant alleles by amplification across the expanded repeat coding sequences, as described earlier. Protein preparations from like genotypes were pooled and separated by 12% denaturing polyacrylamide gel electrophoresis and transferred to nitrocellulose via electroblotting. Membranes were incubated with HOXA13-specific polyclonal antibody as described (24), and primary antibody was detected with goat anti-rabbit antibody HRP. Visualization of protein bands was performed with a Supersignal Chemiluminescent kit (Pierce).

**Whole-mount immunohistochemistry**

Performed as described (33) on wild-type, Hoxa13Ala28+/+ or Hoxa13Ala28/Hoxa13Ala28 embryos collected at E11.5. The anti-HOXA13 and rabbit pre-immune antibodies were used at a dilution of 1:10 000. The secondary antibody, donkey anti-rabbit HRP conjugate (Amersham), was used at a dilution of 1:500.

**In vitro transcription/translation**

TNT-coupled wheat germ extract system (Promega) was used and identical aliquots of the reaction were separated by polyacrylamide gel electrophoresis, electrotransferred and treated as western blots as described earlier. Blots were probed with affinity-purified rabbit anti-HOXA13 antibody (26) at 1:200 or rabbit anti-HOXD13 antibody at 1:1000.

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