The tips of mammalian digits can regenerate after amputation\(^1\)\(^-\)\(^4\), like those of amphibians. It is unknown why this capacity is limited to the area associated with the nail\(^5\)\(^-\)\(^8\). Here we show that nail stem cells (NSCs) reside in the proximal nail matrix and that the mechanisms governing NSC differentiation are coupled directly with their ability to orchestrate digit regeneration. Early nail progenitors undergo Wnt-dependent differentiation into the nail. After amputation, this Wnt activation is required for nail regeneration and also for attracting nerves that promote mesenchymal blastema growth, leading to the regeneration of the digit. Amputations proximal to the Wnt-active nail progenitors result in failure to regenerate the nail or digit. Nevertheless, β-catenin stabilization in the NSC region induced their regeneration. These results establish a link between NSC differentiation and digit regeneration, and suggest that NSCs may have the potential to contribute to the development of novel treatments for amputees.

Digit-tip regeneration in mice and humans involves the coordinated regrowth of the nail organ, including nail epithelial cells, and the terminal phalanx. After regrowth of the nail after amputation of the digit tip, undifferentiated mesenchymal cells, including fate-restricted progenitor cells\(^9\)\(^-\)\(^11\), accumulate under the wound epithelium and form the ‘blastema’. Growth and differentiation of these mesenchymal cells leads to digit regeneration. However, neither the nail nor the digit regenerate when the amputation is proximal to the nail\(^12\)\(^-\)\(^15\) (Supplementary Fig. 2), and it is not known why this limitation exists. Previous studies showed that nail transplantation after amputation at the middle phalanx can induce ectopic digit bone differentiation\(^16\), leading to a hypothesis that the nail epithelium has a special function in digit regeneration. Examination of this hypothesis may provide an understanding of why regeneration is limited to the nail-associated part of digits, and how epithelial cells can influence underlying mesenchymal cells to regenerate digit bone. The role of the nail epithelium in digit regeneration has remained elusive, partly owing to the lack of lineage and molecular analyses of normal nail epithelium.

To locate NSCs, we carried out lineage tracing using K14–Cre-ER; Rosa26\(^{flox\text{-Stop}}\)\(^-\)\(^{LacZ}\) reporter mice (in which the Cre recombinase–mutated oestrogen receptor (Cre-ER) is under the control of the keratin 14 (K14); also known as Krt14) promoter, and LacZ expression is driven by the Rosa26 promoter following Cre-mediated removal of the floxed stop cassette) (Fig. 1a). A single injection of tamoxifen genetically labelled a small subset of K14\(^+\) nail basal epidermal cells, including nail matrix cells and bed cells, with LacZ (Fig. 1b, c). Over time, descendants of the labelled K14\(^+\) nail epithelial cells extended linearly and distally, reflecting the direction of their growth (Fig. 1b). By 3 months after labelling, the number of LacZ\(^+\) colonies (which appeared as streaks) emanating from the distal part of matrix and the bed decreased significantly (Fig. 1d). In contrast, the streaks emerging from the proximal matrix persisted for at least 5 months (Fig. 1b, d). These streaks included the proximal matrix, distal matrix and bed cells (Fig. 1e). The progeny of both proximal matrix and distal matrix migrated vertically to produce individual keratinized layers of the nail plate\(^17\). These results show that the proximal matrix contains self-renewing NSCs that sustain nail growth. LacZ\(^+\) colonies in the nail fold, the epithelium surrounding the nail, were discontinuous from the streaks that produced the nail plate, suggesting that the nail fold did not contribute to the cells for nail growth (Supplementary Fig. 3).

Histological analyses revealed that proximal matrix cells possessed less undifferentiations, characteristic of undifferentiated epidermal cells (Supplementary Fig. 4). Immunohistochemistry with proliferation and epidermal differentiation markers\(^18\) found that proximal matrix cells containing NSCs were highly proliferative (Ki67\(^+\)) and expressed K17 in addition to K14 (Supplementary Fig. 4). Isolated proximal matrix cells, enriched with K14\(^+\)K17\(^+\) expression (Fig. 1f, g), showed the highest colony-forming ability in vitro, a general characteristic of epithelial stem cells (Fig. 1h–j).

To understand the molecular mechanisms underlying NSC differentiation, we generated a microarray of proximal matrix versus distal matrix. Most notably, the analyses revealed that proximal matrix cells enriched with NSCs downregulated the Wnt signalling pathway, which is known to regulate embryonic development of limb and nail organs\(^19\)\(^-\)\(^21\) as well as differentiation of epithelial and melanocyte stem cells\(^22\). Analyses using Wnt reporter mice showed that the Axin2–LacZ signal started from the distal part of the K17\(^-\) NSC region and persisted into the distal matrix, whereas the TOPGAL signal was seen in the K17\(^-\) distal matrix\(^18\). Although these two markers distribute differently\(^13\), both signals were absent in the proximal end of the nail matrix (Supplementary Fig. 5). In addition, Tcf1 (also known as hepatocyte nuclear factor 1x), a nuclear mediator of Wnt signalling\(^23\), and Wls (wntless homologue), required for Wnt ligand secretion\(^24\), were missing in the proximal end of the matrix. Moreover, several keratins that contained a Tcf1 and Lef1 consensus binding site were upregulated in the distal matrix compared with NSC region (Supplementary Table 1)\(^21\)\(^-\)\(^23\), suggesting direct involvement of Wnt signalling in nail differentiation.

To verify the role of Wnt activation in the nail epithelium, we deleted β-catenin, an essential mediator of Wnt signalling, in adult epithelium using K14–Cre-ER/β-catenin\(^{fl/fl}\) conditional knockout mice (Fig. 2a). By 2 months after induction of β-catenin deletion by tamoxifen treatment, nail formation is abrogated (Fig. 2b–e), as revealed by the lack of AE13, a marker for keratinized nail cells\(^25\) (Fig. 2f). Moreover, the entire nail epithelium showed characteristics of the NSC region (K17\(^+\)Ki67\(^+\)) (Fig. 2g–i). Similar defects were observed in another mouse model (K14–Cre-ER;Wntless\(^{fl/fl}\)) that depletes Wls in epithelial cells, confirming the essential role of Wnt signalling in nail differentiation (Supplementary Fig. 6).

Next, to determine how nail differentiation is linked to digit regeneration after amputation, we treated conditional knockout mice with tamoxifen, beginning immediately after digit amputation (Fig. 3a). We focused on digit bone regeneration to evaluate the completeness of the regenerative response, as muscle and tendon are absent at this amputation level\(^5\). In control mice, the nail resumed its original structure by 5 weeks after amputation (Fig. 3b), and the amputated digit bone

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regenerated along with nail regeneration (Fig. 3c–f). In conditional knockout mice, the nail failed to regenerate as expected, owing to the essential role of Wnt signalling in nail differentiation (Fig. 3b, e). Remarkably, bone regeneration in these mice was also blocked completely (Fig. 3c, d, f). Intact non-amputated digits in conditional knockout mice (internal control) maintained similar digit bone length compared with intact digits in control mice at 5 weeks after tamoxifen treatment (Fig. 3f).

Time-course studies showed that β-catenin was clearly depleted in nail epithelial cells of conditional knockout mice by 1 week after tamoxifen induction (Supplementary Fig. 7). Nevertheless, the amputated areas of both control and conditional knockout mice were similar re-epithelialized 2 weeks after amputation. In control mice, the regenerating nail matrix displayed Wnt activation with TOPGAL activity (Fig. 3g), contiguous with the original nail matrix cells, which permitted nail differentiation. Underneath the Wnt-active regenerating matrix, mesenchymal cells were actively proliferating (Fig. 3i). We identified that the majority (approximately 90%) of these proliferating cells express Runx2 (ref. 23), a marker for osteoblast commitment (Supplementary Fig. 8), supporting previous ideas that lineage-restricted progenitor cells contribute to the digit bone regeneration5,6. However, in conditional knockout mice Runx2+ progenitors and Sp7+ osteoblasts were not induced to proliferate, and the expression of Bmp4, which is critical for digit bone regeneration8, was missing in conditional knockout digits (Supplementary Fig. 8), supporting previous ideas that lineage-restricted progenitor cells contribute to the digit bone regeneration5,6. Moreover,
**Figure 2 | Epithelial β-catenin is required for nail differentiation.** 

a, Experimental scheme. Three-week-old K14-Cre-ER;β-catenin<sup>fl/fl</sup> mice and littermates were treated with Tam for 7 days, and analysed at 2 months after Tam treatment. 

b–e, Appearance under a dissecting microscope (b and d) and haematoxylin and eosin (H&E) staining (c and e) of control (b and c) and conditional knockout (d and e) digits. 

f–h, Immunofluorescence for indicated markers at 2 months after Tam treatment. 

i, Summary of immunohistology analysis of f–h. Dashed lines indicate the border between nail basal layer and connective tissue. Lines indicate the outline of nail plate (f–h). Asterisks show nonspecific background. Scale bars, 500 μm (b, c and f).

**Figure 3 | Nail epithelial β-catenin is required for blastema growth and digit regeneration.** 

a, Experimental scheme. Three-week-old K14-Cre-ER;β-catenin conditional knockout (cKO) mice and littermates were treated with Tam for 7 days immediately after distal-tip amputation, and analysed at the indicated time points. 

b, Whole-mount transparent specimen of a regenerated digit 5 weeks after amputation. 

c, Whole-mount alizarin red analysis. 

d, Trichrome staining. 

e, f, Quantitative analyses of the nail length and the bone length 5 weeks after amputation. 

g, Analysis of Wnt activation in regenerating nail epithelium using TOPGAL at 3 weeks after amputation. The lower panel is a schematic illustration of the upper panel. 

h, Quantitative analyses of the distance between nerve tip and wound epidermis and the innervations at 3 weeks after amputation. 

i, Proliferation analyses by Ki67 immunohistochemistry at 3 weeks after amputation. Red bars in h, right panel, indicate the averages. Dashed lines indicate the border between nail epithelium and connective tissue. Asterisks in part h, bottom panel, indicate autofluorescence from blood cells. 

Data are presented as the mean ± s.d. Scale bars, 500 μm (b–d); and 100 μm (h).

semaphorin 5a (Sema5a), an axon-guidance molecule<sup>26</sup>, is upregulated in control nail epithelium at 3 weeks after amputation, but not in that of conditional knockout mice (Supplementary Fig. 10). This may suggest that nerves are attracted to the paracrine factor (or factors) secreted from the Wnt-active nail epithelium, reminiscent of the ability of Wnt-active epithelium to attract nerves, as in the embryonic epidermis<sup>27</sup>. 

To investigate how Wnt-dependent innervations can promote digit regeneration, we removed nerves surgically before amputation. We then found a suppression of blastema growth similar to that in conditional knockout mice (Supplementary Fig. 11). Subsequent microarray analysis showed that fibroblast growth factor (FGF) signalling was significantly downregulated in denervated digits at 3 weeks after amputation when blastema grows in control digits (data not shown). This is particularly interesting, given the vital roles of FGF signalling during amphibian limb regeneration<sup>28</sup>. Immunostaining confirmed that FGF2 was induced in a distal area of regenerating nail epithelium by 3 weeks after amputation (Supplementary Fig. 12). In contrast, FGF2 was not expressed in the nail epithelium of denervated digits (Supplementary Fig. 12). Notably, conditional knockout mice that
showed deficient innervations in the blastema also lacked FGF2 expression in the nail epithelium (Supplementary Fig. 12). Quantitative polymerase chain reaction with reverse transcription (RT–PCR) revealed that FGF receptor 1 (Fgfr1) was expressed in the mesenchymal blastema rather than the regenerating nail epithelium (Supplementary Fig. 12). Consistent with this, phosphorylated ERK (pERK), a downstream mediator of FGF signalling, is detected in the Runx2+ mesenchymal cells of control digits, but not in that of denervated digits and conditional-knockout digits (Supplementary Fig. 12). Similar defects in innervations, FGF2 and pERK induction were observed after deletion of Wls in K14+ epithelial cells, causing failure in nail and digit regeneration (Supplementary Fig. 13).

To test the function of FGF2 signal within blastema, we collected blastema from control mice and allowed their outgrowth in vitro. Addition of FGF2 significantly enhanced the proliferation of blastema cells, whereas this effect is neutralized by RNA interference against Fgfr1 (Supplementary Fig. 14). After exposing blastema cells to bone differentiation media, alizarin red staining became positive, confirming their potential to differentiate into bone (Supplementary Fig. 14). In addition, implantation of FGF2-soaked beads into denervated digits in vivo induced proliferation of Runx2+ mesenchymal blastema, unlike that of control PBS-soaked beads (Supplementary Fig. 14).

The above results suggest that Wnt activation in the nail epithelium performs dual functions to promote both nail regeneration and Runx2+ mesenchymal cell growth through its ability to induce nerve-dependent FGF2 expression. We then asked why digits do not regenerate after amputations proximal to the nail (Supplementary Fig. 2). Careful examination of the amputated digits showed that amputations of the visible nail plate (that is, removal of more than 50% of distal phalanx) do not remove the entire NSC region, although they result in failure to regenerate⁸⁸ (Supplementary Fig. 15). Unlike distal amputations that induce regeneration, these amputations within the NSC region removed the distal matrix expressing Wntless that is required for initiation of Wnt signalling (Supplementary Fig. 15). Consequently, these amputations failed to activate epithelial Wnt signalling, as revealed by the lack of nuclear β-catenin and TCF1 expression after re-epithelialization (Fig. 4b, c), resulting in the failure to regenerate the nail and digit (Fig. 4g and Supplementary Fig. 2).

To test whether stabilization of β-catenin in K14+ epithelium, including the NSC region, can induce digit regeneration, we treated K14–Cre-ER;β-cateninfl/ex3 mice with tamoxifen after completion of re-epithelialization (Fig. 4a). One week after the initial tamoxifen treatment, basal nail epithelial cells, including the NSC region, exhibited nuclear β-catenin (Fig. 4b). In these tissues, NSC progeny expressed TCF1 as they regenerated distal matrix, whereas the proximal end of the NSC region does not express TCF1 (Fig. 4c). Although a transcriptional response to β-catenin stabilization was not evaluated directly, the spatially restricted pattern of TCF1 expression suggests that unidentified mechanisms may be present to cause the disparity in the activation of the pathway that acts downstream of β-catenin stabilization. Nevertheless, it was noteworthy that the regeneration of TCF1+ distal nail matrix in these mutant mice accompanied the formation of a well-innervated blastema, which is not observed in control mice after amputation at this proximal level (Fig. 4d). Consistent with this, we observed nail epithelial FGF2 expression and proliferating Runx2+ mesenchymal cells, leading to digit bone regeneration (Fig. 4e, f and Supplementary Fig. 16). In these mice, nail regeneration was also apparent and nails without amputations did not show any detectable changes (Fig. 4g, i and Supplementary Fig. 17). By contrast, when β-catenin stabilization was induced in K14+ skin epithelial cells after amputation proximal to the NSC region and subsequent re-epithelialization, neither TCF1 expression nor nail formation was observed (Supplementary Fig. 18). This suggests that the skin epidermis and NSCs respond differently after β-catenin stabilization, owing to differences within the intrinsic lineage and/or underlying mesenchyme. Notably, Runx2+ cells and Sp7+ cells were found in the mesenchyme

Figure 4 | Forced Wnt activation in wound epidermis can overcome the limitation of regeneration after proximal amputation. a, Experimental scheme. Three-week-old K14–Cre-ER;β-cateninfl/ex3 (mutant) mice and littermate controls were treated with Tam for 7 days starting from 2 weeks after amputation at the proximal level. b–i, Immunohistochemical analyses with indicated markers 3 weeks after amputation. g, Whole-mount transparent specimen of regenerated digits. h, Whole-mount alizarin red analysis. i, j, Quantification analyses of the nail (i) and bone length (j) 4 weeks after amputation. Red bars in d show the averages. Arrowheads in e and f, bottom panels, indicate TCF1+ proximal matrix and FGF2+ epidermis, respectively. Arrowheads in d point to nerves. Fine dotted lines in b and h indicate the amputation plane. Dashed lines indicate the border between epidermis and connective tissue. Quantified data are presented as the mean ± s.d. Scale bars, 100 μm (b–f); and 500 μm (g and h).

but did not show proliferative activity, resulting in the failure to regenerate the digit (Supplementary Fig. 18). These results show that the distally restricted capacity of digit regeneration is partly due to insufficient...
Wnt induced signals or mechanisms in the nail epithelium, rather than an inherent absence of cells competent to regenerate the digit bone.

By demonstrating the presence of NSCs that undergo Wnt-dependent differentiation into the nail, we have uncovered a unique role of the nail epithelium in digit-tip regeneration. Past studies in amphibians have documented the vital roles of Wnt and FGF signalling in promoting limb regeneration\(^{2,3}\). These studies were limited by their inability to control gene expression in specific cell populations. We used epithelium-specific gene modification and demonstrated the function of epithelial Wnt signalling in digit tip, to open a new avenue to dissect epithelial–mesenchymal interactions that drive organ regeneration in mammals.

The dual function of Wnt signalling in the NSC lineage to direct nail formation and digit regeneration seems to be a key mechanism that coordinates regeneration of epithelial and mesenchymal tissues in mammalian digit-tip regeneration (Supplementary Fig. 1). Further studies of mechanisms regulating NSCs and their interaction with mesenchymal cells may lead to new routes to treat amputees.

**METHODS SUMMARY**

All mice except \(\beta\)-catenin\(^{-/-}\) mice were obtained from the Jackson Laboratory, and maintained in the Smiłow Animal Facility at New York University (NYU). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the NYU School of Medicine. Cre recombination was induced by tamoxifen injection as described previously\(^{4}\). Digit amputation, denervation and bead implantation was carried out according to the method reported previously but with some modifications\(^{5}\). Histology and histochemistry were carried out on paraffin sections. For microarray analysis, basal cells of nail epithelium were isolated by fluorescence-activated cell sorting (FACS). Cells for colony-forming assays were obtained by microdissection followed by enzymatic digestion. Statistical analyses were carried out using Microsoft Excel.

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

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**Author Contributions** M.T. designed and carried out experiments, interpreted data and wrote the manuscript. W.C.C., P.R. and Q.S. performed experiments and interpreted data. M.M.T. generated \(\beta\)-catenin\(^{-/-}\) mice and interpreted the data. C.L. and W.L. performed the colony-forming assays. M.I. designed experiments, interpreted data and wrote the paper. Correspondence and requests for materials should be addressed to M.I. (Mayumi.Ito@nyumc.org).

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**Author Information** Expression information has been submitted to the Gene Expression Omnibus database under accession numbers GSE45484, GSM1105641, GSM1105642 and GSM1105643. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.I. (Mayumi.Ito@nyumc.org).
METHODS

Mice and sample collections. All mice, except β-cateninloxPlox mice, were obtained from Jackson Laboratories and maintained in the Smlow Central Animal Facility at the NYU Langone Medical Center. All animal protocols were approved by the IACUC at the NYU School of Medicine. Cre recombination in K14-Cre;ER, Rosa26loxStoploxLaZ (ref. 32), K14-Cre;ER-β-catenin b/b (ref. 33), K14-Cre;ER-β-catenin b/b and K14-Cre;ER-Wnt5aGFP (ref. 34) mice was induced by tamoxifen injection, as described previously4. For nail sample collections, we killed mice using CO2 narcosis, and collected the middle three digits of the hind limbs.

X-gal staining. Nail samples from K14-Cre;ER,Rosa26loxStoploxLaZ, TOPGAL35 and Axin2loxPlox mice were fixed in 4% PFA at 4 °C for 30 min, rinsed with PBS, and incubated in X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) solution as described previously. After photographing X-gal-stained whole-mount nail samples under a dissection microscope (Zeiss, Discovery V12.), nail samples were incubated in 30% sucrose at 4 °C overnight, embedded into OCT-compound (Sakura), and cut into 10-μm-thick frozen sections.

Immunohistochemistry. Nails were fixed in 10% buffered zinc formalin at 4 °C for 2 nights, and washed in PBS twice. After alcoholysis in 25% formic acid containing 20% sucrose for 2 h, nails were dehydrated through ethanol and xylene, embedded in paraffin, and cut into 6-μm sections. After dehydration, paraffin-sectioned tissues were processed in haematoxylin and eosin, or Masson’s trichrome stain. For immunohistochemistry, antigen retrieval was carried out by microwaving sections for 6 min on the high-heat setting in 1× Tris-EDTA buffer, pH 8.0. Sections were blocked in 10% fetal bovine serum (FBS) or PBS at room temperature for 1 h, then incubated with primary antibodies against K14 (1:500, Covance), K17 (1:500, Abcam), AE13 (1:50, a gift of T. T. Sun), Ki67 (1:50, Abcam), Ctnnb1 (1:400, Sigma), Tcf1 (1:50, Cell signalling), Runx2 (1:100, Sigma), Sp7 (1:100, Santa Cruz) acetylated tubulin (1:500,Sigma), FGF2 (1:100, Santa Cruz), ERK (1:100, Cell signalling; 1:20, Abcam) and MSX1 (1:20, Abcam) overnight, and then incubated with fluororescin conjugated, or biotinylated secondary antibodies at room temperature for 2 h. For biotinylated secondary antibodies, a third amplification step with streptavidin-conjugated TRITC (1:200, Vector) or Horseradish peroxidase (HRP, 1:500, Upstate) was carried out. A diamnobenzidine (DAB) substrate solution (Sigma) was used for developing signals for horseradish peroxidase. All antibodies were diluted in 0.1% Triton-X 100 or PBS.

Transmission electron microscopy. Samples were fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde, and 2% paraformaldehyde for 2 h at room temperature and 4 °C overnight. After post-fixation in 1% osmium tetroxide for 1.5 h at room temperature, samples were prepared using standard methods and embedded in Embed 812 (Electron Microscopy Sciences). Semi-thin (1-μm) sections were cut and stained with 1% toluidine blue to evaluate the quality of preservation. Ultra-thin (60-nm) sections were cut, mounted on copper grids and stained with uranyl acetate and lead citrate. Stained grids were examined under a Philips CM-12 electron microscope (FEI) and photographed with a Gatan (4k × 2.7k) digital camera (Gatan).

Whole-mount visualization of digit bone. Nails were fixed in 4% PFA at 4 °C overnight. After washing in 1% KOH in H2O, digits were incubated serially in 20% glycerol contains 1% KOH for 3–6 h at room temperature, 50% glycerol contains 1% KOH for 4–16 h at room temperature and 100% glycerol overnight at room temperature.

Immunocytochemistry. Dissociated cells were resuspended in 1% FBS or PBS and spun onto glass slides using Cytospin 3 (Shandon). The slide was fixed with acetone at −20 °C for 10 min. After washing in 1× PBS, slides were blocked in 10% FBS or PBS at room temperature for 1 h, then incubated with primary antibodies against K14 (1:500, Covance) at 4 °C overnight, followed by incubation with AlexaFlor 488 conjugated secondary antibody at room temperature for 2 h. After washing in 1× PBS, slides were incubated with primary antibodies against K17 (1:5000, Abcam) at 4 °C overnight, and biotinylated secondary antibodies at room temperature for 2 h, and then with streptavidin-labeled tetramethyl rhodamine isothiocyanate (SA-TRITC) (1:200, Vector) at room temperature for 1 h. Primary antibodies were diluted in 10% FBS or PBS, and secondary antibodies were diluted in PBS.

Colonay-forming assay. Thirty nails from at least five different mice (8- to 10-week-old FVB mice) were collected, and the nail fold overhanging the nail plate was then removed with surgical blades and forceps under a dissection microscope. Dissected fragments were incubated in 0.25% Trypsin for 1 h 45 min at 37 °C, and then in 0.35% Collagenase I and DNAse I for 10 min each at 37 °C. Dissociated cells were resuspended in DMEM or 10% FBS. The percentage of K14+ cells was then determined by cytospin analysis as described below. Cell suspensions containing 1 × 104 K14+ cells were cultured with NIH or 3T3 feeder layers (a gift from A. Mansukhani) in F10:DMEM (1:3) media with 10% new-born calf serum in six-well plates. After 14 days in culture, cells were fixed with 10% buffered formalin and stained with 1% rhodamine B. The number of colonies was counted manually and the size of the colonies was measured using image analysis software (Image J, NIH), and colony-forming efficiency (the number of colonies larger than 3 mm2 per 1 × 104 cells) was calculated. Studies were carried out three times independently.

Gene-expression profiling of NSCs by microarray analysis. Seven- to eight-week-old K14–rtTA:TetO-H2B–GFP mice (Jackson Laboratory) were treated with doxycycline for 7 days to label the entire K14+ matrix cells with green fluorescent protein (GFP). Thirty digits from at least five different mice were collected and single-cell suspensions were prepared as described above. The cells were incubated with APC-conjugated anti–CD49f antibody in 1% FBS or PBS for 15 min at room temperature. Basal nail epithelial cells from each fraction were isolated using FACS based on the GFP label, representing K14 positivity, and expression of CD49f, a general marker of basal cells. To obtain sufficient cells for oligonucleotide gene chip hybridization, we used the Ovation RNA Amplification System V2 (Nugen) for messenger RNA amplification. The amplified mRNA was labelled and hybridized to the Mouse 430.2 microarrays (Affymetrix). Data were analysed with GeneSpring X software, and genes that were regulated differentially at least two-fold were selected for further analysis.

Digit amputation. Digit amputation was carried out according to a method reported previously, but with some modifications. In brief, the central three digits (digits 2, 3 and 4) of hind limbs of 21-day-old mice were amputated at the level of the nail matrix or in the NSC area. Amputated digits were collected at 1, 2, 3 and 5 weeks after amputation, and processed for Alucian blue or Alizarin red, or by immunohistochemistry. More than 10 different digits from 5 mice were used for each time point. Studies were repeated three times.

In situ hybridization. Digoxigenin-labelled RNA probes complementary to Bmp4 (a gift of M. Han and K. Muneoka) were synthesized according to the manufacturer’s instructions (DIG-RNA Labelling Kit, Roche). In situ hybridization was carried out using a method described previously. Studies were repeated three times.

Blastema cell culture and bone-differentiation assays. The digit tip proximal to the terminal phalanx was collected 3 weeks after digit amputation. Mesenchymal blastema cell mass was separated from the nail epidermis by sine forceps and a needle under a dissecting microscope. Isolated blastema cell mass was placed in 24-well plate with DMEM (invitrogen) or 10% FBS (Cellgro), and incubated at 37 °C, 5% CO2. After 1 week in culture, blastema cells were transfected with 50 nM short interfering RNA (siRNA) targeting FGFR1 (Invitrogen, Mss202494 and Mss202495), or a control siRNA, using LipoLNEF (Roche) for RNAiMAX (Invitrogen). Transfected cells were incubated in DMEM (invitrogen) or 10% FBS (Cellgro) with or without 20 ng ml−1 FGFR2 (Sigma–Aldrich) at 37 °C, 5% CO2, for 2 days, and were stained for Ki67 as described above. For bone-differentiation assays, culture media was replaced with HyClone Advance STEM Osteogenesis differentiation medium (Thermo Scientific) after 7 days in culture. After 3 weeks in culture, mineralization was assessed by alizarin red staining. In brief, the cultures were fixed in 10% Zinc buffered formalin at room temperature for 10 min, washed in PBS twice, and stained with 2% alizarin red S (Sigma) in distilled water for 5 min at room temperature. The stained cell layers were washed, rinsed twice with distilled water, and air dried.

Bead implantation. We carried out bead-implantation experiments using a method described previously, but with the following modifications. In brief, Affi-Gel Blue Gel beads (Bio-Rad) were washed with 0.1% BSA or PBS then soaked with recombinant human FGF2 (Sigma) at a concentration of 0.3 mg ml−1 or 0.1% BSA/PBS as a control for 2 h at room temperature. Bead implantation was performed at 2 weeks after digit amputation, after the completion of wound closure was confirmed.

Statistical analysis. Student’s t-test was used to calculate P values on Microsoft Excel, with two-tailed tests and unequal variance.

31. Harada, N. et al. Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. EMBO J. 18, 5931–5942 (1999).
32. Vasioukhin, V., Degenstein, L., Wise, B. & Fuchs, E. The magical touch: genome targeting in epithelial stem cells induced by tamoxifen application to mouse skin. Proc. Natl. Acad. Sci. USA 96, 8551–8556 (1999).
33. Lowry, W. E. et al. Defining the impact of β-catenin/Tcf transactivation on epithelial stem cells. Genes Dev. 19, 1596–1611 (2005).
34. Myung, P. S., Takeo, M., Ito, M. & Atit, R. P. Epithelial Wnt ligand secretion is required for adult hair follicle growth and regeneration. *J. Invest. Dermatol.*, (2013).

35. DasGupta, R. & Fuchs, E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557–4568 (1999).

36. Lustig, B. et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol. Cell. Biol.* **22**, 1184–1193 (2002).

37. Barrandon, Y. & Green, H. Three clonal types of keratinocyte with different capacities for multiplication. *Proc. Natl Acad. Sci. USA* **84**, 2302–2306 (1987).

38. Yu, L. et al. BMP signalling induces digit regeneration in neonatal mice. *Development* **137**, 551–559 (2010).