SHH propagates distal limb bud development by enhancing CYP26B1-mediated retinoic acid clearance via AER-FGF signalling

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SUMMARY

The essential roles of SHH in anteroposterior (AP) and AER-FGF signalling in proximodistal (PD) limb bud development are well understood. In addition, these morphoregulatory signals are key components of the self-regulatory SHH/GREM1/AER-FGF feedback signalling system that regulates distal progression of limb bud development. This study uncovers an additional signalling module required for coordinated progression of limb bud axis development. Transcriptome analysis using Shh-deficient mouse limb buds revealed that the expression of proximal genes was distally extended from early stages onwards, which pointed to a more prominent involvement of SHH in PD limb axis development. In particular, retinoic acid (RA) target genes were upregulated proximally, while the expression of the RA-inactivating Cyp26b1 enzyme was downregulated distally, pointing to increased RA activity in Shh-deficient mouse limb buds. Further genetic and molecular analysis established that Cyp26b1 expression is regulated by AER-FGF signalling. During initiation of limb bud outgrowth, the activation of Cyp26b1 expression creates a distal ‘RA-free’ domain, as indicated by complementary downregulation of a transcriptional sensor of RA activity. Subsequently, Cyp26b1 expression increases as a consequence of SHH-dependent upregulation of AER-FGF signalling. To better understand the underlying signalling interactions, computational simulations of the spatiotemporal expression patterns and interactions were generated. These simulations predicted the existence of an antagonistic AER-FGF/CYP26B1/RA signalling module, which was verified experimentally. In summary, SHH promotes distal progression of limb development by enhancing CYP26B1-mediated RA clearance as part of a signalling network linking the SHH/GREM1/AER-FGF feedback loop to the newly identified AER-FGF/CYP26B1/RA module.

KEY WORDS: Fibroblast growth factor, Retinoic acid, Sonic hedgehog, Mathematical modelling, Mouse limb development, Systems biology

INTRODUCTION

During vertebrate limb bud development, the proximodistal (PD) and anteroposterior (AP) limb skeletal axes develop by proliferative expansion of mesenchymal progenitors in combination with progressive determination and differentiation (Tabin and Wolpert, 2007; Towers and Tickle, 2009; Zeller et al., 2009; Zhu et al., 2008). The apical ectodermal ridge (AER) secretes several fibroblast growth factors (FGFs; FGF4, FGF8, FGFR9 and FGFR17) that control distal outgrowth and patterning in a likely dose-dependent manner [see e.g. Mariani et al. (Mariani et al., 2008)]. Experimental manipulation of chicken limb bud development has provided evidence that retinoic acid (RA) can promote proximal fates (Tamura et al., 1997). In particular, implantation of RA-loaded beads into limb buds upregulates the expression of the Meis transcriptional regulators, which indicates that they mediate RA signalling in the proximal mesenchyme. Meis1 and Meis2 are first expressed throughout the nascent limb bud mesenchyme and their expression becomes restricted proximally during outgrowth. Ectopic expression of Meis1 in chicken and mouse limb buds causes distal to proximal transformations, whereas implantation of FGFR8-loaded beads into the proximal mesenchyme inhibits Meis expression (Capdevila et al., 1999; Mercader et al., 1999; Mercader et al., 2000; Mercader et al., 2009). These results led to the two-signal model for PD limb axis patterning, which states that RA from the limb bud flank and FGF signalling by the AER oppose one another to specify proximal and distal limb identities, respectively (Mercader et al., 2000). However, the potential functions of endogenous RA during limb bud morphogenesis have remained largely elusive. RA synthesis requires the RALDH enzymes, whereas its inactivation occurs by CYP26 enzymes, which belong to the cytochrome P450-type enzyme family [see e.g. Yashiro et al. (Yashiro et al., 2004)]. During limb bud development, Raldh2 is expressed by the flank mesenchyme and genetic analysis provided evidence that it is the RA synthetising enzyme relevant for forelimb bud development (Niederreither et al., 1999). By contrast, Cyp26b1 is expressed predominantly by the distal mesenchyme and non-AER ectoderm (Yashiro et al., 2004). In Cyp26b1-deficient mouse embryos, distal
limb development is disrupted probably owing to the teratogenic effects of elevated RA, which causes cell death and severely truncates the limb skeleton (Yashiro et al., 2004; Zhou and Kochhar, 2004).

Studying the potential requirement of RA for limb bud development has been difficult due to the early lethality of Raldh2-deficient mouse embryos and lack of conditional alleles. However, the early lethality can be circumvented by supplementing Raldh2-deficient embryos with maternal RA during gastrulation (Niederreither et al., 2002; Zhao et al., 2009). This largely restores forelimb bud outgrowth but AP patterning defects remained, which led the authors to conclude that RA is required for morphogenesis of both limb bud axes (Niederreither et al., 2002). Using a similar approach to restore development of Raldh2/3-deficient embryos, Zhao et al. (Zhao et al., 2009) showed that no RA activity is detected in the forelimb bud mesenchyme, although presumed RA targets such as Meis2, Hand2 and Shh were expressed. Therefore, this analysis suggested that RA is not required for patterning of the PD axis and hindlimb bud development (Zhao et al., 2009). However, RA appears to inhibit Fgf8 expression within the trunk mesenchyme, which creates a region competent to form forelimb buds. Prolonged RA-mediated inhibition of Fgf8 signalling within the trunk mesenchyme might be required for correct scaling of the forelimb bud size. In addition, genetic analysis in the mouse has implicated mesenchymal FGF10 and BMP4 signalling in formation of the Fgf8 expressing AER and initiation of limb bud outgrowth (Ohuchi et al., 1997; Benazet et al., 2009).

Sonic hedgehog (SHH) signalling by the limb bud organizer located in the posterior mesenchyme mainly controls establishment of the anteroposterior (AP) limb bud axis, which manifests predominantly in digit identities of the autopod (Chiang et al., 2001; Harfe et al., 2004; Kraus et al., 2001; Zhu et al., 2008). Genetic and experimental manipulation of mouse and chicken limb bud development has revealed the dual role of SHH in the early specification of AP identities and subsequent proliferative expansion (Towers et al., 2008; Zhu et al., 2008). In particular, the descendents of SHH-producing cells in mouse limb buds give rise to the two posterior-most digits and contribute to the middle digit (Harfe et al., 2004). This study provided good evidence that the time cells are exposed to SHH is crucial for specifying posterior identities (digits 3-5), whereas the progenitors of digit 2 are specified by long-range signalling. The upregulation of Shh expression over time depends on the BMP antagonist gremlin 1 (GREM1), which is key to establishment of the SHH/GREM1/FGF feedback loop that operates between the mesenchyme and AER (Michos et al., 2004; Zuniga et al., 1999). In fact, GREM1-mediated reduction of BMP4 activity defines a key node in the self-regulatory signalling system that interlinks the SHH, GREM1/BMP4 and AER-FGF signalling pathways in the distal mesenchyme (Benazet et al., 2009; Verheyden and Sun, 2008). Altogether, these studies have revealed that these feedback loops are part of a more complex, as yet only partially identified signalling system that coordinates limb bud axes development (Zeller et al., 2009).

We have investigated the transcriptome alterations between Shh-deficient and wild-type forelimb buds around E10.5 (35-36 somites), which corresponds to the period during which limb bud development largely depends on SHH signalling (Benazet et al., 2009; Zhu et al., 2008; Panman et al., 2006). Unexpectedly, this analysis revealed that forelimb buds of Shh-deficient mouse embryos were molecularly proximalised as the expression of several proximal genes was upregulated and their domains extended distally. Molecular analysis of limb buds of Shh-/-, AER-Fgf8-/- and Cyp26b1-/- deficient mouse embryos indicated that the decrease in Cyp26b1 expression and the likely increase in RA activity are the cause for the observed molecular proximalisation of Shh-deficient limb buds. Normally, SHH signalling enhances RA inactivation indirectly via AER-FGF-mediated upregulation of Cyp26b1 expression. These interactions form an integral part of the signalling system that controls distal progression of limb bud development. To gain insight into the potential complexity of the SHH/AER-FGF/RA pathway interactions, a computational model using differential equations based on the observed genetic and molecular interactions was developed. The consistency of the simulations with the in vivo observations could be improved by introducing terms that describe the potential of high RA activity to restrict Fgf8 expression within the AER. The existence of this antagonistic AER-FGF/CYP26B1/RA signalling module was established experimentally. This antagonistic signalling module is established prior to activation of Shh expression and becomes dependent on SHH signalling as the SHH/GREM1/FGF feedback loop is initiated. Therefore, both SHH-mediated upregulation of AER-FGF signalling and CYP26B1-mediated RA clearance are key to distal progression of limb and autopod development.

MATERIALS AND METHODS

Ethics statement

All studies involving mice were performed in strict accordance with Swiss law after being approved by the Joint Commission on Experiments involving Animals of the Cantons of Argovia and both Basel. All animal experiments were classified as grade zero, which implies minimal suffering of mice and the 3R principles were strictly implemented as required by Swiss law.

Mouse forelimb bud isolation and Affymetrix Gene Chip analysis

Shh-deficient mouse embryos were produced using the previously generated Shh loss-of-function mutation (St-Jacques et al., 1998). For the transcriptome analysis, the Affymetrix GeneChip MoGene-1_0-st-v1 was used, which covers over 28,000 well-annotated mouse transcripts. Three individual pairs of mouse forelimb buds at embryonic day E10.5 of the same genotype and sex were used to isolate total RNA by the RNeasy micro kit (Qiagen). Total RNAs (200 ng) were used to synthesize target cRNA using the WT expression kit (Ambion). cRNAs (10 µg) were used to generate ~8.0 µg (~0.1 µg) of cDNAs. The WT Terminal Labelling Kit (Affymetrix) was used to fragment the cDNAs. The hybridization cocktail (85 µl) containing fragmented and biotin-labelled cDNAs (25 ng/µl) was transferred into Affymetrix GeneChip MoGene-1_0-st-v1 cassettes, which were incubated at 45°C inside a hybridization oven by rotating them at 60 rpm for 17 hours. Then the GeneChip arrays were washed and developed using the Hybridization Wash and Stain Kit in a Fluidics Station 450. The GeneChip arrays were read using the GeneChip Scanner 3000 7G and DAT image files were generated using the GeneChip Command Console (Affymetrix). Quality control established that the only significant source of variation in expression levels were the genotypes, which provided the experimental basis for validating the significance of the observed differences (data not shown).

The Partek Genomic Suite 6.5 was employed to further validate the GeneChip results. Three completely independent samples for both genotypes were used to determine significant differences in expression levels. All Affymetrix CEL files were normalized using the RMA method and all data were log2 transformed. To identify differentially expressed genes, two-way ANOVA analysis based on the "method of moments" was used. Differentially expressed genes were further filtered using a P-value threshold of 0.03 or less. These validated data sets were interrogated further using Ingenuity Pathway analysis in combination with manual curation of particular classes of genes. Microarray data have been submitted to MIAMExpress (Accession Number E-MEXP-3142).
Real-time quantitative PCR
Pairs of forelimb buds were collected and cDNAs synthesized using Superscript III (Invitrogen). Transcript levels were quantified by real-time PCR using the BIO-RAD CFX96 Real Time System in combination with the iQ-SYBR Green Supermix (BioRad; primer sequences available upon request). Relative transcript levels were normalized using the levels of the hydroxymethylbilane synthase gene as an internal standard. Transcript levels in mutant forelimb buds were calculated relative to wild-type levels (average set to 100%). All results shown are based on analyzing 7–10 pairs of forelimb buds per genotype (±s.d.). The statistical significance of all results was assessed using the two-tailed, non-parametric Mann-Whitney test.

Culturing of mouse limb buds
Mouse forelimb buds were cultured as described (Panman et al., 2006). AG1-X2 beads were loaded with RA (1 mg/ml, dissolved in DMSO) or the retinoid antagonist BMS493 (2 mg/ml, dissolved in DMSO; InnoChemie). Heparin beads were loaded with recombinant FGF4 (1 mg/ml in PBS/0.1% BSA; from R&D Systems). FGF signal transduction was blocked by supplementing the culture medium with 10 μM SU5402 (dissolved in DMSO) (Zuniga et al., 2004).

Whole-mount RNA in situ hybridization
Digoxigenin-labelled antisense riboprobes were used for whole-mount RNA in situ hybridization (Panman et al., 2006). Minimally three, but often many more, independent embryos were analysed and yielded comparable results for all results shown.

The mathematical approaches underlying the spatio-temporal simulations of the signalling systems interactions (Figs 7, 8) are described in Appendix S1 (see supplementary material).

RESULTS
Transcriptome alterations in Shh-deficient mouse limb buds
Recent studies have revealed the dual function of SHH in specification and proliferative expansion of the autopod primordia (Zhu et al., 2008). To gain insight into how SHH regulates gene expression during limb bud development, we have profiled the transcriptome in Shh-deficient mouse forelimb buds (St-Jacques et al., 1998) using Affimetrix GeneChip Mouse Gene Arrays. Forelimb buds of Shh-deficient mouse embryos at E10.5 (35-36 somites) were chosen because they are of similar size to wild-type limb buds and display neither morphological changes nor massive cellular apoptosis. At this developmental stage, the progression of limb bud development and proliferative expansion of the autopod primordia are mainly controlled by the SHH/GREM1/FGF signalling system (Benazet et al., 2009; Panman et al., 2006). Statistical evaluation of the primary results revealed that ~800 transcripts were altered at least 1.2-fold in Shh-deficient forelimb buds (P<0.03) (see Tables S1, S2 in the supplementary material). The cut-off was set at 1.2, as such differences were reliably detected by RNA in situ hybridization and real-time PCR analysis (Figs 1, 2). This group contained a large number of known transcriptional targets of SHH signalling [such as Ptc1, Ptc2, Hhip, Gli1; the 5’Hoxd genes, Grem1, Hand2, Prdm1 and AER-Fgf4, Fgf8, Fgf9 (see Table 1); Gas1, Boc, Cdon and Disp1 (see Table 2) (Chiang et al., 2001; te Welscher et al., 2002; Vokes et al., 2008; Zuniga et al., 1999)]. In addition, ~400 genes were downregulated ≥1.2-fold in Shh-deficient limb buds, consistent with a positive role of SHH in gene expression. Systematic annotation in combination with RNA in situ hybridization analysis showed that the majority of these genes are normally expressed in the posterior and/or distal limb bud mesenchyme or the AER (Table 1, Fig. 1A-C). In Shh-deficient limb buds, their expression was lowered rather than the spatial distribution altered, as illustrated for selected genes such as Wnt5a (Fig. 1A) (Yamaguchi et al., 1999), Spry4 (Fig. 1B) (Minowada et al., 1999) and Sall1 (Fig. 1C) (Kawakami et al., 2009). This rather general reduction of transcription in the distal mesenchyme and AER (Table 1) preceded the massive apoptosis in Shh-deficient limb buds (data not shown). Furthermore, the expression of components of the cell-cycle and proliferation gene networks were altered in a manner consistent with the G1 cell-cycle arrest observed in Shh-deficient limb buds [Zhu et al. (Zhu et al., 2008) and data not shown].

Table 1. Classification and spatial distribution of genes downregulated in Shh-deficient limb buds

<table>
<thead>
<tr>
<th>A</th>
<th>Posterior</th>
<th>Posterior-distal</th>
<th>Distal</th>
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<th>Gene</th>
<th>FC</th>
<th>P-value</th>
<th>Distribution</th>
<th>Gene</th>
<th>FC</th>
<th>P-value</th>
<th>Distribution</th>
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<td>Hoxa13</td>
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<td>≤4.8×10⁻⁵</td>
<td>Distal</td>
<td>Rspo3</td>
<td>2.1</td>
<td>≤3.7×10⁻⁵</td>
<td>Post-distal</td>
</tr>
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<td>≤6.2×10⁻⁵</td>
<td>Distal</td>
<td>Evx1</td>
<td>1.3</td>
<td>≤5.9×10⁻³</td>
<td>Post-distal</td>
</tr>
<tr>
<td>Sall3</td>
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<td>≤1.2×10⁻²</td>
<td>Distal</td>
<td>Fmn1</td>
<td>1.2</td>
<td>≤7.7×10⁻²</td>
<td>Post-distal</td>
</tr>
<tr>
<td>Cy26b1</td>
<td>1.7</td>
<td>≤1.4×10⁻³</td>
<td>Distal</td>
<td>Ptc2</td>
<td>5.7</td>
<td>≤5.9×10⁻⁵</td>
<td>Posterior</td>
</tr>
<tr>
<td>Sall4</td>
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<td>≤1.4×10⁻²</td>
<td>Distal</td>
<td>Ptc1</td>
<td>4.4</td>
<td>≤6.8×10⁻⁶</td>
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<tr>
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<td>Distal</td>
<td>Gli1</td>
<td>3.8</td>
<td>≤1.2×10⁻⁵</td>
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<td>Distal</td>
<td>Hhip</td>
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<td>Distal</td>
<td>Hand2</td>
<td>2.6</td>
<td>≤1.6×10⁻⁴</td>
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<tr>
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<td>≤3.0×10⁻³</td>
<td>Distal</td>
<td>Cyp1b1</td>
<td>1.8</td>
<td>≤7.0×10⁻⁴</td>
<td>Posterior</td>
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<tr>
<td>Wnt5a</td>
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<td>≤2.8×10⁻³</td>
<td>Distal</td>
<td>Bmp2</td>
<td>1.3</td>
<td>≤1.8×10⁻²</td>
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<td>Post-distal</td>
<td>Fgf8</td>
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<tr>
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<td>Post-distal</td>
<td>Fgf9</td>
<td>1.5</td>
<td>≤1.0×10⁻²</td>
<td>AER</td>
</tr>
</tbody>
</table>

(A) The limb bud schemes define graphically what are considered ‘posterior’, ‘posterior-distal’ and ‘distal’ expression domains. (B) Known transcriptional targets of the SHH signalling pathway are underlined. FC, fold change; post, posterior; prox, proximal.
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Expression was distally extended (middle panel, Fig. 2A) and levels of genes were increased ~50% (right panel, Fig. 2A), which indicated that RA activity might be increased in Shh-deficient limb buds. Indeed, the expansion of Rarb expression was paralleled by Meis2, an established transcriptional target of RA signalling in the proximal limb bud mesenchyme (Fig. 2B) (Mercader et al., 1999; Mercader et al., 2000). In Shh-deficient limb buds, Meis2 expression extended distally and levels were increased by ~40% (Fig. 2B). In addition, we identified two novel potential RA target genes whose expression is normally restricted proximally in limb buds (Fig. 2C,D; see Fig. S1 in the supplementary material). Pbx1 is expressed in the proximal-anterior part of wild-type limb buds (Selleri et al., 2001). In Shh-deficient limb buds, its expression extended both distally and posteriorly (Fig. 2C). Pkdcc encodes a putative protein kinase (Imuta et al., 2009) and in wild-type limb buds its expression was restricted proximally, while it was distally extended in Shh-deficient limb buds (Fig. 2D; similar to Meis2). In addition, Pbx1 and Pkdcc transcript levels were significantly upregulated in Shh-deficient limb buds (Fig. 2C,D). To determine if these transcriptional alterations could be a consequence of increased RA activity, RA-loaded beads were not hallucinated.

Upregulation of genes expressed in the proximal and proximal-anterior mesenchyme in Shh-deficient limb buds

Initially rather unexpected, the expression of a roughly equal number of genes was upregulated in Shh-deficient limb buds at E10.5. Annotation revealed that at least 36 of these upregulated genes were expressed proximally, and about half were restricted to the proximal-anterior mesenchyme domains in wild-type limb buds (Table 2). The expression of anteriorly restricted genes extended posteriorly in Shh-deficient limb buds as shown for Gria2, Ibx3 and Hoxc6 (Fig. 1D-F) (Houweling et al., 2001; Jia et al., 1996; Nelson et al., 1996). Furthermore, ALCAM is expressed in two distinct mesenchymal domains in wild-type limb buds (Bowen et al., 1995), but this restricted localization was lost in Shh-deficient limb buds (Fig. 1G). The distally extended expression of Cxcr7 (Maksym et al., 2009) is representative for the spatial alterations of many proximal genes in Shh-deficient mouse limb buds (Fig. 1H). In wild-type limb buds, Mab2111 (Wong et al., 1999) is expressed in a medial mesenchymal stripe (left panel, Fig. 1I). This indicated that the Mab2111 expression domain could mark the presumptive zeugopodal domain like Hoxa11 (Zakany and Duboule, 2007). In Shh-deficient limb buds, Mab2111 expression was upregulated and distally extended (right panel, Fig. 1I). Taken together, this analysis reveals that the expression of proximal genes was distally extended in Shh-deficient limb buds, which shows that SHH signalling is required for the correct proximal restriction of their expression domains during limb bud outgrowth.

Proximalization of Shh-deficient limb buds is paralleled by increased RA activity

The molecular alterations described so far indicated that Shh-deficient limb buds (E10.0-E10.5) were molecularly proximalized. One of the affected genes was the RA receptor Rarb (Fig. 2A), which serves as a sensor for RA activity as its expression is positively regulated by RA (see e.g. Zhao et al. (Zhao et al., 2009)). Norm ally, Rarb expression is restricted to the proximal part of the limb bud with highest levels in the most anterior and posterior mesenchyme (left panel, Fig. 2A). In Shh-deficient limb buds, its expression was distally extended (middle panel, Fig. 2A) and levels were increased ~50% (right panel, Fig. 2A), which indicated that RA activity might be increased in Shh-deficient limb buds. Indeed, the expansion of Rarb expression was paralleled by Meis2, an established transcriptional target of RA signalling in the proximal limb bud mesenchyme (Fig. 2B) (Mercader et al., 1999; Mercader et al., 2000). In Shh-deficient limb buds, Meis2 expression extended distally and levels were increased by ~40% (Fig. 2B). In addition, we identified two novel potential RA target genes whose expression is normally restricted proximally in limb buds (Fig. 2C,D; see Fig. S1 in the supplementary material). Pbx1 is expressed in the proximal-anterior part of wild-type limb buds (Selleri et al., 2001). In Shh-deficient limb buds, its expression extended both distally and posteriorly (Fig. 2C). Pkdcc encodes a putative protein kinase (Imuta et al., 2009) and in wild-type limb buds its expression was restricted proximally, while it was distally extended in Shh-deficient limb buds (Fig. 2D; similar to Meis2). In addition, Pbx1 and Pkdcc transcript levels were significantly upregulated in Shh-deficient limb buds (Fig. 2C,D). To determine if these transcriptional alterations could be a consequence of increased RA activity, RA-loaded beads were
implanted into wild-type mouse forelimb buds, which indeed suffices to distally extend the expression domains of *Meis2, Pbx1* and *Pkdcc* (see Fig. S1 in the supplementary material).

To further substantiate the hypothesis that increased RA pathway activity could underlie the proximalization of *Shh*-deficient forelimb buds, the transcriptome alterations were interrogated for changes in components of the RA pathway and target genes. Indeed, significant alterations were seen (see Table S3 in the supplementary material). In particular, the expression of *Cyb26b1* was reduced ~1.7 fold in the distal mesenchyme of *Shh*-deficient mouse limb buds (Fig. 2E). CYP26B1 converts RA to its hydroxylated, inactive form (MacLean et al., 2001). This decrease in *Cyb26b1* expression provides a plausible mechanistic explanation for the suspected increase in RA activity and distally extended expression domains of RA responsive genes in *Shh*-deficient limb buds. Furthermore, beads loaded with a RA antagonist (RAA) (Mercader et al., 2000) were implanted into *Shh*-deficient forelimb buds (E10.5; 34-36 somites) were cultured for 16-20 hours following implantation of a carrier bead loaded with 2 mg/ml RA antagonist (RAA, dotted circle). The non-grafted contralateral forelimb bud is shown in the left panels. Expression of the proximal genes (*Rarb* (n=7/8), *Pbx1* (n=5/6) and *Pkdcc* (n=5/6) is reduced following implantation of an RAA bead.

**Cyb26b1 expression in the distal limb bud depends critically on AER-FGF signalling**

Next, we sought to determine the cause of the decreased *Cyb26b1* expression in *Shh*-deficient limb buds. In particular, we considered the possibility that this decrease could be linked to the ≥50% overall reduction of AER-*Fgf* expression (Table 1) (Chiang et al., 2001; Zuniga et al., 1999). Indeed, culturing of wild-type forelimb buds in the presence of an inhibitor of the FGF receptor tyrosine kinase (SU5402) (Zuniga et al., 2004) resulted in almost complete loss of *Cyb26b1* expression (Fig. 4A). Conversely, the implantation of FGF4-loaded beads into *Shh*-deficient limb buds resulted in striking restoration of *Cyb26b1* expression (Fig. 4B). By contrast, the implantation of FGF4-loaded beads into wild-type limb buds caused only a modest increase, which indicates that *Cyb26b1* is probably expressed at close to maximal levels in wild-types (Fig. 4C). These manipulations established that increased FGF signalling was sufficient to restore *Cyb26b1* expression in the absence of SHH, which reveals that the reduction in AER-FGF signalling (Table 1) (Zuniga et al., 1999) is the likely cause of reduced *Cyb26b1* expression in *Shh*-deficient limb buds.

To further investigate the role of AER-FGFs in regulation of *Cyb26b1* expression, we analyzed forelimb buds with reduced AER-FGF signalling. In AER-*Fgf*mut limb buds, the *Fgf8* and *Fgf4* loci are conditionally inactivated in the AER by the *Msx2-Cre* transgene. In addition, AER-*Fgf*mut limb buds are heterozygous for a *Fgf9*-null allele. In the AER of mutant forelimb buds there is early but transient expression of both *Fgf4* and *Fgf8*, which supports the development of proximal but not distal skeletal structures (Mariani et al., 2008). This significant genetic reduction in AER-*Fgf* expression results in much more distally restricted expression of *Cyb26b1* than in wild-type and *Shh*-deficient forelimb buds (Fig. 5B; compare with Fig. 5A and 5C), which corroborates the proposed positive regulation of *Cyb26b1* by AER-FGFs. Furthermore, the expression of the RA transcriptional targets *Rarb* and *Pbx1* was extended distally in AER-*Fgf*mut forelimb buds (Fig. 5E,H) compared with wild-type and *Shh*-deficient forelimb buds (Fig. 5D,F,G,I).
In Cyp26b1-deficient limb buds (Yashiro et al., 2004), the expression of all RA targets investigated was extended very distally (Fig. 5J-L). The extent of molecular proximalization was rather similar in Cyp26b1-deficient and AER-Fgf8mut forelimb buds, but less severe in Shh-deficient limb buds. In particular, the distal expansion of Rarb expression in Cyp26b1-deficient and AER-Fgf8mut limb buds was indicative of ectopic RA activity in the distal mesenchyme (Fig. 5E,J, compare with 5D). Taken together, these results indicate that distal progression of limb bud development not only depends on AER-RGF and SHH signalling as part of the SHH/GREM1/AER-RGF feedback loop, but also on CYP26B1-mediated RA clearance.

### The early activation of Cyp26b1 indicates molecular specification of a distal domain within the nascent limb bud mesenchyme

Next, we assessed the temporal relationship between the expression of Rarb, Cyp26b1 and Fgf8, the first AER-RGF expressed during limb bud development and investigated when the regulation of Cyp26b1 expression by AER-RGFs would become SHH dependent. The expression of Rarb was first detected throughout the nascent limb bud mesenchyme, but decreased concurrent with activation of Cyp26b1 expression in the distal-most mesenchyme (Fig. 6A,B). This suggested that the onset of CYP26B1-mediated RA inactivation creates a ‘RA-free’ domain within the distal limb bud mesenchyme. Fgf8 is expressed by the nascent AER (Fig. 6C) from the initiation of forelimb bud development onwards (embryonic day ≤E9.0) (Sun et al., 2002) together with its transcriptional target Spry4 in the mesenchyme (Fig. 6D) (Minowada et al., 1999). In contrast to Spry4, Cyp26b1 expression was always rather distally restricted (Fig. 6B,D). Cyp26b1 expression increased in parallel to AER-RGF signal transduction and remained complementary to the Rarb expression domain (≤E9.5; Fig. 6E-H). These expression patterns delineate a proximal (Rarb) and distal (Cyp26b1) domain, even during early initiation of limb bud outgrowth. In parallel, SHH signalling is activated (Platt et al., 1997), but no alterations in Rarb, Cyp26b1/AER-RGF expression were detected in Shh-deficient limb buds at these early stages (Fig. 6I-L). Therefore, the initial upregulation of Cyp26b1 expression and molecular definition of proximal and distal domains are independent of SHH signalling. The expression of Rarb, Cyp26b1 and AER-Fgf8s started to be altered in Shh-deficient limb (see Fig. S2 in the supplementary material) only during the time period when the SHH/GREM1/AER-RGF signalling system becomes operational in wild-type limb buds (>E10.0) (Michos et al., 2004; Chiang et al., 2001; Zuniga et al., 1999).

### Spatiotemporal simulations of the genetically verified interactions reveal an antagonistic signalling module

The simplest signalling module that could be derived from the genetic and functional analysis is shown in Fig. 7A. Mathematical simulations were used to determine if this module would be sufficient to explain the molecular alterations observed in Shh-deficient limb buds. Previously, we had succeeded in simulating the temporal dynamics of the SHH/GREM1/AER-RGF feedback signalling system (Benazet et al., 2009). However, as spatial
Pbx1 and (K) Cyp26b1-projected onto the growing 2D limb bud domain (Fig. 7B). The domains, the simulated spatial distributions and activity levels were enable easier comparison with the in vivo gene expression over time and are shown at specific developmental time points. To bud domain (for details see Appendix S1 in the supplementary reaction-diffusion type partial differential equations on a growing forelimb buds are of the following genotype: Fgf8mut and (2) AER inhibition in chicken limb buds (Tickle et al., 1989); observations: (1) beads loaded with high levels of RA were able to establish that RA is indeed able to inhibit AER-Cyp26b1-deficient mouse limb buds (Yashiro et al., 2004). To (I-L) expression of the same genes in Shh-deficient forelimb buds at E9.5. The complementary domains of Rab and Cyp26b1, and the expression of AER-Fgf8 and Spry4 are not yet altered in Shh-deficient forelimb buds. Asterisks indicate the anterior and posterior margins of forelimb buds.

Fig. 6. Molecular evidence for the early and SHH-independent establishment of a distal limb bud mesenchymal domain. (A-H) Expression of Rab, Cyp26b1, Fgf8 and Spry4 in wild-type forelimb buds at E9.25 (A-D; 23 somites) and E9.5 (E-H; 25-26 somites). Cyp26b1 expression is already detected in the distal-most mesenchyme during initiation of limb bud development (E9.25, B). (I-L) Expression of the same genes in Shh-deficient forelimb buds at E9.5. The complementary domains of Rab and Cyp26b1, and the expression of AER-Fgf8 and Spry4 are not yet altered in Shh-deficient forelimb buds. Asterisks indicate the anterior and posterior margins of forelimb buds.

was reduced throughout the AER (Fig. 8D). Furthermore, the expression of Spry4 was also reduced (Fig. 8D). These results corroborate the proposal that high RA activity inhibits AER-Fgf expression and the antagonistic nature of the RA/AER-FGFS interactions. The antagonistic AER-FGF/CYP26B1/RA signalling module (Fig. 8A) probably serves (1) to restrict AER-Fgf8 expression and (2) to define molecularly a distal RA-free mesenchymal region during initiation of limb bud outgrowth (Fig. 6).

When we incorporated the ~12 hours delayed input of the SHH/GREM1/AER-FGF feedback loop (right panel, Fig. 6B; see Fig. S2 in the supplementary material) than observed in Shh-deficient mouse limb buds (Fig. 8F; compare with Fig. 2; Shh-deficient forelimb buds at E9.5). The complementary domains of Rab and AER-Cyp26b1-deficient mouse limb buds (Fig. 8F; compare with Fig. 2; S2 in the supplementary material). In particular, Cyp26b1 expression was reduced concurrent with the disruption in the expression of AER-Fgfs in simulations of Shh-deficient limb buds. This resulted in distal expansion of Rab expression (Fig. 8F), which reproduced the observed increase in RA activity and molecular proximalization of Shh-deficient limb buds (Fig. 2, Table 2). When Cyp26b1 expression was set to zero to simulate the Cyp26b1 deficiency, Rab levels were more increased and AER-Fgf levels more decreased (see Fig. S3 in the supplementary material) than observed in Cyp26b1-deficient mouse limb buds (Fig. 5-J-L). Yashiro et al. (Yashiro et al., 2004) suggested that precocious activation of Cyp26a1 could to some extent compensate the Cyp26b1 deficiency. Indeed, when the expression of the RA-inactivating enzymes was reduced to only 45% to mimic partial functional compensation, the simulations of

Fig. 5. Similar alterations in the expression of selected RA pathway genes in Shh, Fgf and Cyp26b1 mutant limb buds. Comparative expression analysis of (A-C) Cyp26b1, (D-F) Rab, (G-I,L) Pbx1 and (K) Pkdcc in wild-type (Wt), AER-Fgfmut, Shh-deficient and Cyp26b1-deficient forelimb buds (E10.5; 34-35 somites). AER-Fgfmut forelimb buds are of the following genotype: Msi2-Cre<sup>tg+</sup>-Fgf4<sup>fl<sup>ox</sup></sup>; Fgf8<sup>tg+</sup>tg<sup>fl<sup>ox</sup></sup>; Fgf9<sup>fl<sup>ox</sup></sup>.
Cyp26b1-deficient limb buds matched the in vivo observed alterations much better. For example, the expression of AER-Fgf activity was initially normal, but reduced later (see Fig. S3 in the supplementary material), which mimics the observed reduction of Fgf4 during mouse limb bud outgrowth (Yashiro et al., 2004). These simulations indicate that the partial redundancy among Cyp26 family members might be of functional relevance to limb bud development, as is the case during primary body axis development (Uehara et al., 2009). Taken together, these simulations (Fig. S3 in the supplementary material) underscore the importance of the antagonistic AER-FGF/CYP26B1/RA signalling module for regulating the spatiotemporally restricted gene expression along the PD limb bud axis during both initiation and SHH-dependent distal development.

**DISCUSSION**

We combine transcriptional profiling with genetic analysis and mathematical modelling to analyse the gene networks regulated by SHH signalling. Although the present study agrees with a previous analysis of the impact of SHH signalling on transcriptional regulation (Vokes et al., 2008), additional conclusions can be reached as the transcriptome of Shh-deficient limb buds was analysed at an earlier developmental stage. By embryonic day E10.5, SHH is the major driving force in the feedback signalling system that interlinks the SHH, BMP and FGF signalling pathways (Benazet et al., 2009). However, the transcriptome analysis performed here revealed an important additional function of SHH signalling for PD limb bud axis development. In fact, we identified several novel markers for the proximal limb bud mesenchyme owing to their significant upregulation and distal expansion in Shh-deficient limb buds. For example, Mab21l1 and Mab21l2 were identified as genes whose expression domains can serve as additional markers for the presumptive zeugopod.
In Shh-deficient limb buds, the expression of molecular markers for RA activity is distally extended as a likely consequence of reduced Cyp26b1 expression within the distal mesenchyme. Our analysis establishes that SHH-mediated upregulation of AER-FGF signalling not only propagates the SHH/GREM1/AER-FGF signalling system but also the expression of Cyp26b1, which in turn enhances RA clearance. To gain a better mechanistic understanding of the spatiotemporal regulation of gene expression by these interacting pathways, we simulated limb bud development in silico by translating the genetically and molecularly defined interactions into mathematical equations. These simulations reveal the existence of an antagonistic AER-FGF/CYP26B1/RA signalling module that initiates RA clearance and defines a distal domain prior to the onset of SHH signalling. Subsequently, Cyp26b1 expression becomes dependent on SHH-mediated upregulation of AER-Fgfs as part of the self-regulatory SHH/GREM1/AER-FGF signalling system. The spatiotemporal simulations of PD gene expression and activity domains in wild-type and Shh-deficient limb buds were remarkably similar to the normal and altered gene expression domains observed in mouse limb buds.

An antagonistic AER-FGF/CYP26B1/RA signalling module creates distal identity during onset of limb bud outgrowth

We show that the AER-FGF/CYP26B1/RA signalling module is already established during initiation of limb bud development. The key role of AER-FGFs in promoting specification and expansion of distal limb bud progenitors has been established by extensive genetic analysis in mouse embryos (Lewandoski et al., 2000; Mariani et al., 2008; Sun et al., 2002). The increase in AER-FGF signalling due to the sequential activation of Fgf8, Fgf4, Fgf9 and Fgf17 regulates the expansion of the mesenchymal progenitors that will give rise to progressively more distal limb skeletal elements (Mariani et al., 2008). PD limb bud identities are probably determined during progression of limb bud outgrowth round the time their differentiation is initiated (differentiation front mechanism) (Tabin and Wolpert, 2007). The complete inactivation of Fgf8 and Fgf4 disrupts hindlimb bud development at an early stage, while their transient expression in forelimb buds (Sun et al., 2002) suffices to initiate development of distal skeletal elements. The activation of Cyp26b1 expression in the nascent mesenchyme suggests that a molecularly distinct pool of distal cells is already established during initiation of limb bud development and is subsequently expanded. As Cyp26b1 expression depends on FGF signalling (this study) (Gonzalez-Quevedo et al., 2010; Hernandez-Martinez et al., 2009), limb bud mesenchymal cells that are no longer under the influence of AER-FGFs may lose Cyp26b1 expression. In fact, Cyp26b1 expression in the limb bud mesenchyme is more distally restricted than Spry4, which indicates that high levels of AER-FGFs might be required to maintain Cyp26b1. The AER-FGF-mediated upregulation of Cyp26b1 expression and enhanced RA clearance is apparently important for distal limb bud development as increased RA levels are teratogenic and cause distal limb skeletal truncations (Yashiro et al., 2004; Zhou and Kochhar, 2004).

Although our study does not address the issue of whether endogenous RA is involved in specifying proximal limb bud mesenchymal identities, it provides evidence that RA is incompatible with SHH-controlled distal progression of limb bud development. However, RA may be important to restrict the length of the Fgf-expressing AER during the onset of forelimb bud outgrowth. RA may locally inhibit Fgf8 expression and/or AER development via the RA-response element located in the cis-regulatory region of the Fgf8 locus (Zhao et al., 2009). Thus, RA might participate in defining the forelimb field and nascent bud by inhibiting Fgf8 expression both in the trunk mesenchyme (Zhao et al., 2009) and in the overlying ectoderm (this study) by correctly scaling the Fgf8-expressing AER in concert with mesenchymal FGF10 and BMP4 signalling (Benazet and Zeller, 2009; Ohuchi, 1997). The higher and more general sensitivity of AER-Fgf4 expression to RA agrees with the fact that Fgf4 is normally only expressed after CYP26B1 has begun to inactivate RA in the distal mesenchyme (Yashiro et al., 2004). Finally, the distal expansion of proximal genes in Cyp26b1-deficient, Shh-deficient and AER-Fgfmut limb buds may reflect the aberrant RA activity rather than a gain of proximal identities. However, the distal expansion of the expression domains of the newly identified RA-responsive genes Pbx1 and Pkdcc could have direct effects on the PD axis as their genetic inactivation alters PD limb axis development. Pbx1-deficient mice display anomalies of the proximal limb skeleton, while the sizes of the long limb bones are reduced in Pkdcc-deficient mice (Imuta et al., 2009; Selleri et al., 2001).

SHH integrates AP and PD development during limb bud outgrowth

Our study provides mechanistic insights into the role of SHH as a node that integrates AP and PD limb axes development such that coordinated proliferation of the mesenchymal progenitors is ensured (Mariani et al., 2008; Zhu et al., 2008). Genetic evidence indicates that the AP axis is specified early and independent of the PD axis during limb bud development (Zeller et al., 2009; Zhu et al., 2008). Subsequently, the antagonistic AER-FGF/CYP26B1/RA signalling module becomes regulated by SHH as part of the self-regulatory SHH/GREM1/AER-FGF signalling system (Benazet et al., 2009). Such molecular interlinking of the AP and PD signalling systems appears essential for the dynamic and spatially coordinated regulation of gene expression and limb bud outgrowth. Last but not least, our study exemplifies how the combination of mouse genetics with in silico simulations aids in the identification of the signalling systems and interactions that control the development of complex structures such as vertebrate limbs. The possibility to simulate the key signalling interactions in both time and space begins to provide insights into the underlying interaction dynamics. For example, the genetic and functional analysis alone would not have revealed the relevance of local inhibition of AER-Fgf8 expression by RA for restricting AER length.

The importance of FGF signalling for the expression of Cyp26 enzymes has now been established for rather diverse developmental processes such as neurogenesis in zebrafish embryos (Gonzalez-Quevedo et al., 2010), somitogenesis in Xenopus laevis (Moreno and Kintner, 2004) and interdigital cell death in mouse embryos (Hernandez-Martinez et al., 2009). In fact, the FGF/CYP26/RA signalling module has been deployed repeatedly to regulate the temporally and spatially coordinated development of limb buds (this study), the neural tube (Diez del Corral et al., 2003), somites (Goldbeter et al., 2007) and germ cells (Bowles et al., 2010). During somite formation, this antagonistic signalling module has been proposed to generate sharp developmental thresholds and a bi-stable switch that governs segmentation (Goldbeter et al., 2007), which is echoed by its functions during determination of male and female germ cells (Bowles et al., 2010). Thus, this antagonistic signalling module is required for specifying distinct expression domains and/or identities during the development of rather diverse tissues and structures in vertebrate embryos.
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Supplementary material

References


