Patched 1 is a crucial determinant of asymmetry and digit number in the vertebrate limb

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The vertebrate hedgehog receptor patched 1 (Ptc1) is crucial for negative regulation of the sonic hedgehog (Shh) pathway during anterior-posterior patterning of the limb. We have conditionally inactivated Ptc1 in the mesenchyme of the mouse limb using Prx1-Cre. This results in constitutive activation of hedgehog (Hh) signalling during the early stages of limb budding. Our data suggest that variations in the timing and efficiency of Cre-mediated excision result in differential forelimb and hindlimb phenotypes. Hindlimbs display polydactyly (gain of digits) and a molecular profile similar to the Gli3 mutant extra-toes. Strikingly, forelimbs are predominantly oligodactyly (displaying a loss of digits), with a symmetrical, mirror-image molecular profile that is consistent with re-specification of the anterior forelimb to a posterior identity. Our data suggest that this is related to very early inactivation of Ptc1 in the forelimb perturbing the gene regulatory networks responsible for both the pre-patterning and the subsequent patterning stages of limb development. These results establish the importance of the downstream consequences of Hh pathway repression, and identify Ptc1 as a key player in limb patterning even prior to the onset of Shh expression.

KEY WORDS: Patched 1, Sonic hedgehog signalling, Limb development, Prx1-Cre, Shh/Grem1/FGF loop, Mouse

INTRODUCTION

Hedgehog (Hh) signalling is essential for the correct patterning of virtually every vertebrate organ system, including the limb (Hill, 2007; Ingham and McMahon, 2001). In the absence of a hedgehog ligand (Sonic, Shh; Indian or Desert), the transmembrane receptor patched 1 (Ptc1; also known as Pch1) constitutively inhibits the transducer of the Hh signal, smoothened (Smo) (Chen and Struhl, 1996; Marigo et al., 1996a). In this repressed state, proteolytically cleaved Gli transcription factors, primarily Gli3, translocate to the nucleus to repress target genes. Upon binding of a hedgehog ligand to Ptc1, inhibition of Smo is relieved, Gli cleavage is inhibited, and full-length Gli proteins are converted to transcriptional activators. Importantly Ptc1, an inhibitor of the pathway, is transcriptionally upregulated in response to Hh signalling, creating a negative-feedback loop that is crucial for tight spatiotemporal control of signalling levels. Disruption of this feedback loop by inactivation of Ptc1 in mice results in inappropriate ligand-independent activation of the Hh pathway, and early embryonic lethality around 9.5 days post coitum (dpc) (Ellis et al., 2003; Goodrich et al., 1997).

In the limb, Shh is secreted from a group of posterior mesenchymal cells called the zone of polarizing activity (ZPA) (Riddle et al., 1993). The posteriorly biased Shh gradient, together with a temporal gradient of exposure to the Shh signal, specifies digit number and identity (reviewed by McGlinn and Tabin, 2006). Shh successively inhibits the cleavage of full-length Gli3 (Gli3FL) to the repressor form (Gli3R) from posterior to anterior (Wang et al., 2000), and this interplay between Shh and Gli3 is crucial in defining anterior-posterior (AP) limb patterning (Litingtung et al., 2002; te Welscher et al., 2002b). The Shh null mouse hindlimb has a single digit corresponding to the most anterior digit 1 (Chiang et al., 2001), whereas the extra-toes (Gli3Xt/Xt) mouse mutant lacks functional Gli3 and displays extra digits, or polydactyly (Hui and Joyner, 1993). While Gli3R is regarded as the primary effector of AP patterning in the limb, the role of Gli3FL in this process is the subject of some debate (Hill et al., 2009; Wang et al., 2007a; Wang et al., 2007b).

The nascent limb bud is subject to an exquisitely controlled network of gene regulatory loops that pattern the limb bud both before and after expression of Shh. Soon after emergence of the limb bud, an AP pre-pattern is established whereby anterior Gli3R restricts Hand2 expression to the posterior, and Hand2 reciprocally limits Gli3 to the anterior (te Welscher et al., 2002a). At this stage, the posterior restriction of 5'Hoxd genes (those genes at the 5' end of the Hoxd cluster) is crucial to final AP limb asymmetry, as these genes subsequently trigger expression of Shh specifically in the posterior limb (Tarchini et al., 2006; Zakany et al., 2004). Around the same time, Bmp4 induces expression of the BMP antagonist gremlin (Grem1), thus initiating the first of a number of finely tuned regulatory loops linking Shh signalling in the ZPA with FGF signalling from the distal apical ectodermal ridge (AER) (Benazet et al., 2009). Downregulation of Bmp4 by Grem1 enables Shh signalling from the ZPA, thus establishing the Shh/Grem1/FGF loop, whereby Shh induces Grem1 expression in the mesenchyme. This then allows FGF signalling from the AER, which in turn maintains Shh in the ZPA (Kokhka et al., 2003; Zuniga et al., 1999). A recent study suggests that limb outgrowth ceases when the Shh/Grem1/FGF loop drives FGF signalling to high enough levels to inhibit Grem1 expression (Verheyden and Sun, 2008). However, previous studies in the chick indicate that the inability of Shh expressing cells and their descendants to express Grem1 contributes to termination of limb outgrowth, as Grem1-expressing cells move beyond the influence of Shh from the ZPA (Nissim et al., 2006; Scherz et al., 2004).

Ptc1 null mice, or mice in which Ptc1 is ubiquitously inactivated, die prior to the initiation of limb budding (Ellis et al., 2003; Goodrich et al., 1997). Despite elegant studies involving transgenic rescue of
Ptc1 null mice (Milenkovic et al., 1999), the effects of Ptc1 inactivation in the early limb have not previously been investigated. We have used Cre recombinase driven by a Prx1 enhancer (Logan et al., 2002) to conditionally remove functional Ptc1 from the limb mesenchyme. This results in high-level ligand-independent activation of the Hh pathway across the entire limb. Ptc1 inactivation occurs before and after establishment of the ZPA in the forelimb and hindlimb, respectively, and these variations in timing and the subsequent level of Hh pathway activation are thought to underlie the dramatically altered phenotype produced in each limb. Later and lower levels of signalling in the hindlimb lead to polydactyly (gain of digits), while early and high-level pathway activation in the forelimb predominantly and unexpectedly results in oligodactyly (loss of digits). These forelimbs display a molecular expression profile that is largely symmetrical about the midline of the AP axis, reflecting re-specification of the anterior limb to a posterior fate. In addition, activation of the Hh pathway in a pattern that mirrors Prx1-Cre activity suggests that, unlike previous assumptions based on in situ hybridisation analysis of Ptc1, most embryonic mesenchyme is competent to activate Hh signalling if stimulated. This highlights the crucial nature of Ptc1-mediated repression.

MATERIALS AND METHODS
Mouse breeding
All animal experimentation was approved by a University of Queensland Animal Ethics Committee and conformed to relevant ethical guidelines. Prx1-Cre homozygous male mice (Logan et al., 2002) were mated with females homozygous for the Ptc1 conditional allele (Ptc1<sup>f<sup>1</sup></sup>)(Ellis et al., 2003), on a C57/B6/SV129 background. Male offspring heterozygous for both alleles were then backcrossed to Ptc1<sup>f<sup>1</sup></sup> females. The offspring of these crosses were designated wild type (WT; no Prx1-Cre and one or two Ptc1 conditional alleles), heterozygous (Prx1-Cre: Ptc1<sup>f<sup>1</sup></sup>) or homozygous (Prx1-Cre: Ptc1<sup>c<sup>1</sup></sup>)(conditional allele). For analysis of Prx1-Cre activity, females homozygous for the ZAP reporter transgene were mated to Prx1-Cre: Ptc1<sup>f<sup>1</sup></sup> males, and offspring were assayed for alkaline phosphatase expression as previously described (Lobe et al., 1999). PCR genotyping for the Ptc1 conditional transgene was performed as previously described (Ellis et al., 2003). Cre recombinase was detected using the primers: F, 5<sup>'</sup>-GATATCTCAGTACTGACGGTG-3<sup>'</sup>; R, 5<sup>'</sup>-GCTGTTT-CACATCCAGGTTAC-3<sup>'</sup>. For purification and sequencing to ensure correct product amplification.

RNA in situ hybridisation
Whole-mount in situ hybridisation was performed as previously described (Fovles et al., 2003). Probes were: Shh [nucleotides 36-678 of the vertebrate hedgehog sequence NM_017221 described by Roelink et al. (Roelink et al., 1994)], Ptc1 (Hahn et al., 1996), Gli1, Gli3 (C. C. Hui, The Hospital for Sick Children, University of Toronto, Canada), Hand2 (E. Olson, UT Southwestern Medical Center, Dallas, TX, USA), Fgf4, Fgf8 (G. Martin, University of California San Francisco, CA, USA), Mkp3, Spry1, Spry4 (M. Little, The University of Queensland, Australia), Greml1 (R. Harland, University of California, Berkeley, CA, USA), Hoxd11 (C. Tabin, Harvard Medical School, Boston, MA, USA), Hoxd12, Hoxd13 (D. Duboule, University of Geneva, Switzerland), Msx1 (R. Maas, Brigham and Women’s Hospital, Boston, MA, USA), Msx2 (R. Macon, University of Southern California, Los Angeles, CA, USA), Zfp503 (McGlinn et al., 2008), Pax9, Jag1 (McGlinn et al., 2008), and Bmp2 and Bmp4 (E. Robertson, University of Oxford, UK).

Reverse transcriptase (RT)-PCR for exon 3 of Ptc1
Individual limb pairs (9.5-11.5 dpc) were dissected and epithelium removed with 1 unit/ml dispase (Sigma), 10% foetal calf serum (Biowhittaker) in Puck’s Saline A for 1 hour at 37°C. Total RNA was extracted using an RNeasy miniprep kit (QiAGEN). cDNA was reverse transcribed from 1 µg of RNA with pd(Ns) random hexamers and MMLV-RT (Invitrogen) according to the manufacturer’s instructions. To ascertain recombination, a region including the exon flanked by loxp sites in the Ptc1 conditional allele (exon 3) was ampliﬁed using the primers Exon 2-F (5<sup>'</sup>-TGGCTTGTGTGGTCTCCCTCATATT-3<sup>'</sup>) and Exon 6-R (5<sup>'</sup>-CACCTTAAAGAGGCCGTTACCTA-3<sup>'</sup>) to produce 450-bp (WT allele) and 250-bp (deleted allele) products. Actin-positive control products (650 bp) were generated as described by Chen et al. (Chen et al., 2002).

Quantitative real-time PCR
Quantitative real-time PCR on cDNA was performed as previously reported (Bruce et al., 2007), and expression levels normalised against mouse hypoxanthine phosphoribosyltransferase (HPRT) as determined from the ratio of ΔCT values. Primers used were: HPRT (Bruce et al., 2007); Ptc1-F, 5<sup>'</sup>-GGTCTGGACGTTCGTTGTC-3<sup>'</sup>; Ptc1-R, 5<sup>'</sup>-GCCAGAAGCCGAAAGGA-3<sup>'</sup>; Gli1-F, 5<sup>'</sup>-TCAAGGCCAATACATGTCG-3<sup>'</sup>; Gli1-R, 5<sup>'</sup>-AGGACCTCCAGACAGCTTCA-3<sup>'</sup>; Prx2-F, 5<sup>'</sup>-TGTTAATCTCCTGGGCCTC-3<sup>'</sup>; and Prx2-R, 5<sup>'</sup>-GCTCAGATGCGTCGGAG-3<sup>'</sup>.

RESULTS
Inactivation of Ptc1 upregulates Hh signalling and produces variable gain/loss of digits
The Prx1-Cre transgenic drives Cre recombinase activity in the early limb mesenchyme (Logan et al., 2002). Prxl-Cre heterozygotes were mated with mice homozygous for a conditional allele of Ptc1 engineered by flanking exon 3 with loxp sites (Ellis et al., 2003). Heterozygous Prxl-Cre: Ptc1<sup>f<sup>1</sup></sup> mice survive to adulthood and are fertile and viable. Homozygous Prxl-Cre: Ptc1<sup>c<sup>1</sup></sup> embryos survive until 14.0 dpc, allowing analysis of the effects of Ptc1 inactivation in the limb mesenchyme during early limb development. Oedema visible from 13.5 dpc (Fig. 1B, C, arrowheads), and vascular haemorrhage evident at 14.0 dpc (Fig. 1C, arrowheads), are likely to be responsible for the lethality at this stage, although this phenotype has yet to be analysed in detail. Loss of tissue integrity in the distal limb often results in blister formation, which is particularly evident in the interdigital region at 13.5 dpc (arrowhead, Fig. 1H). At these later stages we have also detected defects in chondrogenesis, and characterisation of this phenotype will be presented elsewhere.
Ptc1 inactivation in the mouse limb

Fig. 1. Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> embryos display variable polydactyly/oligodactyly, and do not survive past 14.0 dpc. Wild-type (A,D,G) and Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> (B,C,E,F,H,I) embryos at 13.5 and 14.0 dpc. At 13.5-14.0 dpc, Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> embryos display polydactyly of the cranial mesenchyme, the inter-limb flank region and the maxilla (stars; B,C,I). By 14.0 dpc, embryos display blistering (also in the distal limb at 13.5 dpc, arrowhead, H), and vascular haemorrhage into oedematous areas (arrowheads; C; oedema also visible at 13.5 dpc, arrowhead, B). The majority (76%) of 13.5 dpc Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> forelimbs are oligodactylous (E), while 24% are polydactylous (F). All Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> hindlimbs display asymmetrical polydactyly (H). Scale bars: 500 μM in A-C; 200 μM in D-I.

Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> hindlimbs display polydactyly (Fig. 1H) with expansion of anterior tissue reminiscent of the Gli3<sup>M3X</sup> limb and other Hh gain-of-function mouse models (Hill et al., 2003). Unexpectedly, the forelimb displays an opposing phenotype, with 76% of 13.5 dpc Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> forelimbs analysed showing evidence of fewer digit primordia (oligodactyly; Fig. 1E; n=41). A minority (24%) of 13.5 dpc Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> forelimbs are polydactylous (Fig. 1F), but most retain the symmetry of shape of the oligodactyl forelimb, rather than the anterior expansion observed in the hindlimb (Fig. 1; compare F to H). Mesenchymal overgrowth visible in the dorsal cranium, maxilla and flank (star, Fig. 1B,C,I) correlates with additional sites of Prx1-Cre activity (see below).

We sought to define the spatiotemporal boundaries of Prx1-Cre activity relative to both Ptc1 excision and subsequent activation of the Hh pathway in the Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> limbs. Deletion of exon 3 of Ptc1 results in the upregulation of a transcript that encodes a non-functional protein incapable of dampening pathway activity via negative autoregulation. However, similar to wild-type Ptc1 and Gli1 transcripts (Goodrich et al., 1996; Marigo et al., 1996b), this truncated transcript acts as a robust marker of pathway activation. Using a probe capable of detecting both wild-type and exon 3-deleted Ptc1, whole-mount in situ hybridisation revealed wild-type Ptc1 expression primarily in a restricted domain in the limb bud and in the vibrissae at 12.5 dpc (Fig. 2A). Upon Ptc1 inactivation, expression was visible throughout the limb mesenchyme (Fig. 2B), and across the embryo in a pattern correlating closely with sites of Prx1-Cre activity (Fig. 2, compare B with D), as visualised by alkaline phosphatase expression in the Z/AP reporter assay (Lobe et al., 1999). In addition to the limb, these sites include the mesenchyme of the flank, the distal maxilla and the dorsal cranium, although expression of Ptc1 appears to be less robust in this latter region (Fig. 2B,D; Fig. S1B,E in the supplementary material) (see also Logan et al., 2002). The extent of Hh pathway activation revealed by Ptc1 was confirmed by Gli1 expression (Fig. 2C). A probe directed specifically to the deleted exon 3 of Ptc1 showed no hybridisation to regions of the embryo expressing Prx1-Cre outside the normal domains of Ptc1 expression (data not shown), confirming that the ectopic Ptc1 transcript is the non-functional deleted form.

The precise timing of Ptc1 inactivation was determined by RT-PCR of a region spanning exon 3. Detection of only the exon 3-deleted Ptc1 allele in three out of four 9.5 dpc Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> forelimb samples indicates, within the limits of detection by RT-PCR, complete excision of exon 3 before 9.5 dpc (Fig. 2E). Wild-type Ptc1 was still detectable in the Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> hindlimb at 10.5 dpc, but not at 11.5 dpc, indicating that exon 3 deletion approaches completion in the hindlimb between 10.5 and 11.5 dpc (Fig. 2F). The relationship between Ptc1 exon 3 excision and Hh pathway activation was assessed by quantitative real-time PCR for Ptc1 and Gli1 transcripts. These data confirm that earlier excision in the forelimb (Fig. 2E,F) results in earlier and higher upregulation of Ptc1 and Gli1 in the forelimb compared with in the hindlimb (Fig. 2G,H). This provides a likely cause for the phenotypic differences observed between these limbs. A similar trend in expression of a second pathway inhibitor, patched 2 (Ptch2; also known as Ptc2), was also observed (Fig. 2I), although upregulation in both Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> forelimbs and hindlimbs did not reach significance until 11.5 dpc. Because we observed no obvious attenuation of Hh signalling at the stages analysed, it is unlikely that upregulated Ptc2 is acting redundantly to significantly dampen pathway activity in this context. Taken together, these data confirm that excision of Ptc1 and subsequent upregulation of the Hh pathway correlate closely with sites of Prx1-Cre expression, and demonstrate a delay in these events in the hindlimb compared with in the forelimb.

The Gli3 protein gradient is disrupted in Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> limbs

Based on the negative regulation of both Gli3 transcript and Gli3R production by Shh signalling (Marigo et al., 1996b; Wang et al., 2000), constitutive Hh pathway activation should result in decreased levels of Gli3R. Anterior and posterior halves of 11.5 dpc limbs were assayed for Gli3R (190 kDa) and Gli3R (83 kDa) by western blot. In control limbs, the anterior/posterior gradient of Gli3R (Fig. 2J) was consistent with published results (Chen et al., 2004; Litingtung et al., 2002; Wang et al., 2000). By contrast, this Gli3R gradient was lost in both the Prx1-Cre:Ptc1<sup>1<sup>Cre</sup></sup> forelimb and hindlimb, with Gli3R levels being markedly reduced, particularly in the anterior (Fig. 2J). However, no clear increase in Gli3FL levels was observed in either limb.

Ptc1 inactivation in the hindlimb produces a molecular profile similar to that of Gli3<sup>M3X</sup>

The effect of Ptc1 inactivation on the expression of key limb patterning genes was investigated by whole-mount in situ hybridisation. In the hindlimb, changes in gene expression were most obvious at 11.5 dpc (Fig. 3A-L’). However, expression of Fgf4,
Grem1, Hand2, Hoxd13, Bmp2, Bmp4 and Fgf8 was altered as early as 10.5 dpc (see Fig. S2A-G’ in the supplementary material). Additionally, at this stage ectopic Ptc1 expression was weakly detectable in the anterior limb (Fig. S1B in the supplementary material), although the upregulation of Ptc1 and Gli1 detected by quantitative real-time PCR was not statistically significant at this point (Fig. 2G,H). From 11.5 dpc, Ptx1-Cre:Ptc1c/wt embryos displayed a small anterior domain of ectopic Hand2, Hoxd11-Hoxd13; see also Fig. S2L-M’ in the supplementary material). Expression of the anteriorly restricted gene Pax9 was abrogated in the mutant hindlimb, reflecting loss of Gli3R (Fig. 3F,F’). In addition to gene expression in the mesenchyme, the anterior AER of the hindlimb also displayed expansion of Fgf8, Bmp2, Bmp4; Fig. 3H–I). Consistent with this, the underlying mesenchyme ectopically expressed the downstream FGF target Mkp3 (also known as Dusp6) (Kawakami et al., 2003), suggesting enhanced FGF signalling from the anterior AER (Fig. 3L,L’). In all cases, the anterior AER staining exhibited a sharp boundary, distinct from the anterior tapering normally revealed by Fgf8 expression (Fig. 3K,K’; arrowhead). Discrete anterior expression of the above genes in the AER, together with Shh in the underlying mesenchyme, persisted in Ptx1-Cre:Ptc1c/wt hindlimbs until 12.5 dpc (or 13.5 dpc for Fgf8; Fig. 3M-Q’). Overall, the expression patterns of these genes correlate with their behaviour in the Gli3R limb (Bastida et al., 2004; Hill et al., 2007; Litingtung et al., 2002; McGlinn et al., 2005; te Welscher et al., 2002b), indicating that the two models are largely indistinguishable from one another during AP patterning stages.

### Early Ptc1 inactivation in the forelimb results in symmetrical expression of AP patterning genes

The molecular profile of the Ptx1-Cre:Ptc1c/wt forelimb was markedly different from that of the hindlimb. Shh expression was reduced and often lost from the ZPA (arrowheads, Fig. 4B and inset), and ectopic expression was redistributed along the distal anterior margin in 12 out of the 18 forelimbs analysed at 10.5 dpc (Fig. 4B), and in seven out of seven forelimbs analysed at 11.5 dpc (Fig. 4D). In the remaining six (out of 18) Ptx1-Cre:Ptc1c/wt forelimbs analysed at 10.5 dpc, Shh expression was completely undetectable (Fig. 4B, inset).
Although *Grem1* expression was detected in the anterior of most *Prx1-Cre*:Ptc1<sup>1/c</sup> forelimbs at 10.5-11.5 dpc, the size of the domain was reduced and showed a striking discontinuity (Fig. 4E-H; 4/8 at 10.5 dpc, 6/6 at 11.5 dpc). *Grem1* expression was undetectable in two out of eight mutant forelimbs, and its expression domain was equivalent in size to that seen in wild type in a further two limbs at 10.5 dpc (data not shown). In general, the proportion of 10.5 dpc forelimbs in which *Grem1* expression was reduced correlates loosely with the prevalent oligodactylous phenotype (75%). In accordance with the overall reduction in *Grem1* expression, *Fgf4* expression was not detected in the AER in 10 out of 14 forelimbs at 10.5 dpc (Fig. 4N), whereas in the remaining four limbs, *Fgf4* expression was shifted anteriorly to the central AER (Fig. 4N, inset). No *Fgf4* expression was observed from 11.5 dpc onwards in wild-type or *Prx1-Cre*:Ptc1<sup>c/c</sup> forelimbs (see Fig. S3J,J in the supplementary material).

Coupled with the loss of *Fgf4* from the AER in the majority of *Prx1-Cre*:Ptc1<sup>1/c</sup> forelimbs was a mild decrease in the length of the AER, as shown by *Fgf8* expression (Fig. 4I-L). A reduced domain of *Mkp3* expression was detected in the *Prx1-Cre*:Ptc1<sup>1/c</sup> forelimb at 10.5 dpc (Fig. 4O-P). Together with a similar reduction in other FGF targets, including *Spry1* and *Spry4* (Minowada et al., 1999), at 10.5 dpc (Fig. S3E-F in the supplementary material), this suggests a decreased response to FGF signalling from the AER. Both *Mkp3* and *Grem1* expression were also downregulated at 9.75 dpc (Fig. 4Q-T), and overall these data support an early disruption of the Shh/Grem1/FGF loop in the mutant forelimb.

A striking phenomenon evident in the expression of virtually all of the genes examined in the *Prx1-Cre*:Ptc1<sup>1/c</sup> forelimb from 10.5 dpc was symmetry about the AP midline. Loss of *Pax9*, an anterior marker in the 11.5 dpc limb (McGlinn et al., 2005), suggests a loss of anterior identity (Fig. 5A,I). *Gli3* expression was reduced, although still visible in the anterior limb at 10.5 dpc (Fig. 5B,J), with remaining *Gli3* expression appearing symmetrical by 11.5 dpc (see Fig. S3K-K in the supplementary material). In addition, those genes normally restricted to the posterior limb were expressed in the anterior *Prx1-Cre*:Ptc1<sup>1/c</sup> forelimb (Fig. 5C,K). Expression of genes such as *Fgf4* (4/14; Fig. 4N, inset), *Hoxd11*-*Hoxd13* (4/14; Fig. 5C,K; see also Fig. S3M-O in the supplementary material) and *Hand2* (Fig. 5D,L), showed continuous expansion into the anterior. However, this was distinct from the expansion in the hindlimb as the expression domain was now symmetrical and, in the case of the *Hoxd* genes *Gbx2* and *Mxl4* (arrowheads, H'-K'), *Mlp1* was expanded in the anterior mesenchyme of the *Prx1-Cre*:Ptc1<sup>1/c</sup> hindlimb at this stage. (M-Q) The anterior AER region displays ectopic expression of *Shh* (underlying mesenchyme; M,M', arrowhead), *Bmp2, Bmp4, Fgf4* and *Fgf8* at 12.5 dpc, and *Fgf8* at 13.5 dpc (N-Q', arrowheads). Scale bars: 200 μM.
Fig. 4. ZPA/AER interactions are disrupted in Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs. Whole-mount in situ hybridisation of wild-type and Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs for the indicated genes. (A–D) At 10.5 dpc, Shh expression in Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs is redistributed from the ZPA (marked by arrowheads, A–D) to the anterior margin, with reduced (B) or completely undetectable (B, inset) ZPA expression; compare with wild type in A. At 11.5 dpc, Shh expression is reduced in the ZPA of Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs but is present in the anterior margin (D). (E–H) Gremlin1 expression is anteriorly expanded in Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs but is reduced and discontinuous at 10.5 dpc (F) and 11.5 dpc (H). (I–N) The Fgf8 expression domain is slightly shortened at 10.5 dpc (I) and 11.5 dpc (L) in Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs, and Fgf4 expression is lost in most 10.5 dpc Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs (N), but is shifted centrally in a subset (approx. 28%) of limbs (N, inset). (O–T) Mkp3 expression is reduced at 10.5 dpc (O–P), and Mkp3 and Gremlin1 are reduced at 9.75 dpc (27 somites; Q–T) in Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs. Scale bars: 200 μM.

Importantly, no such distinct anterior mesenchymal duplication of Bmp2, Bmp4 or Zfp503 expression was observed in the Prx1-Cre:Ptc1<sup>Cre</sup> hindlimb (Fig. 3H–I’) (see also McGlenn et al., 2008), which clearly demonstrates the dichotomy between the molecular profiles of the forelimb and hindlimb. Taken together, these data suggest that the anterior Prx1-Cre:Ptc1<sup>Cre</sup> forelimb has undergone posterior re-specification.

To investigate pre-patterning and the generation of asymmetry in the forelimb, expression of Hand2 was examined at 9.25 dpc (23 somites), prior to the establishment of the ZPA at around 9.5 dpc, and was found to be expanded into the anterior of the Prx1-Cre:Ptc1<sup>Cre</sup> forelimb (Fig. 5Q,R). Mice ectopically expressing Hoxd11-Hoxd13 across the forelimb bud displayed symmetrical limbs similar to the Prx1-Cre:Ptc1<sup>Cre</sup> forelimb phenotype (Zakany et al., 2004). We therefore investigated 5’Hoxd gene expression in the early Prx1-Cre:Ptc1<sup>Cre</sup> forelimb, prior to the onset of Shh expression. Hoxd11 was ectopically expressed across the limb bud at 9.25 dpc (23 somites; Fig. 5S,T). Upregulation of Hoxd13 in Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs was also observed at the 27 somite stage (data not shown). Thus, the precocious 5’Hoxd activity following the loss of Ptc1 in the Prx1-Cre:Ptc1<sup>Cre</sup> forelimb provides a likely mechanism for the symmetrical redistribution of Shh, Hand2 and other patterning genes in these limbs.

We next sought to determine whether the changes in gene expression we observed in Prx1-Cre:Ptc1<sup>Cre</sup> limbs were associated with a disruption in the apoptosis/proliferation balance. TUNEL analysis of 10.5 dpc limbs revealed no increase in apoptosis in Prx1-Cre:Ptc1<sup>Cre</sup> limbs compared with that observed in wild type (Fig. 6A–D). This is consistent with a reduction in the Msx1 and Msx2 anterior expression domain at 10.5 and 11.5 dpc (see Fig. S3B–C, ‘H–I’ in the supplementary material), and suggests that neither Cre-mediated toxicity nor early apoptosis plays a major role in the forelimb phenotype. Some evidence of enhanced apoptosis in the central mesenchyme was detected at 12.5 dpc (data not shown), but as this later apoptosis occurs in both Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs and hindlimbs, it is unlikely to specifically affect early forelimb patterning events. At 11.5 dpc, a time when the size difference between mutant and wild-type limbs generally becomes obvious, proliferation levels were significantly increased in Prx1-Cre:Ptc1<sup>Cre</sup> hindlimbs (P<0.001; Fig. 6E–H,I). Although a downward trend in proliferation was detected in the 11.5 dpc forelimb, no statistically significant change was observed (Fig. 6I). Hence neither altered apoptosis nor proliferation is likely to be the major determinant of the oligodactyly observed in Prx1-Cre:Ptc1<sup>Cre</sup> forelimbs.

**DISCUSSION**

We have demonstrated for the first time the effects of unchecked ligand-independent upregulation of the Hh pathway specifically in the early limb mesenchyme by conditional ablation of functional Ptc1. Strikingly, we observe variable decrease or increase in digit number in the forelimb and hindlimb, respectively, together with posterior re-specification of the anterior forelimb. To our knowledge, this forelimb phenotype is unique amongst Hh gain-of-function mouse models, and we propose that this is primarily due to the timing and level of Hh pathway activation. Early inactivation of Ptc1 in the Prx1-Cre:Ptc1<sup>Cre</sup> forelimb reveals a key role for Hh......
pathway repression in early pre-patterning events prior to Shh expression in the ZPA, and results in perturbation of gene expression networks responsible for subsequent patterning (Fig. 7).

**Ptc1 excision activates the Hh pathway**

*Prx1-Cre* is expressed in limb mesenchyme as well as in restricted domains in other regions of the mouse embryo. Hh pathway activation in *Prx1-Cre: Ptc1* embryos, as determined by *Ptc1* and *Gli1* expression, was observed in domains that closely mirror sites of *Prx1-Cre* expression. This unexpected pathway activation outside the normal domains of detectable *Ptc1* expression is consistent with a model in which Hh signalling in these areas is normally inhibited by low-level *Ptc1* transcription, below the limits of in situ hybridisation detection. Upon removal of this functional *Ptc1* transcript, the pathway is activated inappropriately in a more widespread domain than anticipated. Our results, together with those from studies showing derepression of *Ptc1-lacZ* expression in early *Ptc1* null embryos (Goodrich et al., 1997; Milenkovic et al., 1999), suggest that much of the embryonic mesenchyme is competent to respond to Hh signalling. This indicates that, in addition to maintaining the fine balance of signalling mediated by Hh ligands, negative regulation by *Ptc1* in the absence of ligand is essential to maintain pathway repression in otherwise signalling-competent tissue. The finding that an expanded *Shh* domain is likely to underpin the drastic phenotype difference between the bat forelimb and hindlimb, suggests a precedent for evolutionary variation in the spatial restriction of *Shh* signalling as a means of controlling limb patterning to generate diversity (Hockman et al., 2008).

We determined the precise timing of *Ptc1* excision in *Prx1-Cre: Ptc1* limbs and found that, consistent with *Prx1-Cre* expression (Logan et al., 2002), there is a delay in excision in the hindlimb compared with in the forelimb, and this translates to a corresponding delay in pathway activation. The mutant hindlimb is therefore exposed to wild-type *Ptc1* transcript for a considerable time after the establishment of *Shh* expression. By contrast, *Ptc1* excision is virtually complete in most forelimbs prior to normal *Shh* expression, and this appears to lead to defects in the establishment of the ZPA. Our data and published results show that expression of *Prx1-Cre* throughout the forelimb mesenchyme at 9.5 dpc varies between individuals (Logan et al., 2002). Although this variability could explain the occurrence of polydactyly in a minority of forelimbs, we are not able to confirm this with our model.

**Early expression cascades are perturbed in the symmetrical *Prx1-Cre: Ptc1* forelimb**

In contrast to the asymmetry in *Prx1-Cre: Ptc1* limbs, mutant forelimbs appear largely morphologically and molecularly symmetrical about the midline (Fig. 7B). Although embryonic lethality prevents unequivocal determination of digit identity in the *Prx1-Cre: Ptc1* forelimbs, anterior expansion of *Hoxd11* and *Hoxd12* implies that digit 1 identity is lost, as *Hoxd11* and *Hoxd12* expression in the wild-type limb is excluded from the digit 1 primordium (Kmita et al., 2005; Nelson et al., 1996). Importantly, this distinguishes the oligodactyly of the *Prx1-Cre: Ptc1* forelimb from that produced by *Shh* inactivation, in which digit 1 identity is preserved in the absence of all other digits (Chiang et al., 2001). The lack of extensive ectopic anterior cell death at 10.5 dpc, as seen in...
the posteriorised limbs of Msx1/2 mouse mutants (Lallemand et al., 2005), indicates that the symmetrical Prx1-Cre:Ptc1Cre+ limbs phenotype is unlikely to reflect a loss of anterior tissue. This, and the expression of normally posteriorly restricted genes in the anterior limb, suggest that the symmetrical forelimb results from re-specification of anterior tissue to a posterior identity.

Limb asymmetry and establishment of the ZPA is determined by interplay between 5’ Hoxd genes, Gli3 and Hand2 in the early limb bud (Tarchini et al., 2006; te Welscher et al., 2002a; Zakany et al., 2004). Our data show that early inactivation of Ptc1 in the forelimb disturbs these early patterning events, as judged by the anterior expansion of Hoxd11 and Hand2 expression at 9.25 dpc (23 somites), and of Hoxd13 slightly later (27 somites). By contrast, there is no change in expression of these genes between wild-type and Prx1-Cre:Ptc1Cre+ hindlimbs at the equivalent stage. In a previous study, inactivation of Shh using Prx1-Cre produced a hypomorphic phenotype (Lewis et al., 2001), suggesting that this driver does not inactivate gene expression in all cells prior to Shh expression. Indeed, it is likely that a small fraction of cells escape Ptc1 excision prior to the normal onset of Shh pathway activation in the Prx1-Cre:Ptc1Cre+ forelimb. However, our data indicate that a high enough level of Ptc1 inactivation is achieved to drive downstream signalling to a sufficient threshold to perturb pre-patterning.

Following early expansion of Hoxd11 and Hand2 expression across the entire Prx1-Cre:Ptc1Cre+ forelimb paddle, Shh expressing cells are visible around the distal limb periphery. Similar changes in Shh expression are induced by ectopic anterior expression of Hand2 (Mcfadden et al., 2002), and 5’ Hoxd genes (Zakany et al., 2004). In these cases, limbs show a loss of asymmetry and a conversion of anterior digits to posterior identities (McFadden et al., 2002; Zakany et al., 2004). The ectopic Hoxd11-Hoxd13 expression in the latter model also produces variable oligodactyly (Zakany et al., 2004), which further implicates early ectopic 5’ Hoxd gene expression in the generation of the Prx1-Cre:Ptc1Cre+ forelimb phenotype. Although the limb symmetry in these other mouse models is likely to be caused by the distally expanded Shh, this is unlikely to be a major contributing factor in the Prx1-Cre:Ptc1Cre+ forelimb phenotype, because this limb is characterised by widespread high-level ectopic Hh pathway activation. It is more likely that signalling events downstream of the constitutively active Hh pathway underpin the forelimb symmetry, although a contribution of properties intrinsic to the redistributed Shh-expressing cells and their descendants cannot be ruled out.

**The Shh/Grem1/FGF signalling loop is disrupted in Prx1-Cre:Ptc1Cre+ forelimbs**

To date, activation of Hh signalling in both mouse models and human dysmorphologies has generally been associated with extra digits (Hill et al., 2003). Although we observe this phenotype in the Prx1-Cre:Ptc1Cre+ forelimbs, the forelimbs predominantly display an oligodactyly-like phenotype with an average of 3–4 digits. Analysis of Gli3 processing at 11.5 dpc revealed that the Gli3FL:Gli3R gradient is abolished in both forelimbs and hindlimbs in a similar manner. In both cases there is a decrease in Gli3R levels in the anterior limb, consistent with inhibition of Gli3 cleavage by Shh signalling (Wang et al., 2000), although a clear increase in Gli3FL was not detectable by western blotting. These data suggest that the forelimb-specific phenotype is not due to differences in Gli3 cleavage.

Early reduced expression of Mkp3 and other targets of AER-FGF signalling suggests that the mesenchymal response to FGF signalling is attenuated specifically in the Prx1-Cre:Ptc1Cre+ forelimb. This reduced response corresponds to a lack of Fgf4 expression in most limbs, and to a relatively subtle shortening of the Fgf8 expression domain. However, mice lacking Fgf4 do not display limb defects (Moon et al., 2000; Sun et al., 2000), and recent studies demonstrate conclusively that of all the AER FGFs, Fgf8 is sufficient for limb patterning (Mariani et al., 2008). Thus, the reduced expression of Fgf4 expression is unlikely to be the major cause of the Prx1-Cre:Ptc1Cre+ forelimb phenotype, but it is nevertheless indicative of perturbation of the Shh/Grem1/FGF loop (Verheyden and Sun, 2008). Accordingly, we detected evidence of reduced Grem1 expression in the Prx1-Cre:Ptc1Cre+ forelimb very early after limb budding. Although Shh normally maintains expression of Grem1 in the limb, Shh-expressing
cells and their descendants cannot express Grem1, a fact attributed to very high levels of autocrine Shh signalling (Nissim et al., 2006; Scherz et al., 2004). Early high-level autocrine activation of the Hh pathway across the Prx1-Cre;Ptc1c/c forelimb might therefore mediate an early reduced capacity for cells to express Grem1, thus disturbing the Shh/Grem1/FGF loop. We favour this over a more direct effect on an early reduced expression domain of Mkp3 at 9.75 dpc which is consistent with the high levels of FGF signalling required to repress Grem1. Given that a primary feature of the Prx1-Cre;Ptc1c/c forelimb is the widespread upregulation of 5’ Hoxd genes, it may be that high-level constitutive Hh pathway activity is acting through this early ectopic Hox gene expression to downregulate Grem1. This leads to the intriguing but speculative hypothesis that high levels of 5’ Hoxd genes contribute to the refractoriness to Grem1 expression in Shh lineage cells of the wild-type limb.

Like the Prx1-Cre;Ptc1c/c forelimb, Grem1 null limbs are characterised by fewer digits, but they also display reduced Shh signalling and increased apoptosis (Khokha et al., 2003; Michos et al., 2004). By contrast, we see no evidence of enhanced early forelimb-specific apoptosis in the Prx1-Cre;Ptc1c/c model. Furthermore, we demonstrate a reduction in the anterior expression domain of Msx1 and Msx2, which are thought to mediate apoptosis downstream of both Shh/Gli3R and BMP signalling in the limb (Lallemand et al., 2009; Pizette et al., 2001). Although reduced Msx gene expression is not consistent with the observed expanded anterior expression of Bmp2 and Bmp4, it supports a recent suggestion that anterior Msx gene expression might be mediated predominantly by Gli3R (Lallemand et al., 2009). This is further supported by the selective maintenance of Msx gene expression in the most anterior region of the limbs of Bmpr1a conditional-knockout mice (Ovcchinikov et al., 2006). Conversely, despite the duplication of both Bmp2 and Bmp4 expression domains, a decrease in overall BMP signalling in the Prx1-Cre;Ptc1c/c forelimb cannot be ruled out. However, this is less likely in those limbs lacking Fgf4, as it has been shown that inhibition of BMP signalling allows FGF expression in the AER (Capdevila et al., 1999; Pizette and Niswander, 1999; Zuniga et al., 1999).

While much effort has focused on the mechanisms underlying the positive potentiation of the posteriorly biased expression of Shh, our data highlight a previously unappreciated requirement for Ptc1-mediated negative control over the precise timing and levels of Shh signalling in the developing limb. Importantly, the Prx1-Cre;Ptc1c/c forelimb phenotype suggests that failure to prevent activation of this pathway throughout the early pre-patterning stages of limb budding results in perturbations to key gene expression cascades responsible for defining limb asymmetry and digit number.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/20/3515/DC1

References


WT

10.5dpc A

11.5dpc D

Prx1-Cre:Ptc1C/c

B

Prx1-Cre:PtclC/c:
Z/AP+/−

C

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Hindlimb

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