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

Natalie C. Butterfield1, Vicki Metzis1, Edwina McGlinn2, Stephen J. Bruce1, Brandon J. Wainwright1 and Carol Wicking1,*

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 Ptch1) constitutively inhibits the transducer of the Hh signal, smoothened (Smoo) (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 Smoo 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., 2001). 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>loxP<sup>/>loxP</sup>) (Ellis et al., 2003), on a C57Bl/6SV129 background. Male offspring heterozygous for both alleles were then backcrossed to Ptc1<sup>loxP</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>loxP</sup> or homozygous (Prx1-Cre:Ptc1<sup>cre</sup>). For analysis of Prx1-Cre activity, females homozygous for the Z/AP reporter transgene were mated to Prx1-Cre:Ptc1<sup>cre</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'-GATATCTCAGTACTGAGG-3'; R, 5'-CTTGAATTCATACGTT-3'.

RNA in situ hybridisation

Whole-mount in situ hybridisation was performed as previously described (Fowles et al., 2003). Probes were: Shh [nucleotides 36-678 of the vertebrate hedgehog sequence NM_017221 described by Roelink et al. (Roelink et al., 2008), Hoxd12, Hoxd13, Shh, Jag1, Grem1, Gli1, Spry1, Spry4, Gli2, Bmp4, Delta1, Delta3, Ctnnb1, Hes1, Notch1, Fgf4, Fgf8, Gli1-F (Bruce et al., 2007); Ptc1-F, 5'-GCTACCATGGCTGGTCAGG-3'; Ptc1-R, 5'-GCAGGAGGGCC-GGAGAAAG-3'; Gli1-F, 5'-TCAAGGCCCAATACATGCTG-3'; Gli1-R, 5'-AGAACCTCAGCACAGCTTTCA-3'; Ptc2-F, 5'-TGGTATACCTC-TGGTCGCTT-3'; and Ptc2-R, 5'-GTCTACATGCTGTCGAGG-3'. Mean of relative expression ± s.d. was determined from biological replicates (n=2-4 samples of two limbs each). Statistical significance was determined using a Student's t-test (⁎P<0.05, ⁎⁎P<0.01). All ampiclons were gel-purified and sequenced to ensure correct product amplification.

BrdU and TUNEL analysis

Proliferating cells were detected by injecting pregnant mice with 10 μl/g bromodeoxyuridine labelling reagent (BrdU; Zymed). Two hours after injection, embryos were dissected, fixed, processed for paraffin wax embedding and sectioned at 8 μm according to standard protocols. BrdU was detected using a biotinylated α-BrdU antibody (Zymed), Vectorstain Elite ABC and DAB peroxidase substrate kits (Vector Laboratories), according to the manufacturer’s instructions, prior to counterstaining with Nuclear Fast Red (Vector Laboratories). The relative areas occupied by proliferating and non-proliferating cells were quantified using ImageJ software. Mean of relative percentage ± s.e.m. was determined from biological duplicates (>15 technical replicates of <5% variation). Statistical significance was determined using a Student’s t-test (⁎⁎⁎P<0.001). Apoptotic cells in 8 μm limb sections were detected using a Fluorescein In Situ Cell Death Detection Kit (Roche).

Detection of Gli3 protein by western blot

Pooled anterior and posterior 11.5 dpc autopod halves were lysed in RIPA buffer plus complete protease inhibitor cocktail (Roche). Samples in 1×Laemmli sample buffer with 10% β-mercaptoethanol were boiled for 5 minutes prior to electrophoresis through an 8% Tris-glycine gel and transfer onto a PVDF membrane (Millipore) overnight at 30 V. Gli3 protein was detected using a rabbit α-Gli3 antibody (Wang et al., 2000), and α-tubulin by a mouse DIA antibody (Sigma), with HRP-conjugated secondary antibodies (Zymed) and ECL detection reagents (Amersham).

RESULTS

Inactivation of Ptc1 upregulates Hh signalling and produces variable gain/loss of digits

The Prx1-Cre transgene drives Cre recombinase activity in the early limb mesenchyme (Logan et al., 2002). Prx1-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 Prx1-Cre:Ptc1<sup>loxP</sup> mice survive to adulthood and are fertile and viable. Homozygous Prx1-Cre:Ptc1<sup>cre</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 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;Ptc1flox/flox 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;Ptc1flox/flox hindlimb at 10.5 dpc, but not at 11.5 dpc, indicating that exon 3 deletion approaches completion in the hindlimb between 10.5 dpc 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 (Ptc2; also known as Ptc2h), was also observed (Fig. 2I), although upregulation in both Prx1-Cre;Ptc1flox/flox 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;Ptc1flox/flox 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 Gli3FL (190 kDa) and Gli3R (83 kDa) by western blot. In control limbs, the anteroposterior 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;Ptc1flox/flox 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 Gli3flox/flox**

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, Prx1-Cre:Ptcf1 crystalloid hindlimb buds displayed a small anterior domain of ectopic Shh expression, the hallmark of a group of polydactylous mouse mutants that includes Gli3Xt/Xt (Hill, 2007) (arrowhead, Fig. 3A). From 11.5 dpc (21-29 somites), only one hindlimb pair was obtained from a 9.5 dpc embryo. For other stages, 2 (10.5 dpc), 3 (11.5 dpc) or 4 (9.5 dpc forelimb) samples were analysed. (G-I) Quantification of the delayed upregulation of Ptc1, Gli1 and Ptc2 in hindlimbs versus forelimbs (*P<0.05, **P<0.01). The single 9.5 dpc hindlimb sample was excluded from this analysis, and results from individual limb pairs were pooled for statistical analysis. (J) Western blot showing that Gli3R (83 kDa) is present in an AP gradient across the wild-type hindlimb, but is reduced in anterior Prx1-Cre:Ptcf1 crystalloid limbs and hindlimbs. No significant increase in levels of Gli3FL (190 kDa) is evident. α-tubulin (55 kDa) serves as a loading control.

Fig. 2. Hh pathway upregulation at sites of Prx1-Cre activity. (A-C) Whole-mount wild-type and Prx1-Cre:Ptcf1 crystalloid embryos at 12.5 dpc, showing that Ptc1 and Gli1 upregulation in Prx1-Cre:Ptcf1 crystalloid embryos correlates closely with activity of Prx1-Cre (D), shown by alkaline phosphatase expression in a 12.0 dpc Prx1-Cre:Ptcf1 crystalloid/Z/IP+/-embryo (compare B and C with D). Scale bars: 500 μM. (E,F) RT-PCR of Ptc1 exon 3 from wild-type (WT) and mutant (M) limbs detects only exon 3-deleted Ptc1 at 9.5-11.5 dpc in the forelimb (E); the wild-type allele is still detectable in the hindlimb at 9.5-10.5 dpc, but not 11.5 dpc (F). Results are from representative samples of 9.5-11.5 dpc limbs, each consisting of two limbs. Due to variability in budding of the hindlimb at 9.5 dpc (21-29 somites), only one hindlimb pair was obtained from a 9.5 dpc embryo. For other stages, 2 (10.5 dpc), 3 (11.5 dpc) or 4 (9.5 dpc forelimb) samples were analysed. (G-I) Quantification of the delayed upregulation of Ptc1, Gli1 and Ptc2 in hindlimbs versus forelimbs (*P<0.05, **P<0.01). The single 9.5 dpc hindlimb sample was excluded from this analysis, and results from individual limb pairs were pooled for statistical analysis. (J) Western blot showing that Gli3R (83 kDa) is present in an AP gradient across the wild-type hindlimb, but is reduced in anterior Prx1-Cre:Ptcf1 crystalloid limbs and hindlimbs. No significant increase in levels of Gli3FL (190 kDa) is evident. α-tubulin (55 kDa) serves as a loading control.

**Early Ptc1 inactivation in the forelimb results in asymmetrical expression of AP patterning genes**

The molecular profile of the Prx1-Cre:Ptcf1 crystalloid 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) Prx1-Cre:Ptcf1 crystalloid 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>l/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 oligodactyly 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>l/c</sup>* forelimbs (see Fig. S3J’ in the supplementary material).

Coupled with the loss of *Fgf4* from the AER in the majority of *Prx1-Cre;Ptc1<sup>l/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>l/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>l/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 of both *Prx1-Cre;Ptc1<sup>l/c</sup>* hindlimb (Fig. 5C-G,K-O). Expression of genes such as *Hoxd* genes showed continuous expansion into the anterior. However, this was duplicated in the anterior *Prx1-Cre;Ptc1<sup>l/c</sup>* hindlimb (Fig. 5C-G, inset), with remaining *Hoxd* expression appearing symmetrical by 11.5 dpc (see Fig. S3K-K’ in the supplementary material). In addition, those genes normally restricted to the posterior mutant forelimb 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 oligodactyly 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>l/c</sup>* forelimbs (see Fig. S3J’ in the supplementary material).

Coupled with the loss of *Fgf4* from the AER in the majority of *Prx1-Cre;Ptc1<sup>l/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>l/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.

Although *Grem1* expression was detected in the anterior of most *Prx1-Cre;Ptc1<sup>l/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 oligodactyly 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>l/c</sup>* forelimbs (see Fig. S3J’ in the supplementary material).

Coupled with the loss of *Fgf4* from the AER in the majority of *Prx1-Cre;Ptc1<sup>l/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>l/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.

### Fig. 3. Gene expression in *Prx1-Cre;Ptc1<sup>l/c</sup>* hindlimbs.

Whole-mount in situ hybridisation of wild-type and *Prx1-Cre;Ptc1<sup>l/c</sup>* hindlimbs for the indicated genes. (A-E) Expression of Shh, Grem1, Hand2, Hoxd13, and Jag1 is expanded into the anterior 11.5 dpc *Prx1-Cre;Ptc1<sup>l/c</sup>* hindlimb. (F-G’) Expression of Pax9 is abrogated in the *Prx1-Cre;Ptc1<sup>l/c</sup>* hindlimb (F,F’). (I,J) This was further reinforced by the expression of the transcriptional repressor Zfp503, which we have previously shown to be downregulated in the anterior of both *Gli3<sup>V503X</sup>* and *Prx1-Cre;Ptc1<sup>l/c</sup>* hindlimbs (McGlinn et al., 2008; McGlinn et al., 2005). The distinct proximal domain of *Zfp503* expression visible at the autopod/zeugopod boundary of the posterior limb was duplicated in the anterior *Prx1-Cre;Ptc1<sup>l/c</sup>* forelimb, whereas the more distal anterior autopod expression was lost (Fig. 5H,P).
Important, no such distinct anterior mesenchymal duplication of Bmp2, Bmp4 or Zfp503 expression was observed in the Prx1-Cre;Ptc1<sup>1/c</sup> forelimbs (see also McGlinn 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>1/c</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 9.5 dpc, and was found to be expanded into the anterior of the Prx1-Cre;Ptc1<sup>1/c</sup> forelimbs (Fig. 5S,R). Mice ectopically expressing Hoxd11-Hoxd13 across the forelimb bud displayed symmetrical limbs similar to the Prx1-Cre;Ptc1<sup>1/c</sup> forelimb phenotype (Zakany et al., 2004). We therefore investigated 5′Hoxd activity following the loss of Ptc1 in the Prx1-Cre;Ptc1<sup>1/c</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>1/c</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>1/c</sup> 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-1′ 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;Ptc1<sup>1/c</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>1/c</sup> hindlimbs (P<0.001; Fig. 6E-H,1′). 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>1/c</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 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>1/c</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:Ptc1c/c 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:Ptc1c/c 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:Ptc1c/c forelimb**

In contrast to the asymmetry in Prx1-Cre:Ptc1c/c hindlimbs, 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:Ptc1c/c 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:Ptc1c/c 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:Ptcl<sup>fl/fl</sup> forelimb 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:Ptcl<sup>fl/fl</sup> 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 Hh pathway activation in all cells prior to Shh expression. 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:Ptcl<sup>fl/fl</sup> 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'Hox gene expression in the generation of the Prx1-Cre:Ptcl<sup>fl/fl</sup> 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:Ptcl<sup>fl/fl</sup> 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:Ptcl<sup>fl/fl</sup> 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:Ptcl<sup>fl/fl</sup> hindlimbs, 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:Ptcl<sup>fl/fl</sup> 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 reduction in Fgf4 expression is unlikely to be the major cause of the Prx1-Cre:Ptcl<sup>fl/fl</sup> 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:Ptcl<sup>fl/fl</sup> forelimb very early after limb budding. Although Shh normally maintains expression of Grem1 in the limb, Shh-expressing
cells and their descendants cannot express Greml1, a fact attributed to very high levels of autocrine signalling (Nissim et al., 2006; Scherz et al., 2004). Early high-level autocrine activation of the Hh pathway across the Prx1-Cre;Ptc1+/c forelimb might therefore mediate an early reduced capacity for cells to express Greml1, thus disturbing the Shh/Greml1/FGF loop. We favour this over a more direct effect on the later FGF/Greml1 inhibitory loop (Verheyden and Sun, 2008), as the early reduced expression domain of Mkp3 at 9.75 dpc is inconsistent with the high levels of FGF signalling required to repress Greml1. Given that a primary feature of the Prx1-Cre;Ptc1+/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 Greml1. This leads to the intriguing but speculative hypothesis that high levels of 5’Hoxd genes contribute to the refractoriness to Greml1 expression in Shh lineage cells of the wild-type limb.

Like the Prx1-Cre;Ptc1+/c forelimb, Greml1 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 signalling and increased apoptosis (Khokha et al., 2003; Michos et al., 2004). Early high-level autocrine activation of the Hh pathway across the Prx1-Cre;Ptc1+/c forelimb might therefore mediate an early reduced capacity for cells to express Greml1, thus disturbing the Shh/Greml1/FGF loop. We favour this over a more direct effect on the later FGF/Greml1 inhibitory loop (Verheyden and Sun, 2008), as the early reduced expression domain of Mkp3 at 9.75 dpc is inconsistent with the high levels of FGF signalling required to repress Greml1. Given that a primary feature of the Prx1-Cre;Ptc1+/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 Greml1. This leads to the intriguing but speculative hypothesis that high levels of 5’Hoxd genes contribute to the refractoriness to Greml1 expression in Shh lineage cells of the wild-type limb.

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;Ptc1+/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.

Acknowledgements
We thank C. Tabin, P. Koopman, M. Little and D. Ovchinnikov for kindly providing probes with permission from cited sources, M. Logan for the Prx1-Cre mouse line, and B. Wang for the Gli3 antibody. We also thank C. C. Hui, R. Villani and T. Ellis for helpful discussion, and A. Hardacre, J. Conway and T. Davidson for assistance with mouse husbandry. This work was supported by the National Health and Medical Research Council of Australia (NHMRC). N.C.B. was a recipient of an Australian Postgraduate Award, and C.W. is an NHMRC Senior Research Fellow.

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

References


WT

Prx1-Cre;Ptc1^{Cre}/^{Cre}

Prx1-Cre;Ptc1^{+/+};Z/AP^{+/-}

10.5dpc

11.5dpc

A B C

D E
<table>
<thead>
<tr>
<th></th>
<th>Fgf4</th>
<th>Grem1</th>
<th>Hand2</th>
<th>Hoxd13</th>
<th>Bmp2</th>
<th>Bmp4</th>
<th>Fgf8</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hoxd11</th>
<th>Hoxd12</th>
<th>Msx1</th>
<th>Msx2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>M</td>
<td>N</td>
<td>O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Gli3</th>
<th>Msx1</th>
<th>Msx2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>I</td>
<td>J</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L'</td>
<td>M'</td>
<td>N'</td>
<td>O'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Shh

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H'</td>
<td>I'</td>
<td>J'</td>
<td>K'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10.5dpc

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

11.5dpc

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>M</td>
<td>N</td>
<td>O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx1-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bmp2</td>
<td>Bmp4</td>
<td>Bmp7</td>
</tr>
<tr>
<td>-----</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td>12.5dpc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td>13.5dpc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Prx1- Cre;Ptc1c/c</td>
<td></td>
<td></td>
<td>12.5dpc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D'</td>
</tr>
</tbody>
</table>

Hindlimb

<table>
<thead>
<tr>
<th></th>
<th>Bmp4</th>
<th>Bmp7</th>
<th>Msx1</th>
<th>Msx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>I</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>I'</td>
<td>L'</td>
</tr>
<tr>
<td>Prx1- Cre;Ptc1c/c</td>
<td></td>
<td></td>
<td>K</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K'</td>
<td>M'</td>
</tr>
</tbody>
</table>

Hindlimb