Polydactyly in the mouse mutant Doublefoot involves altered Gli3 processing and is caused by a large deletion in cis to Indian hedgehog

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ABSTRACT

The mouse mutant Doublefoot (Dbf) shows preaxial polydactyly with 6–9 triphalangeal digits in all four limbs and additional abnormalities including a broadened skull, hydrocephalus, and a thickened, kinked tail. The autopod undergoes a characteristic expansion between late embryonic day (E) 10.5 and E11.5, following the onset of ectopic Indian hedgehog (Ihh) expression in the entire distal mesenchyme, except for the zone of polarising activity (ZPA), at E10.5. We show here that limb prepattern, as indicated by expression of Gli3 and Hand2 at E9.5 is unaffected by the mutation. As both Sonic hedgehog (Shh) and Ihh expression are present in Dbf limb buds at E10.5, we generated Dbf\textsuperscript{+/+};Shh\textsuperscript{-/-} mutants to analyse the effects of different patterns of Hedgehog activity on the limb phenotype and molecular differentiation. Dbf\textsuperscript{+/+} embryos lacking Shh showed postaxial as well as preaxial polydactyly, and the Ihh expression domain extended posteriorly into the domain in which Shh is normally expressed, indicating loss of ZPA identity. Differences in gene expression patterns in wild type, single and compound mutants were associated with differences in Gli3 processing: an increased ratio of Gli3 activator to Gli3 repressor was observed in the anterior half of Dbf\textsuperscript{+/+} limb buds and in both anterior and posterior halves of compound mutant limb buds at E10.5. To identify the cause of Ihh misregulation in Dbf\textsuperscript{+/+} mutants, we sequenced 20 kb of genomic DNA around Ihh but found no pathogenic changes. However, Southern blot analysis revealed a 600 kb deletion disrupting or deleting 25 transcripts, starting 50 kb 5' of Ihh and extending away from the gene. The large deletion interval may explain the wide range of abnormalities in Dbf\textsuperscript{+/+} mutants. However, we did not detect analogous deletions in cases of Laurin–Sandrow syndrome, a human disorder that shows phenotypic similarities to Dbf.

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1. Introduction

The Dbf mutant, which arose spontaneously in the 3H1 (C3H/HeH × 101/H F1 hybrid) genetic background at Harwell (UK), is a polydactyloous mouse that exhibits semidominant inheritance. Mice heterozygous or homozygous for Dbf have 6–9 digits in all four limbs; the extra digits are all triphalangeal and arise preaxially (Lyon et al., 1996; Hayes et al., 1998a). Dbf\textsuperscript{-/-} mice also show malformation of the tibia, a broadened skull, hydrocephalus, a thickened kinked tail, and reduced fertility and viability. Homozygotes additionally exhibit a midline facial cleft but cannot be recovered alive beyond embryonic day (E) 14.5.

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Polydactyly has been described in many mouse mutants, all except two of which show a discrete anterior domain of Sonic hedgehog (Shh) expression (Masuya et al., 1995; Hill et al., 2003). The Extra-toes (Xt) mutant has an extended Shh domain due to functional inactivation of Gli3 (Hui and Joyner, 1993), whereas Dbf mice exhibit ectopic Indian hedgehog (Ihh) expression in the distal limb bud mesenchyme (Yang et al., 1998). Ectopic Ihh upregulation is first detectable at E10.5 (Crick et al., 2003), the stage at which hyperexpansion of the autopod begins; downstream targets of Shh signalling are ectopically upregulated (Hayes et al., 1998b; Yang et al., 1998). However, the molecular mechanism by which the polydactyly arises from ectopic Ihh expression has not been investigated.

The polydactyly phenotype of the Xt mutant was originally thought to result from the enlarged Shh expression domain (Hui and Joyner, 1993). However, Shh−/−;Gli3−/− mutants exhibit polydactyly in a similar pattern to Gli3−/− mutants, suggesting that the polydactyly of Gli3-deficient mice is independent of Shh (te Welscher et al., 2002). In wild type (wt) limb buds, digital number and identity are regulated by interaction between Shh and Gli3 (Litingtung et al., 2002). In the presence of Shh, Gli3 remains as a 190 kDa activator species, Gli3A, that upstream-regulates Hedgehog (Hh)-responsive gene expression, while in the absence of Shh, Gli3A is processed to a smaller 83–86 kDa repressor form, Gli3R, which negatively regulates expression of Gli3 and its target genes (Dai et al., 1999; Shin et al., 1999; Sasaki et al., 1999). Litingtung et al. (2002) suggested that in wt limb buds the Gli3A:Gli3R ratio controlled by Shh limits the polydactyly growth of the autopod, imposing pentadactyl constraint. This is supported by the localization of Shh protein in wt limb buds, which extends anterior to the zone of polarising activity (ZPA) in a domain coincident with Patched1 (Ptc1) expression (Gritli-Linde et al., 2001), resulting in a posterior-to-anterior increase of the Gli3A:Gli3R ratio (Wang et al., 2000). Consistent with these observations, the Gli3 mutant throughout Shh−/− limb buds is mainly processed to Gli3R (Litingtung et al., 2002). Recently, the mutation underlying the polydactylyous chicken talpid2 mutant has been reported to be a novel gene and has also been shown to result in abnormal Gli3 processing (Davey et al., 2006). Given the evidence of involvement of abnormal Gli3 processing in the Xt, Shh−/− and talpid2 mutants, it is possible that the polydactylyous present in Dbf mice also results from aberrant Gli3 processing. This hypothesis is supported by evidence that Gli3 acts downstream of Ihh during endochondral skeletal development (Hilton et al., 2005; Koziel et al., 2005).

To investigate the mechanism by which polydactyly arises in Dbf we have analysed gene expression in Dbf+/+ limbs, where there is an excess of Hedgehog (Hh) signalling, and compared this to Shh−/− limbs, where there is none. Since Shh and Dbf are located on different chromosomes (5 and 1, respectively) (Blake et al., 2003; Hayes et al., 2001), we have been able to generate mutant mice that carry two copies of the disrupted Shh allele and are heterozygous for the Dbf mutation. To further dissect the mechanisms underlying the limb malformations in both Shh and Dbf mutants, we have analysed the effects of the ectopic Ihh expression associated with Dbf limb abnormalities in the Shh-null background by correlating altered patterns of gene expression with the phenotype of single and double mutants. Differences in Gli3 processing between each genotype suggest that Hh-Gli3 interactions govern the observed differences in digital number, and that postaxial polydactyly results from expression of Ihh, but not Shh, in the posterior ZPA mesenchyme.

Previous attempts to identify the Dbf mutation have been unsuccessful. Hayes et al. (2001) constructed a high resolution genetic map and localized the mutation to a 0.4 cM interval on mouse chromosome 1. This region contained 35 genes including several plausible candidates for the Dbf mutation. However, despite the sequencing of three of these genes, the Dbf mutation remained unidentified. Based on the misregulation of Ihh expression in Dbf, we sequenced ~20 kb of the surrounding genome but found no obvious pathogenic changes. To investigate whether a genomic rearrangement could be responsible, we used the mouse genome sequence to design a Southern blotting strategy to systematically screen the regions 5’ and 3’ of Ihh for copy number changes. We identified a ~600 kb deletion starting ~50 kb 5’ of Ihh, which removes or interrupts 25 known and predicted transcripts. This raises the possibility that additional abnormalities seen in Dbf/Dbf mice arise from loss of function of deleted genes, in addition to Ihh misregulation.

2. Results

2.1. The prepattern of Dbf limb buds is unaffected

Expression of Hand2 and Gli3 has been implicated in patterning the limb bud prior to Shh expression, and has been shown to be affected later by the absence of Shh (Chiang et al., 2001; te Welscher et al., 2002). We assayed expression of these two genes before (E9.5) and after (E11.5) the onset of ectopic Ihh at E10.5 in Dbf+/+ mutant embryos (Fig. 1). Gli3 expression is restricted to the anterior portion of the limb bud in wt embryos at E9.5 (Fig. 1A) and this expression pattern is not altered in the limb buds of Dbf+/+ mutants (Fig. 1B). Hand2 is expressed throughout the flank of wt embryos prior to formation of the limb bud, then becomes limited to the posterior region of the limb bud as it is initiated (Fig. 1C); this pattern is not altered in Dbf+/− embryos at E9.5 (Fig. 1D). At E11.5, expression of Gli3 in Dbf+/+ limb buds differs from that in wt embryos in extending more distally; the domain is also broader although this probably simply reflects the greater breadth of the limb bud (Fig. 1F). Expression of Hand2 is limited to the proximal posterior margin in wt E11.5 limb buds (Fig. 1G); in contrast, the Hand2 domain in Dbf+/+ limb buds extends anteriorly and distally (Fig. 1H). Hence the limb prepattern as indicated by the expression of Hand2 and Gli3 at E9.5 is unaffected in Dbf+/+ limb buds, but the expression domains of both genes are altered in association with the presence of ectopic Ihh expression at E11.5 (Fig. 3H).

2.2. Altered limb phenotype of Dbf mutants in the absence of Shh

As Shh-null embryos die perinatally, gross morphological examination of wt, Shh−/−, Dbf+/+ and Shh−/−;Dbf+/+ embryos was conducted at E13.5 and alcian blue staining of the limb bones was carried out at E17.5 (Fig. 2). Both forelimb and hindlimb autopods of Shh−/−;Dbf+/+ embryos resemble those of Dbf+/+
expression domains of Shh and Ihh in limb buds immediately prior to (E10.5) and after (E11.5) the period of expansion. In both wt and Dbf/+ limb buds at E10.5, Shh is expressed at the posterior margin (Fig. 3A and B), defining this region as the ZPA (Riddle et al., 1993). In wt mice Ihh is not expressed in limbs prior to E12.5 (St-Jacques et al., 1999) while in Dbf/+- mutant mice, Ihh expression is present in the distal mesenchyme of the limb bud at E10.5 (Fig. 3C). This ectopic Ihh domain extends throughout the area anterior to the ZPA and may correspond to the progress zone. Its absence from the ZPA was confirmed by double in situ hybridization to show nonoverlapping juxtaposed Shh and Ihh expression (Fig. 3D). Expression of Ihh in E10.5 Shh/C0/Dbf/+ mutant limb buds extends throughout the distal mesenchyme including the posterior margin, i.e. the domain in which Shh is expressed in Dbf/+ embryos (Fig. 3E).

At E11.5, Shh expression continues in the posterior margin of wt and Dbf/+ limb buds (Fig. 3F and G). Expression of Ihh in Dbf/+ mutant limb buds at E11.5 is progressively down-regulated from posterior to anterior, until it remains only in the anterior margin (Fig. 3H); in contrast, in Shh/C0/Dbf/+ mutant limbs, down-regulation of Ihh expression begins mid-distally, remaining strong in both the anterior and posterior mesenchyme (Fig. 3I).

2.4. Gene expression is altered in Dbf limb buds lacking Shh

To gain insight into the mechanisms underlying the different patterns of polydactyly generated in the presence of different sources of Hh signalling in Dbf/+ and Shh/C0/Dbf/+ limbs, we examined the expression of genes implicated in Shh signalling and limb patterning in wt, Dbf/+, Shh/C0/ and Shh/C0/Dbf/+ limb buds at E10.5 (Fig. 4); as shown in Fig. 3, this is the stage at which Ihh expression is first detected. Expression of the transcriptional targets of Hh signalling, Ptc1 and Gli1, is expanded anteriorly in Dbf/+ limbs; interestingly, expression of these genes is broader in the proximal mesenchyme of Dbf/+ limbs lacking Shh, suggesting expansion of the domain of Hh signalling in these limb buds. Conversely expression of Gli3, which is thought to be repressed by Hh signalling (Takahashi et al., 1998), shows a reduced expression domain in Dbf/+ limbs. As expected, Gli3 is expressed throughout Shh/C0/ limbs at E10.5, but in the presence of Ihh in Shh/C0/Dbf/+ mutants it is dramatically down-regulated and required a prolonged colour development time for detection.

In wt and Dbf/+ limbs at E10.5 there is a strong expression of Hand2 in the posterior mesenchyme, with a graded lower expression anteriorly, similar expression is seen in Shh/C0/ limbs. However, in Shh/C0/Dbf/+ limbs there appears to be a second strong anterior domain of Hand2 expression, consistent with the extended expression seen at E11.5 (Fig. 1H). As reported previously (Hayes et al., 1998b; Yang et al., 1998), the Hoxd13 domain is expanded anteriorly in Dbf/+ limb buds; in Shh/C0/Dbf/+ limb buds, the domain shows even greater expansion, consistent with the more regular digital fan seen in these mutants. Expression of Fgf8 throughout the AER of expanded Dbf/+ and Shh/C0/Dbf/+ limbs indicates that in both mutants Hh signalling between the mesenchyme and ectodermal AER is intact. Ectopic anterior expression of Fgf4 in

### Figure 1
Expression of Gli3 and Hand2 in E9.5 (A–D) and E11.5 (E–H) wt (A, C, E, G) and Dbf/+ (B, D, F, H) forelimb buds. Gli3 is expressed in the anterior region of both wt and Dbf/+ at E9.5, and Hand2 in the posterior region (arrows indicate the limits of the expression domains). At E11.5 Gli3 is expressed in a broader anterior domain in Dbf/+ limb buds, and Hand2 expression extends more anteriorly in Dbf/+ than in equivalently staged wt limb buds. Scale bars 0.2 mm.

2.3. Ihh and Shh expression in compound mutant limbs is mutually exclusive

The expansion that characterizes the Dbf/+ autopod takes place from late E10.5 to E11.5. We therefore analysed the
the expanded limb buds of both mutants is consistent with their ectopic Ihh expression. Bmp4 expression in the progress zone was slightly down-regulated in Dbf/+ limbs but up-regulated proximally; like wt limbs, it was absent from the AER. In contrast, Shh−/−;Dbf/+ limbs, which showed further down-regulation of Bmp4 in the mesenchyme of the progress zone, showed ectopic expression throughout the AER. Explanation for this pattern requires further investigation.

2.5. The Dbf mutation affects the limb bud Gli3 ratio

The action of Gli3 protein as a transcriptional activator relies on its maintenance as Gli3A, which requires Hh signalling (Dai et al., 1999; Sasaki et al., 1999; Shin et al., 1999). To determine the effect of differential Hh signalling on Gli3 processing in mutant limb buds, we used a Gli3 antibody combined with Western blot analysis to assess the comparative levels of Gli3A and Gli3R in the anterior and posterior halves of E10.5 limb buds of all four genotypes (Fig. 5). As reported previously, wt limbs have a higher ratio of Gli3R to Gli3A anteriorly than posteriorly (Wang et al., 2000, and Fig. 5B and C). Dbf/+ limb buds have a reduced level of the repressor relative to the activator, especially in the anterior half, where levels of the two forms of Gli3 are similar. In Shh−/−;Dbf/+ mutant showing a femur of approximately normal length and a rudimentary zeugopod with the proximal tibia and attached putative fibula, plus a single short digit. (L) Shh−/−;Dbf/+ hindlimb (upside-down compared to I–K) showing a femur of normal length, full-length zeugopod and an autopod exhibiting both preaxial and postaxial polydactyly (9 triphalangeal digits, one of which is duplicated distally). Scale bars 1 mm.

Fig. 2 – Morphology of E13.5 (A–H) and E17.5 (I–L) embryos and limbs, genotypes as indicated. (A and B) The preaxial polydactyly characteristic of Dbf/+ embryos (B) can be seen clearly in the hindlimb, which has 8 digits. (C) Shh−/− embryos show holoprosencephaly, oedema and highly truncated limbs. (D) Shh−/−;Dbf/+ embryos show only minor modification of the Shh−/− head phenotype; the limbs resemble those of Dbf/+ embryos except that the autopods show both preaxial and postaxial polydactyly; the whole embryo shows hypervascularization. (E–H) Cartilage preparations of E13.5 forelimbs. (E) wt forelimb, (F) Dbf/+ forelimb showing preaxial polydactyly. (G) The forelimb zeugopod and autopod of Shh−/− fetuses are represented by single elements and there is no elbow joint. (H) In the Shh−/−;Dbf/+ forelimb an elbow joint and radius are visible. (I) Wild type E17.5 hindlimb. (J) Dbf/+ hindlimb showing preaxial polydactyly (7 triphalangeal digits) and medial rotation (fusation) of the autopod due to the hypoplastic tibia. (K) Hindlimb of Shh−/− mutant showing a femur of approximately normal length and a rudimentary zeugopod with the proximal tibia and attached putative fibula, plus a single short digit. (L) Shh−/−;Dbf/+ hindlimb (upside-down compared to I–K) showing a femur of normal length, full-length zeugopod and an autopod exhibiting both preaxial and postaxial polydactyly (9 triphalangeal digits, one of which is duplicated distally). Scale bars 1 mm.
both parental strains, but found no pathogenic changes (data not shown). Subsequently we sought genomic rearrangements using a systematic Southern blotting strategy to interrogate the mouse genome sequence (http://genome.ucsc.edu/), which initially identified the absence of a polymorphic 8 kb SpeI fragment in \textit{Dbf} (see Section 4.5). Characterization of the breakpoint by Southern analysis and subsequently by inverse PCR led to identification of the centromeric breakpoint at position 75,098,488 bp on chromosome 1 (Fig. 6). Analysis of sequence 3' to this in \textit{Dbf}/+ DNA revealed the telomeric breakpoint to be at position 75,694,480 bp on chromosome 1. The deleted region therefore appears to be 595,992 bp; however this figure is not precise because the deletion encompasses a ~16 kb gap in the current mouse genome sequence (mm9 assembly) present between 75,102,130 and 75,118,131 bp. We confirmed the deletion by PCR using primers flanking the breakpoint and further demonstrated that three different loci distributed within the putatively deleted region were present only in a single copy in \textit{Dbf}/+ mutant DNA (see Section 4.5). Analysis of the wt sequences at the two breakpoints showed that the sequence at the centromeric breakpoint is unique, lying within the gene \textit{Non-homologous end joining factor 1} (Nhej1). However, a hexanucleotide motif CCAAAC present at the breakpoint is repeated 17 nucleotides upstream, separated by four copies of a trinucleotide CCT motif. The telomeric breakpoint resides within the 3' terminal region of a B1 repetitive element at the endpoint of a very T-rich motif (35 thymine residues in 47 bases) which is likely to represent the complement of an ancestral poly(A) tract related to the B1 element and does not disrupt any known gene. There is a third nucleotide ambiguity in the position of the breakpoint as the sequence ACA is present on both sides of the deletion (Fig. 6). In addition to disrupting Nhej1, the deletion completely removes 24 known and predicted genes (Fig. 6, Supplementary Table 2 and Section 3).

2.7. Laurin–Sandrow syndrome does not result from large deletions 5' of IHH

Laurin–Sandrow syndrome (LSS) (MIM 135750) is rare human developmental disorder characterized by triphalangeal preaxial polydactyly of the hands and feet, with variable involvement of the proximal limb elements. It has been previously suggested that LSS shares many similarities with \textit{Dbf} and may also arise from ectopic IHH expression (Innis and Hedera, 2004). To investigate the possibility that \textit{Dbf} and LSS share a common etiology, we screened five patients diagnosed with LSS for copy number variation at 23 sites between IHH and EPHA4 using multiplex ligation-dependent probe amplification (see Supplementary Information). No copy number variation was detected (data not shown).

3. Discussion

3.1. Ectopic IHH expression in \textit{Dbf}/+ is modified in the absence of Shh and is associated with loss of ZPA identity

Although we have previously shown that expression of ectopic IHH in \textit{Dbf}/+ limb buds coincides with the onset of limb bud expansion at E10.5 (Crick et al., 2003), it was not known whether the prepattern of \textit{Dbf} limbs might be affected by the mutation prior to IHH expression. However, no differences were detected in the expression of \textit{Gli3} or \textit{Hand2} in wt and \textit{Dbf}/+ embryos at E9.5 or E10.5, consistent with the hypothesis that ectopic IHH expression represents the primary pathogenic event. By E11.5, expression domains of both \textit{Hand2} and \textit{Gli3} were more extensive in \textit{Dbf}/+ than wt limb buds, suggesting that IHH signalling is able to modify their expression.

In \textit{Dbf}/+ limb buds, IHH and Shh are expressed in discrete adjacent domains. Exclusion of IHH from the Shh domain is
reminiscent of the exclusion of the Hh-inducible gene Gremlin from this domain; Scherz et al. (2004) suggested that the effect may be due to high levels of intracellular autocrine Shh signalling. The loss of identity of the ZPA resulted in a abnormal expansion of the posterior limb bud mesenchyme in Shh-/-;Dbf/+ mice leading to the additional postaxial polydactyly seen in these mutants.

3.2. Abnormal gene expression leading to an aberrant Gli3 ratio underlies Dbf polydactyly

To elucidate the limb patterning underlying Dbf/+ polydactyly and to investigate the generation of the broader, more regular fan of digits seen in Shh-/-;Dbf/+ mutants, we studied the expression of a range of limb patterning and development genes at E10.5. Dbf/+ mutant limbs show an anterior expansion of the positive regulators of Hh signalling Ptc1, Gli1 and the downstream targets Hoxd13 at E10.5 and Hand2 by E11.5. Dbf/+ limbs also show a reduction of Gli3 expression, which is thought to be negatively regulated by Hh signalling. Conversely, due to the complete lack of Hh activity in Shh-/-;Dbf/+ mutant limbs prior to E10.5, Gli3 is ubiquitously expressed in these limb buds until this stage, when it is down-regulated in Shh-/-;Dbf/+ but not Shh-/- mutants. Gli3R is thought to repress expression of Hoxd13 and Hand2 and Fgf4 in the anterior of wt limb buds while Gli3A induces the expression of Gli1 in the posterior region (reviewed in Tickle, 2006). Therefore, the postaxial polydactyly seen in Shh-/-;Dbf/+ mutants may be due to the loss of identity of the ZPA with concomitant posterior extension of the Ihh domain. In contrast, the preaxial polydactyly that is present in both Dbf/+ and Shh-/-;Dbf/+ mutants is correlated with ectopic Gli3A-induced Hh signal transduction together with lack of repression of posterior patterning genes by Gli3R in the anterior of the limb bud. We suggest that the discrepancy between the very low level of Gli3 mRNA (Fig. 4) and the Gli3 protein detected in Shh-/-;Dbf/+ limb buds at E10.5 (Fig. 5) indicates the perdurance of protein after the gene has been down-regulated.

3.3. Identification of the Dbf mutation

The interpretation of the mechanism of the Dbf mutation has been hampered previously by the failure of attempts to identify the underlying mutation. Using a Southern blotting and inverse PCR strategy we have demonstrated that a ~600 kb deletion underlies the Dbf phenotype. The presence of simple sequences at both breakpoints may have predisposed them to breakage; the lack of significant similarity between the breakpoints (except for a 3 nucleotide identity at the breakpoints themselves) suggests that the rearrangement is likely to have involved nonhomologous end joining (NHEJ). Further analysis of sequence at the breakpoints revealed that the distal breakpoint resides within the degenerate poly(A), tract of a short retrotransposon (SINE) of the rodent B1 family, which, like human Alu repeats originate from 7SL RNA (Vasetzky et al., 2003).

The deleted region in Dbf is relatively gene-dense and completely deletes 24 known and predicted transcripts as well as interrupting Nhej1 at the centromeric breakpoint. Several of these genes have previously been implicated in abnormal mouse phenotypes or human disease; information on the known expression patterns and functions of these genes is summarized in Supplementary Table 2. Abnormalities associated with genes in the deleted region may contribute to additional aspects of the heterozygous Dbf phenotype such as the broadened skull, hydrocephalus, reduced viability and fertility, thickened tail and supernumerary hair follicles. However, none of the homozygous null phenotypes resulting from specific targeting of the Ptpn, Des, Inha or Slc4a3 genes is lethal in late embryogenesis so the cause of death at E14.5 in Dbf homozygotes remains unclear. This could be attributable to loss of function of any of the genes within the interval for which homozygous mice have not yet been described, and/or to homozygosity for the ectopic Ihh expression defect. Interestingly a recent study reported a human fetus with a balanced de novo translocation t(2;7)(q36;p22) with the chromosome 2 breakpoint interrupting the orthologue of Nhej1 at a position similar to the start of the Dbf deletion (Cantagrel et al., 2007). The consequence of this translocation, as in Dbf, would be to isolate the human Ihh gene from possible regulatory sequences present on the opposite side of the NHEJ1 breakpoint. Although the terminated fetus exhibited syndactyly of all four limbs, polydactyly was not present, suggesting that the translocation did not result in ectopic Ihh expression.

We have presented evidence that the prepattern of Dbf limb buds is unaffected and that the preaxial polydactyly is attributable to a reduction in Gli3R resulting from ectopic Ihh expression. It is interesting that preaxial polydactyly, the most striking aspect of the Dbf phenotype, is unlikely to result directly from haploinsufficiency of any of the genes in the deleted region. Rather, the deletion appears to affect a cis-acting regulatory element of Ihh, which could be a repressor located within the deletion, or an enhancer beyond the deleted region. Other examples of regulatory mutations acting at a distance have been reviewed by Kleinjan and van Heyningen (2005). Pinpointing the regulatory sequences involved at a distance have been reviewed by Kleinjan and van Heyningen (2005). Pinpointing the regulatory sequences involved requires a major challenge, one notable success being the identification of the ZPA sequence regulatory sequence (ZRS) which lies ~1.0 Mb upstream of Shh and regulates its expression in the ZPA; mutations in the ZRS lead to ectopic Shh expression resulting in preaxial polydactyly (Lettice et al., 2002). However, owing to the large size of the Dbf deletion and the large number of genes and highly conserved non-coding elements within it, it will be challenging to delineate the precise mechanism underlying ectopic Ihh expression in the Dbf mouse.

4. Materials and methods

4.1. Generation and identification of mutant mice

Mice heterozygous for the Shh null allele (Chiang et al., 1996) on the C57BL/6J background were mated to Dbf/+ mice on the 3H1 background. The Shh mutant allele was detected as previously described (Chiang et al., 1996). Homozygous Shh-/- embryos were identified by their phenotype.
Fig. 4 – Regulation of limb development genes in E10.5 wt, Dbf+/−;Shh−/− and Shh−/−;Dbf−/− forelimb buds. The anterior margin of limb buds is uppermost. Ptc1 and Gli1 expression domains are extended more anteriorly in Dbf−/− limbs than in wt, and are broader in Shh−/−;Dbf+/− limbs, but absent from Shh−/− limb buds. Gli3 expression is present in the anterior region of wt limbs; the domain is reduced in Dbf+ limbs, and extended throughout Shh−/− limb buds; expression is at a very low level in Shh−/−;Dbf+ limb buds (extended colour development time). Expression of Hand2 is unaltered in Dbf−/− limbs, but is present more anteriorly in Shh−/−;Dbf−/− limb buds. Fgf8 is expressed throughout the AER of limb buds of each genotype. Bmp4 expression is similar to wt in Dbf−/− limbs but up-regulated in Shh−/− limbs; in Shh−/−;Dbf−/− limbs expression appears to be lower in the mesenchyme but high in the AER (shown in bright field). Expression of Hoxd13 is extended anteriorly in Dbf−/− limbs and both anteriorly and posteriorly in Shh−/−;Dbf−/− limbs, but is absent from Shh−/− limbs. Fgf4 is expressed in the posterior region of the AER in wt (posterior to the arrowhead), throughout the AER in Dbf−/−, is absent from Shh−/− and present in the anterior and posterior AER of Shh−/−;Dbf−/− limb buds. Scale bar 0.2 mm.
To genotype Dbf/+ embryos (prior to the identification of the causative deletion), Dbf/+ mice were crossed with wt Mus musculus castaneus and the Dbf F1 progeny were bred with C3H wt mice. Embryos were genotyped using primers which amplify the marker D1Mit46 located ~2.3 cm from Ihh (P1 5'-AGTCAGCTAGGGCTA CAGTATG-3', P2 5'-CCAGGGTGCTACTTGTAGAA-3'). This produces amplification products of 276 bp and 320 bp on the C3H and Mus musculus castaneus backgrounds respectively.

4.2. Whole mount in situ hybridization

Doubly heterozygous Shh+/−;Dbf/+ were crossed with Shh+/− mice and embryos of all six possible genotypes were collected for analysis of gene expression domains and morphology: wt, Shh+/−, Shh−/−, Dbf+/−, Shh−/−;Dbf+/− and Shh−/−;Dbf−/−. To ensure consistency between developmental stages, only forelimb buds were analysed and a minimum of two samples were examined with each probe. Timing of embryos was by the vaginal plug method: 12.00 noon on the day on which the plug was observed was regarded as E0.5. Pregnant females were sacrificed on the appropriate day by cervical dislocation and the embryos were dissected from the uterus in ice cold phosphate buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 10 mM Na,HPO4, 2 mM KH2PO4) followed by immersion in cold tissue fixative in accordance with the Animals (Scientific Procedures) Act, 1986. Where necessary, yolk sacs were removed for genotyping and embryos were fixed by immersion overnight at 4 °C in 4% paraformaldehyde in PBS. Embryos were dehydrated by sequential washing in 25%, 50%, 75% ethanol in PBT (PBS + 0.1% Tween 20) and finally by two washes in 100% ethanol. They were stored at −20 °C until required.

Single stranded digoxygenin-UTP labelled antisense riboprobe were generated from linearized plasmids containing cDNAs. Whole mount in situ hybridization was carried out essentially as described by Wilkinson (1992).

4.3. Skeletal preparations

Embryos for skeletal staining were dissected and fixed in 95% ethanol for 1–3 days. They were immersed in alcian blue stain binding to a 190 kDa band and two 83–86 kDa bands, immunoblotted and incubated with a Gli3-specific antibody. (A) Representation of an E10.5 limb bud showing the anterior and posterior regions dissected for protein extraction. (B) Approximately 3 μg of protein extracted from forelimb buds was used in each lane; protein bands were immunoblotted and incubated with a Gli3-specific antibody binding to a 190 kDa band and two 83–86 kDa bands, corresponding to full-length (Gli3A) and processed repressor forms (Gli3R), respectively. A, anterior; P, posterior. (C) The histogram shows the relative intensity of each band in arbitrary units following quantification by densitometry.

4.4. Western Blotting

The polyclonal antibody specific for the amino terminus of Gli3 was a gift from Dr. Chin Chiung (Litingtung et al., 2002). Three μg of protein lysate derived from the anterior and posterior halves of ~6 E10.5 forelimb buds were resolved on 4–12% polyacrylamide gels. Gli3 protein was detected using anti-N-terminal Gli3 (1:300) primary antibody and biotinylated anti-rabbit immunoglobulin–secondary antibody (1:1000). Protein bands were visualized by incubation with a streptavidin-peroxidase conjugate followed by an enhanced chemiluminescence detection method (Amersham).

4.5. Characterization of the Dbf deletion

To determine whether genomic rearrangements were associated with Dbf, single copy probes labelled with 32P-dCTP were synthesized and used to hybridize Southern blots of DNA isolated from heterozygous Shh+/− (C3H × H101 hybrid) Dbf/+ and wt mice from both background strains. We used the mouse genome mm9 sequence release (July 2007) for all analyses presented in this paper. A probe corresponding to 75,099,314–75,099,651 bp revealed the absence of a polymorphic 8 kb Spel fragment in Dbf, found in the C3H parental strain, suggesting the existence of a deletion. A further Southern blot using a probe corresponding to 75,097,987–75,098,255 bp revealed a 1.2 kb BspHI fragment present only in Dbf. This 1.2 kb breakpoint fragment was isolated by inverse PCR. Briefly, genomic DNA from Dbf was digested with BspHI, diluted to 10 ng/μl and T4 DNA ligase was added to promote intramolecular ligation. Religated DNA was used directly in an inverse PCR using the primer pair: 5’-GCATTTGAGAT TGAGACAAGCACTCTCCACAC-3’ and 5’-ACGGGCTAGACACGAGAAA GCCGTGTTGCT-3’. DNA sequencing revealed 228 bp of unknown sequence that was shown by BLAST analysis to originate from a region of chromosome 1, ~596 kb telomeric from the breakpoint. PCR amplification with primers designed either side of the breakpoint (5’-TGGTCTGGAGAGACAGCTCGTCCAGAG-3’ and 5’-GAGTC TAAACACTCTCCGCAACAC showdown to SYABGAT-3’) was employed to confirm the site of the deletion.

To confirm the Dbf lesion was a true deletion, primers were designed to amplify three regions within the deletion predicted to contain polymorphisms variable between the C3H and H101 back-
ground strains. These regions were located at ~150 kb intervals within the deleted region. The primer pairs used were site 1, 5'-GCCCTCATGCTTGAGTACCTTGCTGTGAT-3' and 5'-GTCCTCCAGGGGGCTGAGCAGAGTG-3'; site 2, 5'-TAGACTGAGCACCCGGCTACATGCTC-3' and 5'-TGTGTCATCCACCCGGTGCCTCTGACT-3'; site 3, 5'-TAGAATTCCACCTGGGTCCACCCACTC-3' and 5'-CATA CATCCGTGTACATGCTACTGTCAGT-3'. Amplification products were digested with appropriate restriction endonucleases to discriminate between the alleles. Site 1 contained a novel TACC insertion polymorphism and was digested with HphI, site 2 contained a known C/T polymorphism (rs31657679) and was digested with AvaI and site 3 contained a known polymorphism (rs3049959) and was digested with Hpy8I. In each case only the H101 allele was present indicating that the C3H chromosome carried the deletion. The presence of both background strains was confirmed on the centromeric side of the Dbf deletion by sequence polymorphisms observed during Southern blotting (data not shown). Both background strains were shown to be present on the telomeric side of the deletion by AseI restriction digest of a fragment containing a novel informative C/T polymorphism which was amplified using the primer pair 5'-CAACAAAGCCCATCAATTCACTCAGGCCGTG-3' and 5'-CACCCTGCCTCAACCTC-3'.

Fig. 6 – (A) Genomic map of the region around the Dbf deletion showing the genes flanking the deletion. The box encloses the deleted region shown at a higher scale. All nucleotide positions refer to the mm9 assembly. (B) DNA sequence chromatogram showing breakpoint sequence and alignment of this sequence compared to wt sequences on either side of the breakpoint. The arrows indicate the range of possible positions of the breakpoint within the three nucleotide identity shared by both normal sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2008.01.001.

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