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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*/+;*Shh*$^{-/-}$ mutants to analyse the effects of different patterns of Hedgehog activity on the limb phenotype and molecular differentiation. *Dbf*/+ 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*/+ 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*/+ 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*/+ mutants. However, we did not detect anologous deletions in cases of Laurin–Sandrow syndrome, a human disorder that shows phenotypic similarities to *Dbf*.

1. Introduction

The *Dbf* mutant, which arose spontaneously in the 3H1 (C3H/HeH × 101/H F1 hybrid) genetic background at Harwell (UK), is a polydactylous 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*/+ 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^J) 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 polydactylous phenotype of the Xt^J 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 up-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 *Shh* 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 polydactylous potential 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 present throughout $Shh^{-/-}$ limb buds is mainly processed to Gli3R (Litingtung et al., 2002). Recently, the mutation underlying the polydactylous chicken $talpid^3$ mutant has been reported to be in 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^J, $Shh^{-/-}$ and $talpid^3$ mutants, it is possible that the polydactyly 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 $Dbf/^+$