Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning

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The ability of signalling proteins to traverse tissues containing tightly packed cells is of fundamental importance for cell specification and tissue development; however, how this is achieved at a cellular level remains poorly understood. For more than a century, the vertebrate limb bud has served as a model for studying cell signalling during embryonic development. Here we optimize single-cell realtime imaging to delineate the cellular mechanisms for how signalling proteins, such as sonic hedgehog (SHH), that possess membrane-bound covalent lipid modifications traverse long distances within the vertebrate limb bud in vivo. By directly imaging SHH ligand production under native regulatory control in chick (Gallus gallus) embryos, our findings show that SHH is unexpectedly produced in the form of a particle that remains associated with the cell via long cytoplasmic extensions that span several cell diameters. We show that these cellular extensions are a specialized class of actin-based filopodia with novel cytoskeletal features that have not been previously described. Notably, particles containing SHH travel along these extensions with a net anterograde movement within the field of SHH cell signalling. We further show that in SHH-responding cells, specific subsets of SHH co-receptors, including cell adhesion molecule downregulated by oncogenes (CDO) and brother of CDO (BOC), actively distribute and co-localize in specific micro-domains within filopodial extensions, far from the cell body. Stabilized interactions are formed between filopodia containing SHH ligand and those containing co-receptors over a long range. These results suggest that contact-mediated release propagated by specialized filopodia contributes to the delivery of SHH at a distance. Together, these studies identify an important mode of communication between cells that considerably extends our understanding of ligand movement and reception during vertebrate tissue patterning.

The regulated movement of key signalling proteins within tissues is a central feature of metazoan development that remains poorly understood at the cellular level. Several mechanisms have been proposed to distribute signalling molecules, including free diffusion, transcytosis and directed transport of signalling receptors via filopodia, including those termed cytonemes within invertebrate embryos. An example of a key signalling protein is SHH, which is instrumental in patterning the early embryo. During limb development, SHH is produced by the zone of polarizing activity (ZPA), a small group of mesenchymal cells at the posterior margin of the limb bud, and it acts over a long range to specify the number and identity of digits produced. How tight control in SHH distribution across several cell diameters is established remains poorly understood. Here we combine unique genetic and live-cell imaging approaches to investigate the mechanisms of long-range cell signalling underlying tissue patterning during vertebrate embryonic development.

To image living chick embryos under the regulatory control of specific spatial and temporal regulatory elements, we developed a piggyBac transposon-mediated stable integration approach. This genetically tractable expression system, coupled with optimized confocal microscopy, enabled imaging of embryos (Methods) at single-cell and subcellular resolution (Fig. 1a and Supplementary Fig. 1). At first, we expressed membrane-tethered fluorescent proteins that illuminate individual mesenchymal cells within the developing limb bud in a mosaic fashion (Fig. 1b). Through this approach we uncovered an unexpected, intricate network of thin cellular extensions present on these cells spanning several cell diameters, which orient in many directions from the cell body along the anterior–posterior, proximal–distal and dorsal–ventral axis (Fig. 1c–e and Supplementary Fig. 2). All mesenchymal cells possess several cellular extensions up to 150 μm long, with an average length of 34.27 ± 9.6 μm (mean ± s.e.m.) (n = 24), revealing a surprising morphology to these cells (Fig. 1c–e and Supplementary Video 1). These extensions are remarkably fine, approximately 200 nm in diameter, at the resolving limit of conventional microscopy and can be labelled with membrane-bound (Fig. 1) but not cytoplasmic (Supplementary Fig. 3) fluorescent proteins. They are capable of elongating, at a maximum rate of 150 nm s⁻¹, retracting, and traversing the complex three-dimensional extracellular matrix of the limb bud (Fig. 1g, h, Supplementary Fig. 2 and Supplementary Videos 2 and 3). Importantly, despite their dynamic nature, they form highly stabilized long-range interactions between cells, thereby revealing a new and complex landscape of cell–cell interactions mediated through cytoplasmic extensions within embryonic tissues (Fig. 1f, Supplementary Fig. 2 and Supplementary Videos 4 and 5). These extensions are not amenable to conventional fixation, which severely disrupts their structure and may have precluded their previous identification (Supplementary Fig. 5).

Actin-associated markers—including the high-affinity F-actin probe utrophin calponin homology domain (UCHD) fused to enhanced green fluorescent protein (UCHD–eGFP) and moesin–eGFP—decorate the entire length of cytoplasmic extensions, revealing that these structures are actin-based filopodia (Fig. 2a and Supplementary Fig. 4a). Unlike actin markers, tubulin cytoskeleton markers, such as microtubule-associated proteins TAU (also known as MAPT) and EB3, only label the proximal base of a subset of mesenchymal filopodia (data not shown). We next examined the localization of a plus-ended actin motor, myosin-X. Notably, myosin-X–eGFP moves to the distal tips of the filopodia, where it accumulates, thereby revealing that actin motors can move along these structures (Fig. 2b and data not shown).

Limb bud mesenchymal cytoplasmic extensions also possess distinct cytoskeletal features compared to typical filopodia, commonly characterized as actin-based linear extensions of the cell membrane, with limited lengths of up to 10 μm (ref. 10). For example, LifeAct is a highly specific marker of filopodia in eukaryotic cells, but it unexpectedly labels only the proximal base of limb mesenchyme cytoplasmic extensions (Fig. 2c) and not their distal tips, reflecting a distinguishing feature. This is consistent with the fact that LifeAct does not label certain forms of highly modified actin, or actin that is highly coiled with binding proteins. Additional specific features are also evident in the actin depolymerization factor coflin, which has mainly been implicated in extending lamellipodial protrusions. Notably, coflin–eGFP rapidly accumulates to the...
Figure 1 | Mesenchymal cells of the developing limb bud possess long and highly dynamic cytoplasmic extensions. a, Left, HH14 chick embryo indicating the site of DNA injection. The red dashed line indicates the cross-sectional plane. Right, microinjected DNA in the coelom is shown in green; the electodes position is indicated (LPM). NC, notochord; NT, neural tube. b, Diagram of piggyBac transposon system resulting in integration of transposon inverted terminal repeat (ITR)-flanked expression cassettes by piggyBac transposase. Cre recombinase flanked by loxP sites (black triangles) results in mosaic labelling from a loxP-containing reporter construct, containing membrane-palmitoylated mKate2 (a far-red fluorescent protein) or pmeGFP expressed via the tetracycline responsive element (TRE). Dox, doxycycline. CAGp denotes a ubiquitous promoter; 3G denotes an inducible transactivator protein. c, Confocal z-series acquired in vivo from an HH21 limb bud reveals an intricate network of cellular extensions (Supplementary Video 1). d, Single x–y plane, from c, highlighting the network of long cytoplasmic extensions among mesenchymal cells. e, A representative long extension (75 μm) from c, marked by dotted line. f, Example of an interaction between two cytoplasmic extensions. Cytoplasmic extensions emanating from two cells that are initially separated (left panel, yellow and white arrows) then extend until they interact and overlap (right panel, yellow and white brackets) to form stabilized interactions over a long-range (see also Supplementary Video 4). Time in mins. g, Speed distribution of extending (black) and retracting (grey) cytoplasmic extension velocities; n = 8. h, Extension dynamics. Grey bars represent net length change in micrometres. Red line represents the mean velocity (nm s⁻¹). The x axis tick marks denote 1-min intervals. Scale bars, 10 μm (c–e), 3 μm (f).

tips of limb mesenchymal filopodia, and its subsequent retraction back to the cell soma prefigures the rapid and dynamic retraction of filopodia extensions (Supplementary Fig. 4b and Supplementary Video 6). Cofilin-eGFP is also frequently localized to specific microdomains along these filopodia that are interrupted rather than labelling the entire process that may account, at least in part, for the greater dynamics of these filopodia, including movement and bends in specific sub-regions as they traverse extracellular space (Fig. 2d). Fascin, which enhances cofilin severing², also labels filopodial extensions (Supplementary Fig. 4c). Together, these findings demonstrate that limb mesenchyme filopodia possess unique cytoskeletal features reflecting specialized properties, which include their considerable lengths, highly dynamic behaviours, and complex geometries. Our initial attempts to perturb mesenchymal filopodia formation using known molecular pathways (Methods) have proven ineffective, for example, through conditional inactivation of cell division cycle 42 (CDC42) in the limb bud (data not shown).

To determine the functional role of mesenchymal filopodia, we used genetic strategies to label specific cellular populations with membrane-bound fluorescent proteins along the anterior–posterior axis of the mouse limb bud with respect to SHH signalling. This revealed that SHH-producing cells within the limb bud ZPA extend long filopodia (Supplementary Fig. 6a and Supplementary Video 7), which can orient along the anterior–posterior axis as well as the proximal–distal axis, with a further bias towards the apical ectodermal ridge that maintains the SHH and fibroblast growth factor (FGF) feedback loop¹⁵ (Supplementary Fig. 7a, b). Moreover, mesenchymal cells within the anterior limb bud that respond to SHH also extend similar filopodia (Supplementary Fig. 7a, b). Additionally, mesenchymal cells within the anterior limb bud reveal an intricate network of cellular extensions (Supplementary Video 1).
Supplementary Fig. 8e). Notably, expression of these SHH fusion proteins under doxycycline-inducible control does not perturb endogenous SHH signalling as revealed by PTC1 expression, nor limb development or skeletal patterning (Supplementary Fig. 8d).

Imaging of SHH<sub>N</sub>–eGFP under native regulatory control in the ZPA reveals that it is unexpectedly produced in the form of a particle approximately 200 nm in size (Fig. 3a, b). These particles are not observed in the extracellular space, but remain associated with the SHH-producing cell

Figure 2 | Limb mesenchymal cytoplasmic extensions are a class of specialized actin-based filopodia. a, UCHD–eGFP-localization staining demonstrating that membrane-labelled pmKate2 filopodia extensions contain actin filaments. b, Myosin X–eGFP (MYOX–eGFP) is localized to each pmKate2-labelled filopodium and is concentrated at the distal tip.

c. LifeAct-mKate2 marks only the proximal aspect of pmeGFP-labelled filopodia and does not label the entire extension, shown by the bracket. d, Cofilin–eGFP is present in interrupted domains along the filopodia, negative regions shown with brackets. Scale bars, 3 μm (a), 5 μm (b–d).

Figure 3 | Live-cell imaging of SHH ligand production and transport within the limb bud. a, Schematic of piggyBac-mediated integration of transposon-flanked expression cassettes (ITR). The G. gallus SHH minimal promoter (SHHp) and ZRS element direct spatial expression in the limb ZPA of doxycycline-inducible transactivator protein (3G), which in turn allows for the temporal control of SHH<sub>N</sub>–eGFP or SHH<sub>P</sub>–eGFP and pmKate2. b, Left, a representative SHH-producing cell containing multiple long filopodia, with SHH<sub>P</sub>–eGFP present in discrete particles as well as in a more diffuse form localized along these extensions. Right, SHH<sub>N</sub>–eGFP is produced as a particle visualized within the cell soma (arrows) as well as along the filopodia.

c, Representative timelapse images showing anterograde SHH<sub>N</sub>–eGFP particle movement (arrows) that accumulates at the tip of the certain filopodia but not others (indicated by double-headed arrow) (Supplementary Video 8). Time in mins, interval is four frames s<sup>−1</sup>. d, Particle dynamics graph demonstrating the movement of SHH<sub>N</sub>–eGFP particles relative to the filopodium; normalized distance to filopodia base is 0, and to filopodia tip is 1. e, Net particle movement graph demonstrating the net vectors of particle (n = 38) displacement represented as a percentage of the total filopodia length that particles traverse. Green denotes anterograde, blue denotes no displacement (<5%), and red denotes retrograde displacement. The relative thickness of each vector reflects the percentage of particles within each category. There is a statistically significant net anterograde movement of SHH particles away from the cell soma; P < 0.002. f, SHH-containing filopodia (red lines) are statistically more stabilized than filopodia without SHH (black lines); P < 0.001, n = 200 time points. All scale bars, 3 μm.
via long filopodial extensions. Imaging of SHH<sub>2</sub>–eGFP also reveals that cholesterol-modified SHH is similarly produced as a particle associated with filopodial extensions, and also displays more uniform localization along these extensions (Fig. 3b and Supplementary Fig. 8c). Importantly, such particles are an intrinsic property of the SHH molecule, as they are not formed by expression of cytoplasmic, palmitoylated or cholesterol-modified eGFP (data not shown). SHH particles only travel to specific subsets of filopodia emanating from the same cell, revealing tight selectivity and regulation over this process (Fig. 3c).

High-speed, real-time imaging showed that SHH<sub>2</sub>–eGFP particles move in both anterograde and retrograde directions along filopodia (Fig. 3c, d and Supplementary Video 8), with a statistically significant net anterograde movement away from the cell body (P < 0.0002) (Fig. 3e and Supplementary Fig. 9). The maximum velocity of anterograde particle movement, 120 nm s<sup>−1</sup>, is consistent with actin-based myosin motors. Moreover, filopodia containing SHH particles are stabilized and less dynamic than non-particle-containing filopodia (Fig. 3f; P < 0.001). These findings reveal that filopodia can distribute SHH ligand at a distance from the cell body. It remains to be determined whether the SHH cholesterol modification, which has been proposed to either promote<sup>18</sup> or restrict<sup>16</sup> the spread of SHH, has additional functions in filopodial transport. To our knowledge, this is the first in vivo demonstration of SHH ligand production and movement, revealing an unexpected role for filopodia in this process.

To determine the precise localization of SHH<sub>2</sub>–eGFP within filopodia, we used an optimized split GFP complementation system, consisting of the spGFP<sub>1–10</sub> and spGFP<sub>11</sub> non-fluorescent GFP fragments that reconstitute a fluorescent GFP signal. Notably, SHH<sub>2</sub>–spGFP<sub>11</sub> can physically interact to reconstitute an extracellular leaflet-associated glycosphingolipidylinositol (GPI)-anchored spGFP<sub>1–10</sub>–producing a GFP signal along the extracellular surface of limb mesenchyme filopodia in vivo (Supplementary Fig. 10a, d). We also cultured mesenchymal cells after electroporation of SHH<sub>3</sub>–spGFP<sub>11</sub> and applied a synthesized spGFP<sub>1–10</sub> peptide exogenously to the media (Methods) that similarly produced a GFP fluorescent signal along the filopodial membrane, but not when cells do not express SHH<sub>3</sub>–spGFP<sub>11</sub> or express a control GFP<sub>1–10</sub> cytoplasmic fragment (Supplementary Fig. 10b–d). In addition, ectopic expression of SHH<sub>3</sub>–spGFP<sub>11</sub> tethered to the membrane as a result of its interaction with GPI–spGFP<sub>1–10</sub> leads to the ectopic expansion of PTCP expression (Supplementary Fig. 10e). Although the split GFP complementation system displays remarkable antibody affinity for GFP fragments<sup>20</sup>, we cannot exclude the possibility that ectopic activation of PTCP may also derive from a freely diffusible form of SHH<sub>3</sub>–spGFP<sub>11</sub> that we cannot detect. Collectively, these experiments suggest that SHH<sub>2</sub>–eGFP is localized to the extracellular leaflet of the filopodial membrane, where it is competent to signal and interact with SHH receptors.

We next visualized additional SHH signalling components involved in the reception of SHH in vivo. In addition to PTCP, which serves as the identified receptor for SHH<sup>21</sup>, additional co-receptors of SHH, including the transmembrane proteins CDO and BOC, are necessary for long-range SHH signalling<sup>22,23</sup>. Interestingly, in the limb bud these co-receptors are only expressed in SHH-responding cells<sup>24</sup>. In contrast to other components of the SHH signalling pathway, such as PTCP–yellow fluorescent protein (YFP) and smoothened (SMO)–GFP that can localize to primary cilia (Supplementary Fig. 11), our live imaging reveals that CDO–GFP and BOC–GFP do not. Instead, they exhibit marked localization to discrete microdomains along long filopodial (average size 2.4 ± 1.5 μm; range 0.6–8.5 μm) that remain static and display little lateral movement (Fig. 4a, b). Moreover, there is substantial co-localization of these co-receptors to these microdomains in only a subset of filopodia emanating from individual SHH-responding mesenchymal cells, reflecting tight spatial regulation (Fig. 4c). There is a statistically significant stabilization of the filopodia containing SHH co-receptors (Fig. 4d; P < 0.001). The molecular roles of CDO and BOC in transducing long-range signalling have been poorly understood, although their binding to SHH is independent from PTCP (ref. 25). The unexpected co-localization of these co-receptors on microdomains along filopodia suggests that they may participate in relaying activation of the pathway at a distance from the cell soma.

To mark SHH-producing and -responding cells simultaneously and precisely, we further designed a hybrid GLI3 enhancer and promoter element (Methods and Fig. 4e). Using this dual-expression system, we find that filopodia of SHH-producing cells directly interact with filopodia of SHH-responding cells that contain microdomains of BOC–eGFP (Fig. 4f). Similar stabilized interactions are evident between filopodia containing SHH ligand and SHH-responding cells that have undergone pathway activation as revealed by SMO–blue fluorescent protein (BFP) localization to cilia (Fig. 4g). Together, these results reveal that SHH-producing and -responding cells interact at a distance through filopodial membrane-to-membrane contacts containing SHH ligand and co-receptors.

**Figure 4** | Filopodia on SHH-responding cells display an exquisite distribution and co-localization of SHH co-receptors that interact with SHH-producing filopodia. a, b, Live imaging of CDO–GFP (a) and BOC–GFP (b) expression in defined microdomains along the filopodial membrane, within subsets of filopodia but not others (arrows). Higher magnification images (a, b, right) show multiple positive microdomains of co-receptor localization (brackets) interspersed along the filopodia membrane. c, DCD–GFP and BOC–mKate2 are co-localized along microdomains of filopodia (arrows) labelled with membrane-associated near-infrared fluorescent protein (pmRFP). d, BOC-containing filopodia (red lines) are significantly more stabilized than filopodia without BOC (black lines); P < 0.001, n = 160 time points. e, Expression system to label SHH-producing (left) and SHH-responding (right) cells specifically in the same limb bud. GLI3e/p denotes GLI3 enhancer/promoter. f, Representative three-dimensional image of a filopodia from a SHH-producing cell (pmKate2, red) that interacts with domains of BOC–GFP (green) along the filopodia membrane (pmRFP, fuscia) of an SHH-responding cell. Arrows show interaction along BOC microdomains (brackets). g, SHH-producing cell, indicated by pmKate2 and marked by bracket, with a long filopodium containing SHH<sub>2</sub>–eGFP particles (arrows) that contacts a smoothened–positive cell (SMO<sup>+</sup>; outlined by a dashed line). Smoothened–BFP localization to the cilium is a marker of SHH pathway activation. All scale bars, 3 μm.
Our live imaging studies have identified a specialized class of filopodia with distinct cytoskeletal features that localize and transport components of the SHH signalling pathway, uncovering an important mechanism for the distribution of signalling molecules within tissues. The highly stabilized interactions between filopodia containing SHH ligand and those containing SHH co-receptors strongly suggest that long-range activation of signalling may be mediated through direct receptor–ligand interactions between cell membranes at a distance. Indeed, SHH-producing and -responding cells can extend filopodia as long as 150 μm in length encompassing the entirety of the 300-μm field of SHH signalling within the limb bud. Interestingly, mesenchymal filopodia may share certain properties with those of cellular extensions previously described within invertebrate embryos, and these findings are consistent with earlier studies including electron microscopy carried out more than 30 years ago describing the presence of fine cellular extensions on limb bud mesenchyme. Future studies will be required to determine whether such ‘specialized filopodia’ are an inherent feature of many, additional cell types that may have escaped previous detection in fixed and stained samples. Moreover, an outstanding question is whether they rely on unique cellular machinery for their generation, composition and ability to transport signalling molecules. For example, our findings revealing that SHH is produced in the form of a particle, which travels along filopodial extensions in a highly directional manner, suggest that a yet unidentified molecular motor may be responsible for the movement of SHH along these structures. It is intriguing to speculate that the movement of proteins, and perhaps other molecules such as nucleic acids, along specialized filopodial networks offers a new mechanism for controlling the precise delivery of molecular information among cells during vertebrate embryonic development, regeneration and pathological processes such as cancer metastasis. Considering the diverse cellular milieus in which signalling molecules have been shown to act, specialized filopodia may be a more adapted feature of certain signalling centres that operate alone or together with other proposed models for ligand distribution, including free diffusion through extracellular space.

METHODS SUMMARY

A spatially and temporally regulated piggyBac transposition system was developed to express fluorescent fusion proteins in a heritable fashion within the developing chick embryonic limb bud. The regulated expression of introduced transgenes was stable throughout embryogenesis and did not perturb normal limb development. Cellular morphology and characterization of specialized filopodia was assessed through the use of membrane-associated fluorescent proteins and selected markers of the actin and tubulin cytoskeleton. Molecular components of the SHH signalling pathway were visualized as fusion proteins under endogenous regulatory control. High-resolution live imaging of the embryonic limb bud was performed on custom-designed spinning disk confocal systems, allowing for the visualization of multiple fluorescently coupled proteins in real time, at high spatial resolution.

Full Methods and any associated references are available in the online version of the paper.

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METHODS

Plasmid expression constructs. For extended expression during chicken embryogenesis, the piggyBac transposition system was used. The parental plasmid pCAG-ERNXN was used as the minimal 5' (314 base pairs (bp)) and 3' (242 bp) ITRs of the piggyBac transposon28 was used as the plasmid backbone for expression experiments. PBX contains the cytomegalovirus (CMV) enhancer chicken beta-actin (CAG) promoter expression cassette modified to include the Invitrogen Gateway RIA cassette, allowing for phiC31-mediated recombination from Gateway Entry vectors. For doxycycline-inducible expression, a modified TRE-3G enhancer minimal promoter element (Clontech) replaced the CAG cassette of PBX to generate PBTREX. For the transposase-mediated insertion of the piggyBac transposon cassette, the improved piggyBac transposase28 was expressed via the CAG promoter in the plasmid CAGEN (C. Cepko, Addgene plasmid 31857; ref. 34) were selected based on their spectral characteristics as well as their suitability for live imaging of fusion proteins. Inner leaflet membrane-associated palmitoylated fluorescent proteins were generated by the addition of the 17-amino-acid sequence of rat GAP-43 (McClain et al. 2005) to N-terminus of individual fluorescent protein through sequential PCR amplification; these constructs are designated pm-XFP.

Membrane-associated fluorescent proteins. For multi-colour labelling of cells and signalling components, the monomeric fluorescent proteins TagBFP (Evrogen), monomeric eGFP (K. Svoboda, Addgene plasmid 18969; ref. 32), superfolder GFP (sfGFP)33, mKate2 (Evrogen) and oligomeric iRFP (V. Verkhusha, Addgene plasmid 31857; ref. 34) were selected based on their spectral characteristics as well as their suitability for live imaging of fusion proteins. Inner leaflet membrane-associated palmitoylated fluorescent proteins were generated by the addition of the 17-amino-acid sequence of rat GAP-43 (McClain et al. 2005) to N-terminus of individual fluorescent protein through sequential PCR amplification; these constructs are designated pm-XFP.

Mosaic expression of membrane fluorescent proteins. For Cre-mediated recombination experiments, alox–SV40 pA stop–loxP cassette (LSL) (D. Stainier, Addgene 24334; ref. 35) was introduced into the parental ubiquitous and inducible promoter constructs (PB and PB-TRE, respectively); pmKate2 and pmGFPl were placed before and after the LSL cassette, respectively, to generate a reporter construct for Cre activity. To generate a self-inactivating Cre recombinease construct, Cre recombinease containing an N-terminal nuclear localization signal was placed between in the PB-LSL vector.

Subcellular markers. To label F-actin, two markers were used that showed improved cell viability relative to eGFP–actin, UCHD–eGFP (D. Mullins) and LifeAct-mKate2. LifeAct-mKate2 was generated by the addition of the 17-amino-acid sequence of Abp140 from Saccharomyces cerevisiae (underlined) and linker sequence MVGADLKIFKEISSKEGDPVAT to the N terminus of mKate2 (refs 36, 37).

Additional markers of the actin cytoskeleton including bovine myosin X-heavy meromyosin–eGFP (R. Chenevix39), human moesin–eGFP (S. Shaw, Addgene plasmid 20671; ref. 39), human cofilin–eGFP (J. Bamburg) and human fascin–eGFP (D. Vignjevic) were used. Markers of the tubulin cytoskeleton included Tau–eGFP (P. Mombaerts) and EB3-Wasabi (Allele Biosciences). Human ARL13B–mKate2 (J. Reiter) was used as a marker of cilia. Cholesterol-modified eGFP was constructed placing amino acids 197–437 of mouse SHH on the carboxy-terminal portion of eGFP. The above fluorescent protein constructs were amplified by high-fidelity PCR, inserted into the pENTR/DTOPO vector and subsequently cloned into the piggyBac expression construct.

SHH signalling pathway fusion proteins. Fluorescent fusion proteins were amplified by high-fidelity PCR or overlap-extension PCR, inserted in the pENTR/DTOPO vector and subsequently cloned into the piggyBac expression construct (see above). Mouse Shh cDNA was provided by A. McMahon. SHHH2–eGFP was generated by placing monomeric eGFP between Ser 196 and Gly 197 of mouse SHH through overlap-extension PCR. We optimized the position of monomeric GFP codons relative to the SHH cholesterol modification site to improve in vivo expression40,41. This resulted in an improved GFP signal in vivo; however, SHHH2–eGFP was less intense than SHH2–eGFP and other fusion proteins, which may reflect inefficient processing or stability issues42. SHHH2–eGFP, a fusion protein lacking the C-terminal proteolytic domain and resulting cholesterol addition, was generated by deleting amino acids 197–437 after the eGFP fusion. CDO–GFP and BOC–GFP were made as C-terminal fusion proteins43. Mouse BOC–mKate2 was generated by replacing the cytoplasmic tail GFP with mKate2. Murine SMO–TagBFP was generated replacing the fluorophore of SMO–GFP (J. Reiter). PTC1–YFP was from J. Reiter.

spGFP complementation system for SHH. The optimized split GFP complementation system described previously44 was used to assess whether SHHH2–eGFP was localized to the extracellular surface of filopodia. SHHH2–spGFP1 was generated by placing the M3 peptide after amino acid 196 of mouse SHH separated by a flexible linker, (GGGGS)3×. SHHH2–spGFP1 and GFP-linked CD14–spGFP10 (ref. 45) were inserted into the PB-TRE expression construct. There was no detectable fluorescence observed with either construct independently consistent with previous studies39.

To address further the subcellular localization of SHH in relation to mesenchymal filopodia, we used the exogenous application of a commercially produced spGFP10 (Sandia Laboratories)45. In brief, after the electroporation of the chick embryo somatic lateral plate mesoderm, ex vivo cultures of limb mesenchymal cells were prepared as described previously46. After plating of these cells, the purified spGFP10 fragment was added exogenously to the live culture and imaged. Positive inter-fragment (spGFP10–spGFP1) and the spGFP10–spGFP1 fragments was observed as a fluorescent signal 4 h after fragment addition. As a control for possible endocytosis of the spGFP10, fragment, a spGFP1 luciferase fusion protein localized to the cytoplasm did not produce a fluorescent signal. G. gallus SHH promoter and enhancer element. The 1.7-kilobase (kb) SHH limb specific enhancer element designated the ZRS, and the 1.1-kb chicken SHH minimal enhancer element47 were cloned into the PBX vector replacing the CAG promoter cassette to generate a Gateway compatible expression construct. Subsequently, pmEGFP or the tetracycline activator protein 3G (Clontech) were inserted into the expression cassette to allow for either constitutive or doxycycline-inducible spatially restricted expression in the ZPA.

GL3 intronic enhancer and promoter element. To allow for fluorescent transgenes to be expressed in SHH–responding cells of the limb bud, a screen for promoter elements that would correctly regulate expression was performed. In brief, several human enhancer elements that were previously identified following chromatin immunoprecipitation DNA sequencing (ChIP-seq) of the enhancer associated p30 protein and mouse transgenic analysis48 were subsequently tested in chick embryos when coupled to various minimal promoter elements, HSP68, E1b, thymidine kinase and minimal CMV. Specific expression was achieved when the human GL3 intronic enhancer element, hs1586 (ref. 48), was coupled with the rat minimal Glb3 promoter49. Subsequently, pmKate2 (Supplementary Fig. 12) or 3G (Clontech) were inserted into the expression cassette to allow for either constitutive or doxycycline-inducible spatially restricted expression in the GL3–expressing and SHH–responding cells of the limb.

Chicken embryo manipulation and electroporation. Fertilized chicken eggs (G. gallus) were purchased from Petulama Farms and subsequently stored at 16 °C. Eggs were incubated in a non-rotary incubator at 38.5 °C until the desired stage according to Hamburger and Hamilton (HH)50. Stage HH13–15 chick embryos were windowed following standard techniques in preparation for electroporation. Embryos were visualized with the assistance of a 470/40 nm band-width emission filter, which provided the necessary contrast for injection. PBS without Ca2+ /Mg2+ was applied to the embryo. The vitelline membrane above the forelimb field was carefully sub-dissected, and additional solution was placed over the embryo. DNA constructs, with a final concentration 1–5 μg μl−1, diluted in endotoxin-free H2O were combined with phenol red (0.1 M final concentration) to aid in visualization. A 1.0-mm inner diameter capillary glass electrode was backfilled with DNA injection solution and a volume of solution was pressure injected (WPI Picopump) into the embryonic coelom, to fill completely the anterior to posterior extent of the forelimb territory. For the negative electrode, a 250-μm diameter platinum rod with a 4-mm length and 2-mm exposed surface (Nepagne) was inserted into the yolk and positioned beneath the forelimb field, approximately 0.5–1 mm below the embryo. A 250-μm diameter platinum rod with a 1-mm exposed tip served as the positive electrode and was positioned above the forelimb field with an approximate distance of 2 mm. A square-wave pulse train consisting of 8 V, three pulses, 50-ms duration with a 1-s interpulse interval was delivered via a Nepa 21 electroporator (Nepagne). This delivery resulted in an approximate current of 8–14 mA with energy of 10–18 μl. Embryos were returned to 37.5 °C for the remainder of the incubation period. In experiments using the piggyBac transposition system, a 1.5–1:10 molar ratio of piggyBac transposase helper plasmid, HpyBase, was combined with the transposon expression construct and electroporated into the chick embryo. For experiments testing the expression in the embryonic limb through embryonic day (E)12, 10 days after electroporation (Supplementary Fig. 1a and unpublished observations). Moreover, limb development and resulting morphology was normal as assessed with Alcian Blue cartilage staining (Supplementary Fig. 1b). For induction of gene expression with the inducible 3G system, 12–24 h before imaging, 50 ng doxycycline (Clontech) in 500 μl in HBSS was injected beneath the embryonic vasculature. For experiments with iRFP as a fluorescent protein, 75 μg blinderxin ( Frontier Scientific) was administered more than 4 h before imaging.

Live imaging of embryos. To facilitate accessibility of the chick embryo to live imaging with minimal perturbation, embryos were cultured ex ovo with improved viability and sustainability51. In brief, eggs after 48 h of incubation at 38.5 °C were prepared in sterile fashion and the embryo was directly transferred to a sterile autoclaved 60-mm diameter 35-mm depth crystallization dish by cracking the egg and allowing the albumin and yolk to fall gently into the vessel. Five millilitres of...
sterile PBS without Ca$^{2+}$/Mg$^{2+}$ containing 10% penicillin/streptomycin solution was added to prevent dehydration. The embryo was subsequently covered with a vented sterile lid and placed at 37.5°C. Electroporation was performed as described above. For live imaging on a confocal Axio examiner system (see below), a custom-heated stage top incubator (BioOptechs) was designed allowing for the insertion of a water dipping objective for continuous imaging while maintaining temperature, humidity and normal growth of chick embryos (Supplementary Fig. 1c).

For imaging with the Zeiss Axio Observer confocal system, mouse embryos or electroporated chick embryos were collected into imaging media (DMEM/F12 with HEPES without phenol red containing 10% heat-inactivated FBS, Invitrogen). Extraembryonic membranes were carefully removed and the entire embryo or the isolated forelimb was transferred and positioned on a 35-mm glass bottom culture dish containing a 14-mm German glass coverslip as its base (MatTek Corporation). A 12-mm coverslip was placed above the embryo secured on a ring of Vaseline that served to elevate the coverslip from the embryo, this placement was done to limit the movement of the limb bud during imaging. The chamber containing the embryo or limb bud was placed in a 37°C heated microscope incubator (Solent Scientific) and imaged as described below.

**Mice.** The mT/mG (ref. 53), ShhCreERT2 (ref. 54) and GliCreERT2 (ref. 55) mice were purchased from Jackson laboratories and maintained on C57BL/6j background. The mT/mG transgenic line is a double-fluorescent Cre reporter mouse that expresses tandem dimer Tomato (Electron Microscopy Sciences) before Cre-mediated excision and membrane-targeted enhanced green fluorescent protein (mG) after excision to mark defined populations of limb mesenchymal cells. For induction with tamoxifen, 4 mg of tamoxifen dissolved in corn oil (Sigma) was delivered via orogastric gavage at E9.5 between percentages of anterior and posterior orientation versus proximal and distal. For the comparison of vector orientations, assuming homogeneous distribution of variances and applying Student’s t-test, background fluorescent intensity values were obtained using Volocity 6.0 Quantification suite. These values were normalized to maximal intensity for the cell to account for differences in protein expression levels. Image presentations were generated in the Velocity 6.0 Visualization suite. For select multicolour confocal acquisitions, deconvolution using the calculated point spread function was applied with the Velocity 6.0 Restoration package.

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