A slow-cycling LGR5 tumour population mediates basal cell carcinoma relapse after therapy

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Basal cell carcinoma (BCC) is the most frequent cancer in humans and results from constitutive activation of the Hedgehog pathway1. Several Smoothened inhibitors are used to treat Hedgehog-mediated malignancies, including BCC and medulloblastoma2. Vismodegib, a Smoothened inhibitor, leads to BCC shrinkage in the majority of patients with BCC3, but the mechanism by which it mediates BCC regression is unknown. Here we used two genetically engineered mouse models of BCC1 to investigate the mechanisms by which inhibition of Smoothened mediates tumour regression. We found that vismodegib mediates BCC regression by inhibiting a hair follicle-like fate and promoting the differentiation of tumour cells. However, a small population of tumour cells persists and is responsible for tumour relapse following treatment discontinuation, mimicking the situation found in humans5. In both mouse and human BCC, this persisting, slow-cycling tumour population expresses LGR5 and is characterized by active Wnt signalling. Combining Lgr5 lineage ablation or inhibition of Wnt signalling with vismodegib treatment leads to eradication of BCC. Our results show that vismodegib induces tumour regression by promoting tumour differentiation, and demonstrates that the synergy between Wnt and Smoothened inhibitors is a clinically relevant strategy for overcoming tumour relapse in BCC.

Vismodegib (GDC0449) is the first Smoothened inhibitor to be approved for the treatment of locally advanced and metastatic BCC. A small fraction of patients does not respond to vismodegib administration: their tumours continue to grow and do not show inhibition of the Hedgehog (Hh) signalling pathway during vismodegib treatment2. This type of vismodegib resistance is frequently associated with genetic mutations that render vismodegib unable to inhibit the Hh pathway6,7. Most patients treated with vismodegib experience clinical benefits2. However, many patients respond only partially: their tumours initially regress under therapy but relapse after vismodegib discontinuation3,5. The mechanisms by which vismodegib induces tumour regression and that underlie non-genetic resistance to vismodegib therapy are unknown.

To study the mechanisms by which vismodegib leads to BCC regression, we induced BCC in mice by deleting Ptch1 or overexpressing the constitutive active form of Smo (SmoM2) in the epidermis using Krt14-CreER8,9. BCCs induced by conditional knockout of Ptch1 (Ptch1Ko) arise mainly from the upper hair follicle (infundibulum) whereas those induced by SmoM2 originate from the interfollicular epidermis (IFE)10,11. Eight weeks after deletion of Ptch1 by tamoxifen administration, mice showing fully formed BCCs were treated daily with vismodegib and analysed at different time points (Fig. 1a). A decrease in tumour burden was observed during the first 5 weeks of vismodegib treatment, followed by stabilization of tumour size from 5 to 12 weeks, together with the appearance of vismodegib-resistant lesions (Fig. 1b, c, Extended Data Fig. 1a–d). Vismodegib administration led to the conversion of the BCCs into pre-neoplastic lesions (hyperplasia and dysplasia), which persisted as drug-tolerant lesions (Fig. 1d, Extended Data Fig. 1e). These results show that vismodegib induces tumour shrinkage and the progressive appearance of drug-tolerant lesions.

Staining for active caspase-3 two weeks after vismodegib administration showed a similar number of apoptotic cells in treated and untreated mice (Fig. 1e, f, Extended Data Fig. 1f, g), indicating that apoptosis is not the main mechanism by which vismodegib induces BCC regression. As quiescence has been described as a mechanism of cancer resistance to therapy12, we assessed the proportion of Ki67+ positive tumour cells and observed a strong decrease in the proportion of proliferative cells in persistent lesions (Fig. 1g, h, Extended Data Fig. 1h, i), suggesting that quiescence contributes to the emergence of drug-tolerant cells.

Lgr5 is expressed by different epithelial stem cells, including hair follicle stem cells (HFSCs)13, and is upregulated during BCC initiation4. In situ hybridization (ISH) showed that Lgr5 was highly expressed in untreated BCCs and its expression persisted, albeit at a lower level, in vismodegib-tolerant lesions (Fig. 2a, Extended Data Fig. 2b).

ISH for Gli1, a transcription factor that relays Hh signalling and a Hh target gene, demonstrated that Gli1 was co-expressed with Lgr5 before treatment and was strongly downregulated in all tumour cells upon vismodegib treatment (Fig. 2a–c, Extended Data Fig. 2b–d), consistent with the strong inhibition of Hh signalling by vismodegib. Drug-tolerant lesions did not present mutations in Smo, the most frequently mutated gene in vismodegib-resistant BCCs5 (Extended Data Fig. 2e), reinforcing the notion that the persistence of drug-tolerant lesions is not mediated by mutations that abrogate vismodegib sensitivity, as it occurs in vismodegib-resistant BCCs that continue to grow during treatment6,7.

Relapse of BCC upon vismodegib discontinuation has been reported in human patients4. Discontinuation of vismodegib administration for 4 weeks in Krt14CreER;Ptch1Ko;Lgr5DTR–GFP mice12 bearing drug-resistant persistent lesions led to the re-growth of BCCs to their pre-treatment size. Moreover, re-administration of vismodegib to mice with relapsing BCCs led to tumour regression (Fig. 2d).

To determine whether the quiescent tumour cell population mediates tumour relapse, we performed BrdU pulse-chase label retention studies by administering BrdU for 3 days in mice with BCC to label proliferative cells, and then monitored the labelling during 5 weeks of vismodegib treatment. We found BrdU label-retaining cells (LRCs) in LGR5+ drug-tolerant lesions, suggesting that persisting tumour cells existed before vismodegib treatment and underwent a phenotype switch from a proliferative to a quiescent state (Fig. 2e, f). Upon discontinuation of vismodegib, relapsed tumours lost the LRCs (Fig. 2e, f), suggesting that quiescent LRCs actively proliferated, diluting the BrdU. To test this possibility directly, we performed BrdU–EdU double-labelling studies. Administration of EdU during vismodegib discontinuation led to EdU incorporation in the majority of the LGR5+ LRCs (Fig. 2g).
resting HFSC signature\(^{16}\) (16.4\%) and LGR5\(^+\) hair follicle signature\(^{17}\) (44.2\%) (Fig. 3a, Extended Data Fig. 3a). The LGR5\(^+\) BCC signature included genes downstream of the HH signalling pathway, such as Ptch1, Ptch2 and Hhip, genes involved in the Wnt signalling pathway, such as Lgr5, Fzd2 and Lef1, transcription factors expressed by EHFPs, such as Runx1 and Lhx2, and genes expressed by HFSCs, such as Tbx1 and Foxc1 (Extended Data Fig. 2b). Immunostaining for LEF1, LHX2, CUX1, TBX1, and ALCAM in Ptch1\(^{K0}\)-induced BCCs confirmed the increased expression of these Wnt signalling, EHF and HFSC markers in LGR5\(^+\) tumour cells (Extended Data Fig. 3c).

To assess whether the LRG5\(^+\) LRIG1\(^+\) population represents a differentiated part of the BCC, we defined genes that were upregulated in LRG5\(^+\) LRIG1\(^+\) tumour cells compared to LGR5\(^+\) LRIG1\(^+\) tumour cells (LRG5\(^+\) signature). Notably, the LRG5\(^+\) signature overlapped significantly with previously reported LRIG1\(^+\) and IFE\(^{16}\) signatures, including markers of IFE or infundibulum differentiation such as Oval1, Notch3, Defb6, Krt1, and Krt10 (Extended Data Fig. 3d, e). PCR analysis performed on FACS-isolated LGR5\(^+\)LRIG1\(^+\) and LGR5\(^+\) LRIG1\(^+\) tumour cells confirmed that both populations had Ptch1 deletion, and staining for the proliferation marker Ki67 showed that the LRG5\(^+\) LRIG1\(^+\) population was more proliferative than the LGR5\(^+\) LRIG1\(^+\) population (Extended Data Fig. 3f, g).

To directly assess whether LGR5\(^+\) LRIG1\(^+\) cells were more differentiated than LGR5\(^+\) LRIG1\(^+\) cells, we performed transplantation assays of FACS-isolated tumour cell populations from Krt14\(^{CreER}\);Ptch1\(^{K0}\), Lgr5\(^{GFP}\);Lgr5\(^{TGR-GFP}\) and Krt14\(^{CreER}\);Ptch1\(^{K0}\);Trp53\(^{K0}\);Lgr5\(^{TGR-GFP}\) mice, which grow faster and form bigger tumours\(^{19}\). Groups of cells resembling early BCC and expressing KRT14, LGR5 and LRIG1 were observed only upon transplantation of LGR5\(^+\)LRIG1\(^+\) cells from Trp53\(^{K0}\)BCCs (three in out of seven mice). By contrast, no tumour cells were observed following the transplantation of LGR5\(^+\) LRIG1\(^+\) cells from Trp53\(^{K0}\)BCCs or in the absence of Trp53 deletion (Extended Data Fig. 4a, b). Tumours found after transplantation of LGR5\(^+\) LRIG1\(^+\) cells mimicked the different cell types present in BCCs: LGR5\(^+\) LRIG1\(^+\), LGR5\(^+\) LRIG1\(^+\) and cells with a flat differentiated morphology expressing keratin-10 (KRT10) (Extended Data Fig. 4b, c). Together, these results show that BCCs contain a more stem-like or progenitor-like tumour cell population (LGR5\(^+\) LRIG1\(^+\)) and a more differentiated population (LGR5\(^+\) LRIG1\(^+\)) of tumour cells. Immunostaining for the primary cilia marker ARL13B and the coactivator MKL1 showed that neither loss of primary cilia\(^{19}\) nor serum response factor (SRF)–MKL1 activation\(^{16}\) is involved in the drug-tolerant phenotype described here (Extended Data Fig. 5a–d).

To define the molecular mechanisms by which vismodegib promotes tumour shrinkage and appearance of drug-tolerant lesions, we compared the transcriptional profiles of FACS-isolated LGR5\(^+\) LRIG1\(^+\) and LGR5\(^+\) LRIG1\(^+\) tumour cells from untreated BCCs and mice that received vismodegib for 8 weeks. We found that the overlap between the LGR5\(^+\) LRIG1\(^+\) signature and the EHFIP\(^{15}\), LGR5\(^+\) hair follicle signature\(^{17}\) and resting HFSC\(^{16}\) signatures was considerably lower in vismodegib-treated cells than in untreated cells (Fig. 3a, b). Vismodegib treatment induced a strong decrease in the expression of Hh target genes such as Gli1, Gli2, Ptch1, Ptch2 and Hhip (Fig. 3c). Only a small part of the reduction in overlap between the vismodegib-treated and EHFIP signatures was driven by Hh target genes such as Hhip1, Ptch2 and Gli1, and the reduction in overlap between the HFSC and vismodegib-treated signatures was not mediated by Hh target genes as the HFSC signature was obtained in the resting state, when Hh signalling is not active\(^{16}\). Genes found in the EHF and HFSC signatures, such as Runx1, Lhx2, Lgr5, Alcam, and Tbx1 were also downregulated following vismodegib administration at the mRNA and protein levels (Fig. 3c and Extended Data Fig. 6a).

The overlap between the LGR5\(^+\) LRIG1\(^+\) signature and the infundibulum\(^{16}\) and IFE\(^{16}\) signatures increased considerably upon vismodegib treatment, with genes such as Oval1, Notch3, Plet1, Defb1, Defb6, Krt1 and Krt10 being strongly upregulated after vismodegib treatment (Fig. 3d–f, Extended Data Fig. 6b), indicating that vismodegib...
promotes the differentiation of BCC into IFE- and infundibulum-like cells, possibly through a Notch-dependent mechanism. LIRGI+ stem cells give rise to infundibulum and sebaceous gland under homeostatic conditions. We performed staining for sebaceous gland markers (SCD1 and adipophilin) and lipids (Oil Red O). Whereas sebaceous cysts were visible in the dermis under untreated conditions, cells expressing sebaceous gland markers were localized within the tumour mass after two weeks of vismodegib treatment and adjacent to the neoplastic lesions after five or eight weeks of treatment (Fig. 3g). We studied the expression of KRT10 and Defensin-36 (Defb6), which are normally expressed in infundibulum and IFE cells. Upon vismodegib administration, KRT10 and Defb6 were strongly upregulated in tumour cells (Fig. 3h), consistent with vismodegib inducing tumour differentiation towards a sebaceous gland/infundibulum/IFE-like fate in Ptch1KO-derived BCCs.

We then assessed whether LGR5+ also promotes differentiation of BCC into IFE in SmoM2-induced BCC. Upon vismodegib administration, SmoM2-expressing cells connected to normal differentiating IFE cells expressed high levels of the IFE differentiation marker keratin-1 (KRT1) (Extended Data Fig. 5f). We studied the effect of vismodegib administration on the survival and morphology of the SmoM2 clones during BCC initiation. Two weeks after SmoM2 expression, mice were treated daily with vismodegib for six weeks (Extended Data Fig. 7a). Vismodegib administration led to a progressive loss of SmoM2-expressing clones in comparison to untreated conditions and to the emergence of clones with normal differentiation, with only a small proportion of the clones progressing into hyperplasia and dysplasia (Extended Data Fig. 7b–d). The normally differentiated clones observed during vismodegib treatment were positive for the differentiation marker KRT10 but did not express LHX2, an HFSC marker that is found in hyperplasias and dysplasias (Extended Data Fig. 7e, f), indicating that vismodegib administration inhibits oncogene-induced hair follicle reprogramming, promotes differentiation of SmoM2-expressing cells into an IFE-like fate and prevents BCC initiation.

To assess whether LGR5+ tumour cells consist of heterogeneous populations in terms of proliferation and differentiation, we isolated LGR5+ LIRGI+ tumour cells on the basis of expression of the proliferation marker CD7110 two weeks after vismodegib administration, when both persistent cells and cells that are responsive to vismodegib co-exist. The CD71+ population expressed higher levels of proliferation (Ki67 and Aurka) and differentiation markers (Krt1, Krt10 and...
Scd1) (Extended Data Fig. 7g), indicating that the more proliferative tumour cells are more prone to vismodegib-induced differentiation.

Immunostaining for the differentiation marker KRT10 in LGR5⁺ tumour cells after BrdU label-retention followed by two weeks of vismodegib administration showed that the majority of BrdU-labelled cells were negative for KRT10, whereas KRT10 was observed in non-LRCs or in LRCs in which the BrdU signal was lower owing to its dilution following cell division (Extended Data Fig. 7h). These results support the notion that vismodegib induces a higher rate of differentiation in the drug-responsive tumour population that actively cycles.

To determine the relevance of our findings to human patients, we analysed biopsies from four patients with locally advanced BCCs before, during or immediately after discontinuation of vismodegib treatment. Vismodegib did not eradicate all tumour cells in these patients, and small tumorigenic lesions expressing LGR5 persisted despite the administration of vismodegib for months (Extended Data Fig. 8a–c). ISH for GLI1 and quantification of GLI1 mRNA dots per tumour cell before, after or during vismodegib treatment showed that there was almost no GLI1 expression in samples from patients during vismodegib treatment but few more GLI1-expressing cells were found shortly after discontinuation of vismodegib treatment (Extended Data Fig. 8d, e), indicating that vismodegib administration efficiently inhibits Hh signalling in these drug-persistent lesions. Ki67 immuno-histochemistry showed that vismodegib-persistent lesions were more quiescent than untreated BCCs, and vismodegib induced the expression of the differentiation marker KRT10 in human tumour cells (Extended Data Fig. 8e, f). Notably, patients 1 and 2 relapsed 6 and 9 months after treatment discontinuation, respectively, and patient 4 had previously relapsed after vismodegib discontinuation, showing that vismodegib-mediated tumour cell persistence is fully reversible upon drug withdrawal and re-inducible upon a new cycle of vismodegib treatment (Extended Data Fig. 8a). Together, these results show that drug-tolerant lesions exist in human BCC, characterized by the expression of LGR5 and relative quiescence.

To assess whether LGR5+ cells mediate tumour growth, we lineage-ablated LGR5+ tumour cells by administering diphtheria toxin for 10 days to Krt14CreER;Ptch1 cKO;Lgr5 DTR–GFP mice and for 15 days to Krt14CreER;Rosa26;Lgr5 DTR–GFP mice (Extended Data Fig. 9a). Diphtheria toxin treatment could not be extended because LGR5 deletion is toxic to normal liver cells. Diphtheria toxin administration led to a substantial elimination of the tumour mass in both BCC models (80% of the initial tumour mass) and to almost total elimination of LGR5-expressing cells in Ptch1 cKO-induced BCC (Extended Data Fig. 9b–g), further demonstrating the importance of LGR5+ tumour cells to sustain BCC growth and maintenance.

To determine whether vismodegib administration together with Lgr5 lineage ablation can eliminate the LGR5-expressing drug-tolerant lesions that are responsible for tumour relapse, we administrated diphtheria toxin for five consecutive days in combination with vismodegib to Krt14CreER;Ptch1 cKO;Lgr5 DTR–GFP mice bearing persistent lesions (Extended Data Fig. 9h). Lgr5 ablation combined with vismodegib administration led to almost total (99.5%) elimination of the persistent LGR5-expressing tumour cells (Extended Data Fig. 9i–k). We did not observe reappearance of LGR5+ cells from the vast majority (94%) of the initial LGR5+ persistent tumorigenic lesions 15 days after discontinuation of treatment with diphtheria toxin and vismodegib (Extended Data Fig. 9i, k, l), whereas HFSCs were replenished by LGR5-expressing cells as previously reported, indicating that there is little plasticity within the LGR5+ LRG1+ BCC cells to revert to LGR5+ tumour cells after treatment with diphtheria toxin and vismodegib. The therapeutic benefit of Lgr5 ablation in BCC is reminiscent of the effect of Lgr5 ablation in a mouse model of colorectal cancer, in which Lgr5 ablation prevents metastasis, and in human colorectal cancer organoids, in which Lgr5 ablation promotes tumour regression and synergises with chemotherapy.

Lgr5 has been identified as a Wnt target gene, and acts as a co-receptor for Krt14CreER, positively regulating the Wnt signalling pathway.

Administration of vismodegib decreased but did not abolish the expression of different members of the Wnt signalling pathway (Fig. 3c). Immunostaining for LEF1, a transcription factor that relays Wnt signalling to the nucleus, showed that both LEF1 and Axin2 increased in LGR5+ persistent lesions. Administration of vismodegib decreased but did not abolish the expression of different members of the Wnt signalling pathway (Fig. 3c).

To assess whether dual Wnt and Hh inhibition can promote the elimination of LGR5+ persistent tumour cells, we administered LGK-974, a porcupine Wnt inhibitor25, and vismodegib for 10 consecutive days to Ptch1 cKO mice bearing LGR5+ persistent lesions (Extended Data Fig. 4c). Combined Wnt and Hh inhibition resulted in the disappearance of LEF1 expression consistent with efficient Wnt inhibition, the elimination of the vast majority (93%) of initial LGR5+ drug-tolerant lesions and a substantial (87%) decrease in tumour burden compared to vismodegib treatment alone (Fig. 4d–f). We found no significant reduction in tumour burden after administration of the Wnt inhibitor alone, showing that although Wnt inhibition can block BCC initiation, it is not sufficient as a monotherapy to induce clinically relevant BCC regression (Extended Data Fig. 10d–f). We then investigated whether rare residual tumour cells could lead to tumour relapse upon discontinuation of dual Wnt and Hh inhibition. Four weeks after discontinuation, which corresponds to the time that it takes for drug-tolerant lesions to regrow to their initial size upon vismodegib discontinuation, no tumour relapse was observed, as shown by the stable number of LGR5+ tumour lesions and tumour burden (Fig. 4d–f).
Together, these results show that the synergy between Hh and Wnt inhibition in BCC leads to the elimination of the vast majority of LGR5+ persistent tumour cells and thereby prevents tumour relapse.

In summary, we have shown that vismodegib induces BCC regression by promoting tumour differentiation and have identified a quiescent tumour cell population expressing LGR5 that persists after vismodegib treatment in different mouse models and human patients, promoting BCC relapse upon treatment discontinuation (Extended Data Fig. 11). The non-genetic mechanism of drug resistance described here differs from the previously described mutations in Smo or other genes that render cells insensitive to vismodegib treatment6,19,20. Administration of vismodegib promotes a switch from a proliferative state that fosters tumour growth to a tumour state characterized by Hh inhibition and slow-cycling properties that is fully reversible upon drug withdrawal and re-inducible upon a new cycle of vismodegib treatment. These persistent LGR5+ tumour cells present residual Wnt signalling activity in both mouse and human BCCs and could be eliminated by dual Wnt and Hh inhibition, leading to tumour eradication in the majority of BCCs (Extended Data Fig. 11). Dual Wnt and Hh inhibition constitutes a clinically relevant strategy to avoid BCC relapse that might also be effective against other cancers, such as medulloblastoma, that are characterized by activation of Hh and Wnt signalling26

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0603-3.

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Competing interests C.B. is a consultant at Genentech (San Francisco, USA).

Additional information
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METHODS

Ethical compliance. This study complied with all relevant ethical regulations regarding experiments involving mouse and human skin samples. Mouse colonies were maintained in a certified animal facility in accordance with European guidelines. Experiments involving mice presented in this study were approved by the ethics committee of Vall d'Hebron Institute of Oncology (VHIO) and by the ethics committee of Hôpital Erasme under protocol number P2012/332. Permission and informed consent were obtained from all the patients in order to use their biopsies in this study.

Mice. Krt14CreER;Ptch1fl/fl;Lgr5DTR–GFP transgenic mice were kindly provided by E. Fuchs, Rockefeller University, USA. Ptk1f/f;mice and RosaSmad2-YFP mice were obtained from the JAX repository. Lgr5DTGR-GFP mice (knockin mice that contain the diphtheria toxin receptor (DTR) fused to an enhanced green fluorescent protein (GFP) under the control of the Lgr5 regulatory region, allowing us to identify LGR5-expressing cells using the GFP reporter and to selectively ablate Lgr5 tumour cells by diphtheria toxin (DT) administration) were kindly provided by Genentech (San Francisco, USA). Tp53fl/fl/mice were obtained from the National Cancer Institute at Frederick. Female and male animals were used for all experiments and equal gender ratios were used in the majority of the analysis. Analysis of the different mutant mice was not blind and sample size was calculated to reach statistical significance. The experiments were not randomized.

Tumour induction. For Ptk1f/fKO deletion, Krt14f/f;Ptch1f/f;Lgr5DTGR-GFP mice and Krt14f/f;Ptch1f/f;Tp53f/f;Lgr5DTGR-GFP mice (1.5 months old) received one intraperitoneal injection of 2.5 mg tamoxifen on each of three consecutive days. For SmoM2 expression, Krt14f/f;RosaSmad2-YFP;Lgr5DTGR-GFP mice (1.5 months old) received one intraperitoneal injection of 1 mg tamoxifen. In the clonal induction experiments, Krt14f/f;Ptch1f/f;Lgr5DTGR-GFP mice (1.5 months old) received one intraperitoneal injection of 0.1 mg tamoxifen.

Vismodegib and LGK-974 administration. Vismodegib (GDC-0449) was kindly provided by Genentech (San Francisco, US) and LGK-974 was kindly provided by Novartis (Bâle, Switzerland). During vismodegib treatment, mice received 150 mg/kg vismodegib by oral gavage daily. Vismodegib was administered in two doses (one every 12 h).

During the 10-day LGK-974-treatment mice received: on each of the first six days, 10 mg/kg LGK-974 by oral gavage; and on each of the last four days one topical application of 100 μl of 2 mg/ml LGK-974 diluted in propylene glycol: ethanol (7:3, v/v). Either orange or blue LGK-974 and vismodegib were dissolved in 0.5% methylcellulose solution containing 0.2% Tween-80.

TPA and retinoic acid administration. TPA and retinoic acid (RA) were used to promote epidermal proliferation11,12. TPA (200 μl of 0.02 mg/ml solution in dimethyl sulfoxide) or retinoic acid (200 μl of 0.5 mM all-trans-RA (Sigma) in dimethyl sulfoxide) was administered daily to shaved mouse back skin for 2 weeks.

Diphtheria toxin administration. For Lgr5 lineage cell ablation, mice received a daily intraperitoneal injection of 50 μg/kg diphtheria toxin (Sigma).

Immunostaining in sections. The tail for the SmoM2 model and ventral skin or back skin for Ptch1f/fKO model were embedded in optimal cutting temperature compound (OCT, Sakura) and cut into 5–8 μm frozen sections using a CM3050S Leica cryostat (Leica Microsystems). Samples were fixed for 30 min in 4% PFA at 4°C and the in situ protocol was performed according to the manufacturer’s instructions (Advanced Cell Diagnostics). The following mouse probes were used: Mm-Lgr5 cat. No. 310991C2, Mm-Gli1 cat. No. 311001C2, and Mm-Smome2 cat no. 430141C3.

Human samples were fixed in 4% formalin and embedded in paraffin. Cut sections were deparaffinized and rehydrated before proceeding to the in situ hybridization, which was performed according to the manufacturer's instructions. The following mouse probes were used: Hs-Lgr5 cat. No. 310991C2, Hs-Lgr5 cat. No. 310102 and Hs-Axin2 cat no. 400241C3. A confocal microscope (LSM-780, Carl Zeiss) and ZEN 2.3 software were used to acquire and analyse the ISH images.

Immunohistochemistry. For Krt14, Ki67, KRT10 and Lef1 immunohistochemistry in human samples, paraffin sections were deparaffinized and rehydrated, followed by antigen unmasking performed for 20 min at 98°C in citrate buffer (pH 6) using the PT module. Endogenous peroxidase was blocked using 3% H2O2 (Merk) in methanol for 10 min at room temperature. Endogenous avidin and biotin were blocked using the Endogenous Blocking kit (Invitrogen) for 20 min at room temperature. Nonspecific antigen blocking was performed using blocking buffer. Mouse anti-Krt14 (rabbit, 1:2,000, Thermofisher), anti-Ki67 (rabbit, 1:400; Abcam, ab15580), anti-Lef1 (rabbit, 1:200, Biologend, ref. 90541) and anti-LEF1 (rabbit, 1:100, Cell Signalling, ref. 2230) were incubated overnight at 4°C. Anti-rabbit biotinylated with blocking buffer, standard ABC kit, and ImmunPACT DAB (Vector Laboratories) was used for the detection of horseradish peroxidase (HRP) activity. Slides were then dehydrated and mounted using SafeMount (Labonord).

FACS isolation of tumour cells and microarray analysis. Isolation of tumour cells was performed as previously described14. In brief, Lgr5DTGR-GFP and Krt14f/f;Ptch1f/f;Lgr5DTGR-GFP mice untreated and upon 8 weeks of vismodegib treatment were killed by decapitation. Back skin was placed in a Petri dish and a sterile scalpel was used to remove the adipose tissue and muscle. The skin tissue was minced with a scalpel (Sigma) for 1 h at 37°C and then a scalpel was used to separate epithelial cells from the dermis. The epidermal tissue was chopped into pieces and resuspended in PBS supplemented with 5% chelated fetal calf serum and filtered with 70 μm and 40 μm cell strainers (BD). Cells were stained using
anti-LRIG1 (goat polyclonal, R&D Systems, AF3688) followed by the secondary antibody donkey anti-goat Alexa 647 (Invitrogen).

LRG5* LRG1+ and LGR5*LRIG1+ cells from untreated or vismodegib-treated (8 weeks) Krt14CreER;Ptc1flo/flo;Lgr5DTR−/− mice were sorted using LRIG1 staining and native LGR5–GFP. Two thousand sorted cells per sample were collected directly in 45 μl lysis buffer (20 mM DTT, 10 mM Tris–HCl pH 7.4, 0.5% SDS, 0.5 μg/ml – 1 proteinase K). Samples were then lysed at 65°C for 15 min and frozen.

RNA isolation, amplification and microarray were performed at the IRB Functional Genomics Core, Barcelona. cDNA synthesis, library preparation and amplification were performed as described. Microarrays using Mouse Genome 430pm strip Affymetrix array were performed and the data were normalized using RMA algorithm. Biological duplicates were performed for all conditions. Genetic signatures were obtained by considering genes presenting a fold change greater or smaller than 2 or~2, respectively, in each replicate.

FACS isolation of CD71+ and CD71− populations of tumour cells, RNA extraction and quantitative PCR. Isolation of tumour cells from mouse skin was performed as described above. Cells were stained using anti-LRIG1 (goat polyclonal, R&D Systems, AF3688) and anti-CD71−PE (rat, BD Biosciences, 553267) followed by the secondary antibody donkey anti-goat-Alexa 647 (Invitrogen). Seven thousand FACS-sorted cells were collected directly in the lysis buffer provided by the manufacturer (MicroAmp MicroKit, Quiagen) and RNA extraction was then carried out according to the manufacturer’s protocol. Purified RNA was used to synthesize the first-strand complementary DNA using SuperScript II (Invitrogen) carried out according to the manufacturer’s protocol. Purified RNA was used to synthesize the first-strand complementary DNA using SuperScript II (Invitrogen) carried out according to the manufacturer’s protocol.

Sequencing of the CCAATGT, Gapdh-R: GTGTAGCCCAAGATGCCC TT, Tbox-F: GTACCGCAGCTGAT, β-Actin-F: GAAGCTGTGCTATGTTGCTCTA, Krt1-R: CTGTGCGTTGGTCCTCTTGT, GCCAACGTGC, Krt10-R: TAGGCTAGCCAGCTCTTTTGT, Krt1-F: ACAACGGCGAACCIAACTT, Krt1-R: CTTCGTTGGTGCTCTTGTG, Scl1-F: ACACATTGGGTTCGAGGTTAT and Scl1-R: AGCTTCCTCGTCTTCTGAGAC. Normalizers: HPRT-F: GCAGTAAGGGCCTAATAAGG, HPRT-R: TCCAAAAAGTGTGGCCTGT, |Actin-F: GAAGCTGTGCTAGTTGTGCTCTA, |Actin-R: CAATAGTGAAGCTGCGCCTG, |2M-F: TACCCCCCAGTGGACGATG, GATG, 2M-R: TCCAGATAGCCGTCTTG, GAPDH-F: CTTGGTTCACCCCTCAATGTG, GAPDH-R: GTGTTACCCCTCCAATGTG, Tbox-F: GTACCGCAGCTTCGAGCTCTTGTG, Tbox-R: AAATCAGCGAGCTTGGCTTCTGGT

Sequencing of the Smo gene in vismodegib-persistent lesions. A total of 200,000 LGR5* LRG1− tumour cells from three Krt14CreER;Ptc1flo/flo;Lgr5DTR−/− mice treated for 8 weeks with vismodegib were FACS-sorted following the protocol described above. Exons 3−12 of the mouse Smo gene were amplified using PCR and the products of the PCR were purified using the Monarch DNA Gel Extraction Kit (ref. T1020). The products of the PCR were sequenced following the Sanger standard using chemistry BigDy31.1, the cycle sequencing technology based on dideoxy chain termination/cycle sequencing and performed on the ABI 3730XL sequencer. SnapGene version 4.1.3 was used for the analysis. Information of the amplification primers and sequencing results can be found in Source Data.

**Grafting experiments.** For transplantation experiments, 100,000 cells that had been FACS-sorted to obtain pure populations of LGR5* LRG1+ and LGR5− LRIG1+ cells were transplanted into the interscapular fat pad of NOD−SCID immunodeficient mice. The 100,000 LGR5* LRG1+ and LGR5− LRIG1+ cells were mixed in a proportion of 1:40 with tumour-associated fibroblasts from the same tumours (FACS-sorted using CD140a marker (clone APAS; ebiosciences)). The tumour cells and fibroblasts were embedded in 50μl matrigel containing ROCK inhibitor (5.3 μM/ml) and transplanted into the fat pad.

**GSEA analysis.** The GSEA program was downloaded from the BROAD institute website (http://www.broadinstitute.org/gsea/). We used the GSEA preranked option with standard parameters of weighted enrichment score calculation to run the GSEA against a user-supplied fold-change-ranked list of genes. The results of the enrichment analysis were plotted using R software.

**Ptc1 deletion.** To determine the deletion of the two Ptc1 alleles in the LGR5* LRG1+ and LGR5* LRIG1+ populations, 200,000 cells were FACS-sorted and DNA was extracted using the QIAamp DNA Mini-Kit (Qiagen). The following primers were used to determine the presence of the floxed/floxed or deleted alleles: Forward: AAGAGATCTCTGTTGGCCAAGG; Reverse: CTATTTCAATTGTGTCGGTCCG.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

Data associated with this study have been deposited in the NCBI Gene Expression Omnibus under accession number GSE117458 (microarray).

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Extended Data Fig. 1 | Vismodegib leads to tumour shrinkage and emergence of vismodegib-persistent lesions in mice. a, Immunostaining for SMOM2, KRT14 and β4-integrin in tail from SmoM2 mice after different durations of vismodegib administration. b, Tumour burden in micrometres (total area occupied by tumours divided by the length of the analysed epidermis) in untreated and vismodegib-treated SmoM2 mice (n = 3 mice analysed per time point and condition). Centre values define the mean. See Source Data for description of the skin length and tumour area analysed per mouse. c, Immunostaining for KRT14 and β4-integrin in ventral skin from Ptch1 cKO mice. d, Immunostaining for SMOM2, KRT14 and β4-integrin in tail skin from SmoM2 mice. e, Quantification (mean ± s.e.m.) of lesion type upon vismodegib treatment in SmoM2 mice (n = 3 mice, total number of lesions analysed per time point indicated in parentheses). f, Immunostaining for active caspase-3 (AC3) and SMOM2. g, Percentage of AC3+ tumour cells (mean ± s.e.m.) in untreated and vismodegib-treated SmoM2 mice (n = 30 lesions analysed from 3 mice). Two-sided t-test. h, Immunostaining for Ki67 and SMOM2. i, Percentage of Ki67+ tumour cells (mean ± s.e.m.) in untreated and vismodegib-treated SmoM2 mice (n = 30 lesions analysed from 3 mice). Two-sided t-test. Three independent experiments per condition were analysed showing similar results (a, c, d, f, h). Hoechst nuclear staining in blue; scale bars, 100 μm (c, d), 50 μm (a, f, h). Dashed line delineates basal lamina. Arrows indicate vismodegib-persistent lesions.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Vismodegib-persistent lesions express LGR5 in mice. a, Immunostaining for LGR5–GFP and β4-integrin at different time points after tamoxifen administration in the Ptch1cKO model. b, In situ hybridization for Lgr5 and Gli1 in untreated and treated tumour cells in SmoM2 mice, c, Percentage (mean ± s.e.m.) of tumour cells (LGR5+ and LGR5−) that express Gli1 in SmoM2 mice (n = 3 mice, total number of cells analysed indicated in parentheses). d, Distribution (mean ± s.e.m.) of the number of Gli1 mRNA dots per tumour cell with and without treatment in SmoM2 mice (n = 104 and 111 total tumour cells from 3 mice per condition and time point). e, Representation of the mouse Smo gene, showing in red the exons (E) in which genetic mutations have been described6,7 (top). Results from the sequencing of exon 7 from vismodegib-persistent lesions obtained by pooling drug-persistent cells from three Krt14 CreER;Ptch1cKO;Lgr5DTR-GFP mice, showing absence of genetic mutations in the exon analysed (bottom). See Source Data for the results of the sequencing of exons 3–12. f, Protocol for BrdU and EdU double-labelling studies in Ptch1cKO-induced BCCs followed by vismodegib administration and discontinuation. g, Immunostaining for LGR5–GFP, BrdU and EdU in Ptch1cKO-derived BCCs following 5 days of vismodegib discontinuation. h, Protocol for treatment with vismodegib and retinoic acid (RA) or TPA. i, Immunostaining for LGR5–GFP, K67 and β4 in the back skin of Ptch1cKO mice treated with vismodegib and RA or TPA. j, Quantification of LGR5+ tumorigenic lesions per length of skin upon treatment with vismodegib or vismodegib with RA or TPA (n = 3 mice, 3 mm of skin analysed per mouse). Two-sided t-test. k, Immunostaining for LGR5–GFP and LRIG1 in untreated and treated Ptch1cKO mice. l, Immunostaining for LGR5–GFP and LRIG1 in untreated and vismodegib-treated (8 weeks) mice before and after enzymatic and physical separation of epidermis from dermis in Krt14 CreER;Ptch1cKO;Lgr5DTR-GFP mice. Note that hair follicles co-expressing LGR5 and LRIG1 and sebaceous cysts remained in the dermal fraction whereas the BCCs were isolated with the epidermal fraction, indicating that normal hair follicles did not significantly contaminate the FACS-isolated tumour cells. m, Cell sorting strategy to isolate LGR5+LRIG1−, LGR5+LRIG1+ and LGR5−LRIG1+ in normal skin and in Ptch1cKO-derived BCCs before and after vismodegib administration. Forward scatter (FSC) and side scatter (SSC) were performed to exclude cell debris and doublets. Living cells were selected by Hoechst dye exclusion. Finally, the different LGR5 and LRIG1 cell populations were isolated by FACS sorting.

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A, area; W, width. n, Proportion of cells (mean ± s.e.m.) expressing LGR5–GFP and LRIG1 determined by FACS (n = 3 independent experiments per condition). These experiments indicate that LRIG1 can be used to discriminate between LGR5+ cells from the HFSC or lower hair follicle (LGR5+LRIG1−) and BCC cells (LGR5+LRIG1+). Three independent experiments per condition were analysed with similar results (a, k, l). Hoechst nuclear staining in blue; scale bars, 50 μm (a, i, k, l) and 25 μm (b). Dashed line delineates basal lamina separating IFE from the dermis. Dotted line delineates BCC. Arrows indicate vismodegib-persistent lesions.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Characterization of LGR5⁺LRIG1⁺ and LGR5⁻LRIG1⁺ tumour cells. a, GSEA showing the enrichment of genes upregulated in the LGR5⁺LRIG1⁺ population compared to the LRG5⁻LRIG1⁺ population from two independent microarray experiments with the EHFP16 (left) in telogen HFSCs15 (middle) and hair follicle Lgr5-expressing cell signatures17 (right), showing that LGR5-expressing BCC cells express many genes of the embryonic and adult hair follicle signatures. The normalized enrichment score (NES) and P value (one-sided test) were calculated using the GSEA program. b, mRNA expression of genes upregulated in LGR5⁺LRIG1⁺ tumour cells compared to LGR5⁻LRIG1⁺ tumour cells in untreated conditions (n = 2 independent microarray experiments). c, Immunostaining for LGR5–GFP with LEF1, LHX2, CUX1, TBX1 and ALCAM in untreated Ptch1 cKO-derived BCCs. d, Venn diagram showing the similarities and differences between genes that were upregulated more than twofold from two independent microarray experiments in LGR5⁻LRIG1⁺ versus LGR5⁺LRIG1⁺ cells compared to IFE18 and LRIG113 signatures. P value calculated using the hypergeometric test for each intersection of two subsets of genes with phyper function in R software. The high overlap indicates that LGR5-LRIG1⁺ cells expressed IFE and infundibulum differentiation markers. e, mRNA expression of genes upregulated in LGR5⁺LRIG1⁺ tumour cells compared to LGR5⁻LRIG1⁺ cells in untreated conditions (n = 2 independent microarray experiments). f, PCR analysis of the recombination of the floxed Ptch1 alleles in control samples and in FACS-isolated tumour-derived LGR5⁺LRIG1⁺ and LGR5⁻LRIG1⁺ populations from Ptch1KO-induced BCCs. Two technical replicates were analysed for each sample with similar results. g, Immunostaining for LGR5–GFP, LRIG1 and Ki67 in Ptch1cKO-derived BCCs shows higher proliferation rate in LGR5⁺LRIG1⁺ than in LGR5⁻LRIG1⁺ tumour cells. Three independent experiments per condition were analysed with similar results (c, g). Hoechst nuclear staining in blue; scale bars, 50 μm.
Extended Data Fig. 4 | Transplantation of LGR5⁺LRIG1⁺ Ptch1;Trp53 double conditional knockout BCC cells leads to the formation of BCC-like structures. a, Table summarizing the number of grafted mice that presented KRT14⁺ BCC-like structures upon transplantation of FACS-isolated LGR5⁺LRIG1⁺ and LGR5⁻LRIG1⁻ cells from BCCs arising in Krt14CreER;Ptch1cKO;Lgr5DTR-GFP and Krt14CreER;Ptch1cKO;Trp53cKO;Lgr5DTR-GFP mice. Three independent experiments per condition were analysed with similar results (b, c). Hoechst nuclear staining in blue; scale bars, 50 μm.

| Krt14CreER;Ptch1cKO;Lgr5DTR-GFP | Number grafted mice presenting KRT14⁺ cells |
|----------------------------------|--------------------------------------------|
| LGR5⁺LRIG1⁺                     | 0/7                                        |
| LGR5⁺LRIG1⁻                     | 0/7                                        |
| Krt14CreER;Ptch1cKO;Trp53cKO;Lgr5DTR-GFP |                                 |
| LGR5⁺LRIG1⁺                     | 3/7                                        |
| LGR5⁺LRIG1⁻                     | 0/7                                        |

b, KRT14 LGR5-GFP LRIG1
c, KRT10 LGR5-GFP

Lgr5DTR-GFP mice. b, c, Immunostaining for LGR5–GFP, KRT14 and LRIG1 (b) and for LGR5–GFP and KRT10 (c) in the BCC-like structures obtained upon transplantation of LGR5⁺LRIG1⁺ cells from Ptch1;Trp53 double conditional knockout BCCs in the dorsal fat pads of NOD/SCID mice. Three independent experiments per condition were analysed with similar results (b, c). Hoechst nuclear staining in blue; scale bars, 50 μm.
Extended Data Fig. 5 | Vismodegib-persistent lesions do not show decreased primary cilia numbers or nuclear localization of MKL1.

**a, b,** Immunostaining for ARL13B and LGR5–GFP in $P^\text{tch1}\text{cKO}$ model (a) and for ARL13B and SMOM2 in $S^\text{mOM2}$ model (b) in untreated and vismodegib-treated lesions. **c, d,** Immunostaining for MKL1 and LGR5–GFP in vismodegib-persistent lesions in $P^\text{tch1}\text{cKO}$ mice (c) and for MKL1 and SMOM2 in vismodegib-persistent lesions in $S^\text{mOM2}$ mice (d) treated for 8 weeks with vismodegib. White boxes are expanded on right. Three independent experiments per condition were analysed with similar results. Scale bars, 25 μm.
Extended Data Fig. 6 | Vismodegib promotes BCC differentiation.

a, Immunostaining for LGR5–GFP, LHX2 and ALCAM in untreated and vismodegib-treated Ptch1<sup>cko</sup>-derived BCCs.
b, GSEA showing enrichment of genes upregulated in LGR5<sup>+</sup>LRIG1<sup>+</sup> vismodegib-treated tumours compared to untreated BCCs with IFE<sup>16</sup> and LRIG1<sup>13</sup> signatures, showing that vismodegib treatment promotes the expression of the IFE and infundibulum signatures. The normalized enrichment score (NES) and P value (one-sided test) were calculated using the GSEA program.
c, Oil Red O and haematoxylin and eosin staining in ventral skin of untreated and vismodegib-treated Ptch1<sup>cko</sup> mice. Arrows indicate areas of sebaceous differentiation.
d, Immunostaining for LGR5–GFP and adipophilin in untreated and vismodegib-treated Ptch1<sup>cko</sup>-derived BCCs. Arrows indicate areas of sebaceous differentiation.
e, In situ hybridization for Lgr5 and Defb6 in untreated and vismodegib-treated Ptch1<sup>cko</sup>-derived BCCs.
f, Immunostaining for KRT1 and SMOM2 in untreated and vismodegib-treated SmoM2 mice. Three independent experiments per condition were analysed with similar results. Hoechst nuclear staining in blue; scale bars, 50 μm.
Extended Data Fig. 7 | Vismodegib promotes differentiation of SMOM2-expressing cells during BCC initiation and in Ptch1<sup>−/−</sup> tumour cells. 

**a**, Protocol for tumour induction and timing of vismodegib administration to Krt14<sup>CreER</sup>;Rosa<sup>SmoM2</sup> mice. **b**, Quantification of surviving SMOM2 clones in the interscale (tail epidermis) in untreated mice and after different durations of vismodegib treatment (n = 3 mice per time point and condition). Centre values show mean. See Source Data for description of total number of clones counted per time point and condition. **c**, Immunostaining for KRT31 and SMOM2 in whole-mount tail skin (left) and orthogonal views of the clones highlighted in the left panel stained for β4-integrin and SMOM2 (right). **d**, Quantification (mean ± s.e.m.) of the type of SMOM2-expressing clones after different durations of vismodegib treatment (n = 3 or 4 mice as indicated in the graph, total number of lesions quantified indicated in parentheses). **e, f**, Immunostaining for KRT10 and SMOM2 (e) and for LHX2 and SMOM2 (f) in untreated and vismodegib-treated mice. Three independent experiments per condition were analysed with similar results. **g**, mRNA expression of genes upregulated in the LGR5<sup>+</sup>LRI1<sup>+</sup>CD71<sup>+</sup> population compared to the LGR5<sup>+</sup>LRI1<sup>+</sup>CD71<sup>−</sup> population obtained by quantitative PCR (n = 3 mice). Bars represent the average fold change over LGR5<sup>+</sup>LRI1<sup>+</sup>CD71<sup>−</sup> cells and error bars the s.e.m. **h**, Immunostaining for LGR5<sup>+</sup>GFP, BrdU and KRT10 in mice that received three injections of BrdU followed by two weeks of vismodegib administration. Three independent experiments per condition were analysed with similar results. Hoechst nuclear staining in blue; scale bars, 100 μm (c) and 50 μm (e, f, h).
Extended Data Fig. 8  |  LGR5 expression in vismodegib-persistent lesions in human BCCs. **a**, Tables summarizing the BCC and treatment characteristics in the patients analysed. **b**, Immunohistochemistry for KRT14 in biopsies before, after and during vismodegib treatment. **c**, In situ hybridization for LGR5 and GLI1 in biopsies from patients before, during and after vismodegib treatment. **d**, Percentage (mean ± s.e.m.) of tumour cells (LGR5+ and LGR5−) that express GLI1 in biopsies from patients, during or after vismodegib treatment (n = 3 samples from different patients (Patients 1–3) or body locations (Patient 4), total number of cells analysed indicated in parentheses). **e, f**, Immunohistochemistry for Ki67 (e) and KRT10 (f) in biopsies before, during and after vismodegib treatment. Hoechst nuclear staining in blue; scale bars, 25 μm.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Lgr5 lineage ablation leads to BCC shrinkage and elimination of vismodegib–persistent lesions. a, Protocol for tamoxifen and diphtheria toxin (DT) administration. b, c, Immunostaining for KRT14 and LGR5–GFP in the Pch1cKO model (b) and for KRT14 and SMOM2 in the SmoM2 model (c) after different durations of DT administration. d, Quantification of tumour burden in untreated mice and after DT administration (n = 3 mice per time point and condition). Centre values define the mean. See Source Data for description of the skin length and tumour area analysed per mouse. e, Number of LGR5–GFP+ tumour cells in untreated conditions and following DT administration (n = 3 mice per time point and condition, 1 mm of skin analysed per mouse). Centre values define the mean. f, Quantification of tumour burden (SMOM2-expressing cells) in untreated conditions and following DT treatment (n = 3 mice per time point and condition). Centre values define the mean. See Source Data for description of the skin length and tumour area analysed per mouse. g, Immunostaining for active caspase-3 (AC3) and LGR5–GFP (top) and for active caspase-3 and SMOM2 (bottom) after five administrations of DT. Three independent experiments per condition were analysed with similar results. h, Experimental strategy for combination of vismodegib treatment and Lgr5 ablation in Krt14CreER;Pch1cKO;Lgr5DTR–GFP mice. i, Immunostaining for LGR5–GFP and KRT14 in Krt14CreER;Pch1cKO; Lgr5DTR–GFP mice upon treatment, Lgr5 ablation and discontinuation. j, Immunostaining for active caspase-3 and LGR5–GFP following administration of vismodegib and DT. k, Quantification of the number of LGR5–GFP+ cells (mean ± s.e.m.) in the different experimental conditions upon treatment and discontinuation (n = 3 mice, 3 mm of skin analysed per mouse). Two-sided t-test. l, Quantification of the number of LGR5+ lesions (mean ± s.e.m.) per length of epidermis (mm) in mice treated with vismodegib and upon discontinuation of treatment with vismodegib and DT (n = 3 mice, 3 mm of skin analysed per mouse). Two-sided t-test. Hoechst nuclear staining in blue; scale bars, 50 μm. Dashed line delineates basal lamina separating IFE from the dermis. Dotted line delineates BCC. Arrows indicate tumorigenic lesions in b, c and indicate vismodegib-persistent lesions in i.
Extended Data Fig. 10 | Wnt signalling is active in vismodegib-persistent lesions in mouse and human BCCs. **a**, ISH for Lgr5 and Axin2 in untreated and vismodegib-treated lesions from Ptch1\(^{cko}\) and SmoM2 mice. **b**, ISH for LGR5 and AXIN2 in biopsies from patients before, during and after vismodegib treatment. **c**, Immunostaining for LEF1 and LGR5–GFP in Ptch1\(^{cko}\)-derived tumorigenic lesion following treatment with vismodegib and LGK-974. **d**, Protocol used for LGK-974 treatment in Ptch1\(^{cko}\) mice. **e**, Immunostaining for LGR5–GFP and KRT14 in BCC treated with LGK-974 for 9 days from the Ptch1\(^{cko}\) model. **f**, Quantification of the tumour burden (mean ± s.e.m.) in mice treated with LGK-974 for 9 days or untreated (\(n = 3\) mice). See Source Data for description of skin length and tumour area analysed per mouse. Two-sided \(t\)-test. Hoechst nuclear staining in blue; scale bars, 25 μm. Dashed line delineates basal lamina separating IFE from the dermis. Dotted line delineates BCC. Three independent experiments per condition were analysed with similar results (\(a, c, e\)) and two technical replicates were performed for each sample with similar results (\(b\)).
Extended Data Fig. 11 | Model. Vismodegib administration promotes tumour cell differentiation leading to BCC regression. However, upon vismodegib treatment a small proportion of LGR5+ BCC cells persists, forming vismodegib-tolerant lesions that are slow cycling and characterized by Wnt signalling activation. Discontinuation of vismodegib treatment results in proliferation of LGR5-persistent lesions that lead to BCC relapse. Vismodegib treatment in combination with Lgr5 lineage ablation or Wnt signalling inhibition results in eradication of BCCs.
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- Clearly defined error bars

State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

**Data collection**

- Microscope (Axio Imager M2) images were generated using Axiovision 4.8.2 software (Carl Zeiss).
- The confocal (LSM 780) images were generated using the ZEN 2 software (Carl Zeiss).
- Flow cytometry data analysis and cell sorting was performed on a FACSAria sorter using the FACS DiVa software (BD Biosciences).

**Data analysis**

- Tumor burden was calculated using AxioVision 4.8.2 software (Carl Zeiss).
- The confocal imaging data-sets generated were analysed with the ZEN 2 software (Carl Zeiss).
- Flow cytometry data analysis was performed on a FACSAria sorter using the FACS DiVa software (BD Biosciences).
- Quantitative PCR analysis was performed using Light Cycler 96 and Light Cycler 96 SW 1.1 software (Roche).
- Mouse Smo gene was sequenced using ABI 3730XL sequencer and SnapGene version 4.1.3 was used for the analysis.
- t-test were performed using Excel and Prism (version 7). p-value calculation for Venn diagrams was calculated using R software.
- The GSEA program was downloaded from the BROAD institute website (http://www.broadinstitute.org/gsea/). We used the GSEA...
preranked option with standard parameters of weighted enrichment score calculation to run the GSEA against a user-supplied fold-change-ranked list of genes. Results of the enrichment analysis were plotted using R software.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data associated with this study have been deposited in the NCBI Gene Expression Omnibus under accession number GSE117458 (microarray analysis).

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical methods were used to predetermine sample size. All experiments were repeated at least three times with similar results, except for microarray for which experiments were repeated twice.

Data exclusions

No animals were excluded from the analysis.

Replication

All experiments were repeated at least three times (3 biological independent experiments/mice) showing similar results, except for microarray analysis for which experiments were repeated twice (2 biological independent experiments/mice). All attempts at replication were successful and are shown, n is described in legends.

Randomization

The experiments were not randomized. The mice included in this study were selected according to their correct genotype. The mice received tamoxifen injection/s when they were 1.5 months, and 8 weeks after they all presented basal cell carcinomas (similar tumor burden). Sex-specific differences were minimized by including similar number of male and female animals.

Blinding

The investigators were not blinded to allocation during experiments and outcome assessment. Blinding was not possible as the same investigator processed the animals and analysed the data.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Unique biological materials |
| ☒   | Antibodies |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq |
| ☒   | Flow cytometry |
| ☒   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Vismodegib/GDC0449 (Genentech, San Francisco, US) and LGK-974 (Novartis, Bâle, Switzerland).
Antibodies

For immunofluorescence and ISH the following primary antibodies were used:

- anti-B4-integrin (Rat, 1:200, BD, clone 346-11A, ref. 553745, lot. 236651-23), anti-Active Caspase-3 (rabbit, 1:600, R&D, ref.AF835, lot.CF23517031), anti-Ki67 (rabbit, 1:1000, Abcam, ref. ab15580, lot.GR3198193-1), anti-Lrig1 (goat, 1:500, R&D, ref.AF3688, lot.ZPH0217111), anti-Lef1 (rabbit, 1:100, Cell Signaling, ref.2230), anti-Lhx2 (goat, 1:500, Santa Cruz, sc-19344, lot.K1615), anti-Cux1 (rabbit, 1:6000, Santa Cruz), anti-Tbx1 (rabbit, 1:100), anti-Lef1 (rabbit, 1:100, Cell Signaling, ref.2230), anti-Lig1 (goat, 1:500, R&D, ref.AF3688, lot.ZPH0217111), anti-Lef1 (rabbit, 1:100, Cell Signaling, ref.2230), anti-Lhx2 (goat, 1:500, Santa Cruz, sc-19344, lot.K1615), anti-Cux1 (rabbit, 1:6000, Santa Cruz), anti-Tbx1 (rabbit, 1:100), anti-Calc (goat, 1:1000, Novus, ref.FAB1172F, lot.AAW011121), anti-Keratin10 (rabbit, 1:3000, Covance, ref. PRB-159P-0100), anti-Keratin1 (rabbit, 1:3000, Covance, ref. PRB-159P-0100), anti-Keratin14 (rabbit, 1:3000, Thermofisher), anti-Scd1 (goat, 1:500, Santa Cruz, ref.SC14719), anti-Adipophilin (guinea pig, 1:5000, Fitzgerald, ref.20R-AP002, lot.P17030911), anti-BrdU (mouse, 1:200, BD, clone 3D4, ref. 560209, lot.4293550), anti-MKL1 (rabbit, 1:200, Sigma, ref. HPA030782, lot.C106712), and anti-ARL13b (rabbit, 1:2000, ref.17711-1-AP, Proteintech, lot.49885).

The following secondary antibodies were used: anti-rabbit, anti-rat, anti-goat, anti-chicken, conjugated to AlexaFluor488 (Molecular Probes) and to rhodamine Red-X and Cy5 (JacksonImmunoResearch).

Validation

As positive control tissues with known expression of the marker were used. As negative control staining omitting the primary was performed.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

K14-CreER transgenic mice were kindly provided by E. Fuchs, Rockefeller University, USA. Ptch1fl/fl mice and Rosa/SmoM2-YFP mice were obtained from the JAX repository. Lgr5-DTR-GFP mice were kindly provided by Genentech (San Francisco, USA). Tp53f/f mice were obtained from the National Cancer Institute at Frederick. Mice included in this study were from mixed genetic background.

Female and male animals have been used for all experiments and equal animal gender ratios have been respected in the majority of the analysis. Mice were included in the study when they were 1.5 months old when the tamoxifen was administered. Mice colonies were maintained in a certified animal facility in accordance with European guidelines. The experiments were approved by the local ethical committee (CEBEA) under protocols #483 and #632. The study is compliant with all relevant ethical regulations regarding animal research. Mice used in this study were of mixed background.

Wild animals

No wild animals were used in this study.

Field-collected samples

No field-collected samples were used in this study.

Human research participants

Policy information about studies involving human research participants

Population characteristics

The study includes analysis of skin biopsies from 4 patients:

- Patient 1 (57 years, man, superficial BCC, 9 months of treatment, relapse following treatment discontinuation)
- Patient 2 (82 years, man, nodular and infiltrative BCCs, 7 months of treatment, relapse following treatment discontinuation)
- Patient 3 (84 years, woman, infiltrative BCC, 9 months of treatment, absence of relapse following treatment discontinuation)
- Patient 4 (80 years, man, multiple BCCs, 13 months of treatment, relapse following treatment discontinuation and currently under treatment).

Recruitment

Patients presenting locally advanced Basal Cell Carcinoma that received vismodegib treatment.

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Sample preparation was performed as described in Jensen et al, Nature Protocols 2010. Briefly, control Lgr5-DTR mice and K14-CreER/Ptch1If/If/Lgr5-DTR bearing BCCs and vismodegib-persistent lesions were sacrificed. Back skin was placed in a petri dish and a sterile scalpel was used to remove the adipose tissue and muscle. The epidermal tissue was incubated in thermolysin (Sigma) for 1h at 37°C and after a scalpel was used to separate epidermis from the dermis. The epidermal tissue was chopped in little pieces and resuspended in PBS supplemented with 5% chelated fetal calf serum and filtered in 70um and 40um cell strainers (BD).
Cells were stained using the anti-Lrig1 (goat polyclonal, R&D) followed by the secondary donkey anti-Goat-Alexa 647 (Invitrogen).

| Instrument       | FACS Aria III (BD Bioscience) |
|------------------|-------------------------------|
| Software         | FACSDiva Software 8.0.1 (BD Bioscience) |
| Cell population abundance | The proportion of the relevant cell populations is depicted in Extended Data Figure 2.m-n. |
| Gating strategy  | Living cells were selected by forward scatter, side scatter, doublets discrimination and by Hoechst dye exclusion. Tumor cell subpopulations were selected based on the expression of Lgr5-GFP and Lrig1-Alexa647. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.