A Wnt-producing niche drives proliferative potential and progression in lung adenocarcinoma

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The heterogeneity of cellular states in cancer has been linked to drug resistance, cancer progression and the presence of cancer cells with properties of normal tissue stem cells1,2. Secreted Wnt signals maintain stem cells in various epithelial tissues, including in lung development and regeneration3–5. Here we show that mouse and human lung adenocarcinomas display hierarchical features with two distinct subpopulations, one with high Wnt signalling activity and another forming a niche that provides the Wnt ligand. The Wnt responder cells showed increased tumour propagation ability, suggesting that these cells have features of normal tissue stem cells. Genetic perturbation of Wnt production or signalling suppressed tumour progression. Small-molecule inhibitors targeting essential posttranslational modification of Wnt reduced tumour growth and markedly decreased the proliferative potential of lung cancer cells, leading to improved survival of tumour-bearing mice. These results indicate that strategies for disrupting pathways that maintain stem-like and niche cell phenotypes can translate into effective anti-cancer therapies.

Stem cells are defined by their capacity to self-renew while also producing differentiated cells. The decision to divide or differentiate is primarily controlled by extrinsic signalling factors, which, together with the cells that produce them, form a niche with a local range of action capable of supporting a limited number of stem cells. Among the niche signals that promote stem-cell phenotypes, secreted Wnt proteins are notable owing to their function in multiple epithelial stem cell compartments3. Wnt growth factors are palmitoylated in cells that produce them by the membrane-bound O-acyltransferase porcupine (encoded by Porcn in mice)3. This posttranslational modification is essential for Wnt secretion and binding to frizzled family receptors3. Wnt binds frizzled, promoting the stabilization, nuclear translocation and transcriptional activity of β-catenin through its interaction with T-cell factor (TCF) family transcription factors. Recently, R-spondin (Rspo) growth factors were found to amplify Wnt signalling by engaging leucine-rich repeat-containing G-protein-coupled receptor (Lgr)4, Lgr5 and Lgr6 (ref. 6). Lgr5 marks stem cells in multiple epithelial tissues and in intestinal adenomas3,6–8. Stem-like cells have recently been described in autochthonous mouse tumour models7,9,10 and in tumour transplants11–13, but evidence for the existence of stem-like cells and particularly their niche in advanced solid tumours in situ has been limited14.

Lung adenocarcinoma (LUAD) is a leading cause of death globally. Tumours driven by oncogenic KRAS account for approximately 30% of LUAD and effective chemotherapies against these tumours are lacking15. Wnt signalling is essential for the initiation and maintenance of Braf-driven lung adenomas in mice16, and forced activation of the pathway promotes progression of Kras or Braf mutant lung tumours16,17. LUAD in humans, in particular tumour metastasis, is frequently associated with increased expression of Wnt-pathway-activating genes and downregulation of negative regulators of this pathway18,19.

Figure 1 | Ligand-dependent Wnt signalling sustains proliferative potential in lung adenocarcinoma. a, 3D cultures of sorted tdTomato+ (red) primary mouse KPT LUAD cells 14 days after plating. Proliferating (EdU+) cells (green, arrowheads). RW, combination of Rspo1 and Wnt3a. Scale bar, 100 μm. b, Quantification of tumour spheroids containing EdU+ cells from two mice (TT5678 and TT3861). n = 8 wells per condition. c, Quantification of KPT LUAD primary transplant tumours in recipient mouse lungs treated with LGK974 or vehicle for eight weeks. d, Quantification of tumour spheroids containing EdU+ cells ten days after plating. Rspo2-a, Rspo3-a and Lgr5-a refer to cells expressing CRISPR-activator (SAM) components driving expression of the indicated gene. n = 8 wells per condition. e, Wnt pathway activity measured by the Topflash assay in cultured KP LUAD cells stably expressing shRNAs targeting Lgr4, Lgr5, both Lgr4 and Lgr5 or a vector control. n = 3 technical replicates per condition, experiment was repeated four times. Data are mean ± s.d.; two-way ANOVA (b, d, e) or Student’s t-test (c); *P < 0.05; **P < 0.01; ***P < 0.001 compared to control, except in d, where the LGK974 group is compared to the same CRISPR-activator line, and in e where the comparison is to Rspo1 stimulation.
We isolated tdTomato+ primary cancer cells from autochthonous Kras\(^{G12D+}\);Trp53\(^{flox/flox}\);Rosa26\(^{tdTomato+}\) (KPT) mouse LUAD, and established low-density three-dimensional (3D) organotypic tumour spheroid cultures. 2.7% (±0.5%) (mean ± s.d.) of the cells gave rise to persistently proliferating spheroids (Fig. 1a, b), suggesting that cells capable of self-renewal comprise a small minority of the tumour. Recombinant Wnt3a, Rap1 or their combination increased the absolute number and ratio of primary mouse KPT LUAD spheroids that contained proliferating cells, and promoted overall cell proliferation (Fig. 1a, b and Extended Data Fig. 1a, b). Conversely, inhibition of ligand-driven Wnt signalling with the porcine inhibitor LGK974 (ref. 20), short-hairpin RNA (shRNA) targeting Porcn or recombinant DKK1 (a Wnt antagonist) suppressed proliferative capacity of primary LUAD cells in 3D spheroids (Fig. 1a, b and Extended Data Fig. 1a–f).

Tumour formation by primary LUAD cells was markedly decreased upon orthotopic transplantation (genetically engineered mouse model-derived allograft, GEMM-DA) into recipient mice that were treated with LGK974 compared to control (Fig. 1c and Extended Data Fig. 1g). Collectively, these data indicate that KP LUAD cells display heterogeneity in their proliferative potential, which is maintained by Wnt signals produced by cancer cells.

Rspo1 alone was most potent in stimulating proliferation and expansion of KPT LUAD spheroids (Fig. 1a, b and Extended Data Fig. 1a), even though, as expected, the combination of recombinant Rspo1 and Wnt3a was most potent in activating the Wnt pathway (Extended Data Fig. 1e, h and Supplementary Information). We next used the CRISPR–Cas9-based synergistic activation mediator (SAM) system\(^\text{a1}\) to robustly drive expression of Rspo2, Rspo3 or Lgr5 in KP LUAD cells (Extended Data Fig. 1i–l). This increased their proliferation and induced Wnt target genes in 3D spheroid culture and both of these effects were suppressed by LGK974 (Fig. 1d and Extended Data Fig. 1m, n). Knockdown of both Lgr4 and Lgr5 was required to fully suppress R-spondin-driven Wnt pathway activation (Fig. 1e and Extended Data Fig. 2a–g), indicating that both Lgr4 and Lgr5 are R-spondin receptors in the KP LUAD model.

We next assessed whether the Wnt pathway is activated in autochthonous KP LUAD tumours in vivo. Using a luciferase or GFP-reporter gene driven by a synthetic \(\beta\)-catenin-sensitive 7TCF promoter, we observed Wnt-pathway activation in a subpopulation of cancer cells, particularly in large autochthonous KP tumours (Fig. 2a, b). In subcutaneous transplants of KP tumour lines, 7TCF promoter activity was suppressed by treatment with LGK974 (Extended Data Fig. 3a–c). Notably, we observed nuclear localization of \(\beta\)-catenin, a hallmark of Wnt signalling, in a subpopulation of cancer cells, only in tumours that had progressed from adenoma to adenocarcinoma (Fig. 2c, d and Extended Data Fig. 3d).

We next performed porcine immunostaining in tumour sections to identify cells that were able to produce Wnt in the LUAD tumours. Porcine localized to cancer cells in close proximity to cells with nuclear \(\beta\)-catenin or expression of the Wnt target gene Axin2 in autochthonous KP LUAD, although rare porcine \(^*\) macrophages were also detected, predominantly in peritumoural areas (Fig. 2c and Extended Data Fig. 3e–i). Furthermore, Porcn gene expression was significantly higher in adenocarcinomas compared to adenomas (Fig. 2e). Notably, we detected similar porcine-or nuclear \(\beta\)-catenin-positive subpopulations and induction of \(PORCN\) expression in human LUAD (Fig. 2f and Extended Data Fig. 3j, k). These findings indicate that the Wnt pathway is activated in a subpopulation of lung adenocarcinoma cells in close proximity to porcine \(^*\) cells that are able to provide the Wnt signal. Porcine localized to bronchiolar epithelium in the normal lung and was restricted to sites of high Wnt signalling and stem cell activity in the intestine and liver\(^*\) (Extended Data Fig. 4a–e), suggesting that porcine is specifically expressed in Wnt-producing cells in normal stem cell niches.

To test the functional requirement for porcine expression in LUAD cells in vivo, we used CRISPR–Cas9 to inactivate Porcn specifically in...
the cancer cells of the KP LUAD model. Notably, targeting Porcn did not affect tumour grade or burden at 12 weeks after tumour initiation, when most tumours are still in the adenoma stage (Fig. 2g and Extended Data Fig. 5a). However, targeting Porcn reduced tumour burden and resulted in a shift to lower grade tumours at 20 weeks compared to control (Fig. 2g, h). Of note, of the 12 tumours that were graded as KP;Lgr5GFP-CreER mice infected with the indicated lentiviral vectors. hU6, human U6 promoter; sU6, synthetic U6 promoter; EFS, minimal E1α promoter. n = 6 (sgTom), n = 7 (sglgr4 + sglgr5). e, mRNA ISH for the indicated transcripts (purple) in consecutive sections of a similar region of a KP lung adenocarcinoma. Scale bars, 2 mm (d) and 100 μm (b, c, e). Data are mean ± s.d.; *P < 0.05, **P < 0.01; Student’s two-sided t-test (a–d).

Lgr5 is a Wnt target gene functionally involved in amplification of Wnt signalling and stem cell maintenance in multiple tissues3,6. When compared to adenomas, KP adenocarcinomas had an increased level of Lgr5 transcripts, which localized to a subpopulation of adenocarcinoma cells (Extended Data Fig. 6a, b). Lgr4 expression in KP LUAD tumours was more widespread compared to Lgr5, much like in normal intestinal crypts6, but was enriched in the Lgr5+ cells (Extended Data Fig. 6b–d). Furthermore, in GEMM-DAs established from KP LUAD tumours containing an Lgr5GFP-CreER+ reporter allele, the Lgr5+ cells were localized in close proximity to porcupine+ cancer cells (Fig. 3a). Sorted primary Lgr5+ KP LUAD cells were more efficient at forming persistently proliferating spheroids in vitro and orthotopic KP LUAD GEMM-DAs tumours in vivo than the Lgr5– cells (Fig. 3b and Extended Data Fig. 6e–g), suggesting that these cells have a high proliferative potential.

To investigate whether the Lgr5+ cells also display stem-like properties in situ in established tumours, we established subcutaneous KP LUAD GEMM-DAs containing Lgr5CreER and Rosa26LSL-Cas9-2a-GFP mice infected with the indicated lentiviral vectors. hU6, human U6 promoter; sU6, synthetic U6 promoter; EFS, minimal E1α promoter. n = 6 (sgTom), n = 7 (sglgr4 + sglgr5). e, mRNA ISH for the indicated transcripts (purple) in consecutive sections of a similar region of a KP lung adenocarcinoma. Scale bars, 2 mm (d) and 100 μm (b, c, e). Data are mean ± s.d.; *P < 0.05, **P < 0.01; Student’s two-sided t-test (a–d).

A subpopulation of Wnt-pathway–positive cells that have stem-like properties has recently been described in pancreatic ductal...
In this study, we observed the emergence of porcupine+ niche cells and Lgr5+ stem-like cells as KP lung adenomas progress to adenocarcinomas. This transition is also associated with amplification of Lgr4 and Lgr5, we investigated the requirement for these genes in the KP model. CRISPR–Cas9-based combined inactivation of Lgr4 and Lgr5 in the KP model led to reduced lung tumour burden and prevented progression of adenomas to adenocarcinomas (Fig. 3d), similar to what was observed when targeting Porcn (Fig. 2g). Tumours that progressed into adenocarcinomas predominantly contained wild-type alleles or small non-frameshift mutations in Lgr4 and Lgr5 (Extended Data Fig. 8f–h). These data are consistent with a key role for Lgr4 and Lgr5 in the progression into adenocarcinomas. We detected expression of the Lgr4/Lgr5 ligands Rspo1 and Rspo3 in KP LUAD; these ligands localized predominantly to endothelial cells in the tumours (Extended Data Fig. 9a, b). Analogously, endothelial cells expressing R-spondin form a part of the niche for liver stem cells.23

To identify the Wnt ligands and their frizzled (Fzd) receptors involved in LUAD, we performed qPCR on 84 Wnt-pathway-related genes in sorted KPT lung adenocarcinoma cells and their stroma. This analysis revealed very little expression of Porcn or Wnt ligands in the stroma, consistent with the cancer cells being the predominant source of Wnt ligands in LUAD (Extended Data Fig. 9c, d and Supplementary Table 2). Out of the 19 Wnt genes, Wnt7a, Wnt5a and Wnt7b were robustly expressed in LUAD (Extended Data Fig. 9c–e). In situ hybridization (ISH) showed that expression of these three Wnt ligands in KP LUAD was found in regions with Wnt pathway activation (Fig. 3e). Increased levels of WNT5A, WNT3, WNT5B and WNT10A in subpopulations of patients and, in particular, WNT7B were observed in human LUAD when compared to normal lung in the TCGA dataset (Extended Data Fig. 9f and Supplementary Table 3). We detected robust expression of 8 out of the 10 Fzd receptors and their Lrp5 and Lrp6 co-receptors in sorted KP LUAD cells (Extended Data Fig. 9g). Expression of Fzd1, Fzd4 and Fzd6 was increased in KP lung adenocarcinomas when compared to adenomas (Extended Data Fig. 9h). Of note, each of these receptors can be engaged by at least one of the three Wnt ligands identified in the current study.24

We next explored inhibition of ligand-dependent Wnt signalling as a potential therapeutic strategy in LUAD. Treatment with LGK974 suppressed Wnt target genes, inhibited tumour growth, proliferation and prolonged survival of mice with advanced autochthonous KP LUAD tumours (Fig. 4a–d and Extended Data Fig. 10a, b). Furthermore, treatment of tumour donor mice with LGK974 markedly suppressed the tumour-forming ability of transplanted cells and reduced numbers of proliferative tumours in recipient mice (Fig. 4e and Extended Data Fig. 10c–e), suggesting that inhibiting Wnt can disrupt stem-like cells in LUAD (Fig. 4f).

Our results indicate that a subset of Kras and p53 mutant LUAD cells acts as a Wnt-producing niche for another cancer cell subpopulation that responds to the Wnt signal and has robust proliferative potential (Fig. 4g). Inhibiting porcupine disrupts Wnt secretion and activity in the niche, suppressing stem cell function in tumours, which ultimately translates into therapeutic benefit (Fig. 4f). Inhibitors of Wnt signalling or the R-spondin–Lgr5 axis have also shown efficacy in patient-derived xenograft models of LUAD.25,26 We identified specific components of the Wnt–frizzled and R-spondin–Lgr5 signalling pathways that may serve as entry points for therapeutic approaches aimed at disrupting the interactions between niche cells and stem-like cells in LUAD (Fig. 4g).

Adenocarcinoma (PDAC) cell lines12. In line with these findings, we detected porcupine+ cells in close proximity to a subpopulation of Lgr5+ cells, which had increased proliferative potential in a PDAC GEMM (Extended Data Fig. 7c–f). Furthermore, Lgr5+ stem-like cells have been described in intestinal adenomas.7 We detected porcupine+ cells in close proximity to the Lgr5+ cells in ApcΔ/Δ; Lgr5GFP,CreER+ mouse intestinal adenomas and porcupine expression in human colorectal carcinomas (Extended Data Fig. 7g, h). These results suggest that paracrine Wnt signals may maintain subpopulations of cancer cells in a stem-like state in other epithelial cancers.

To explore the relevance of increased Wnt signalling in human non-small cell lung cancer, we examined gene expression patterns in The Cancer Genome Atlas (TCGA) LUAD dataset. A ligand-stimulated Wnt gene expression signature22 correlated with poor survival and higher tumour grade, and was independently prognostic in human LUAD, but not in squamous cell lung cancer (Extended Data Fig. 8a–c and Supplementary Table 1). We then extended this analysis to 34 additional human cancers within the TCGA dataset, and found similar correlations in PDAC and in mesothelioma (Extended Data Fig. 8d, e).

Given that KP LUAD cells respond to R-spondins through Lgr4 and Lgr5, we investigated the requirement for these genes in the KP model. CRISPR–Cas9-based combined inactivation of Lgr4 and Lgr5 in the KP model led to reduced lung tumour burden and prevented progression of adenomas to adenocarcinomas (Fig. 3d), similar to what was observed when targeting Porcn (Fig. 2g). Tumours that progressed into adenocarcinomas predominantly contained wild-type alleles or small non-frameshift mutations in Lgr4 and Lgr5 (Extended Data Fig. 8f–h). These data are consistent with a key role for Lgr4 and Lgr5 in the progression into adenocarcinomas. We detected expression of the Lgr4/Lgr5 ligands Rspo1 and Rspo3 in KP LUAD; these ligands localized predominantly to endothelial cells in the tumours (Extended Data Fig. 9a, b). Analogously, endothelial cells expressing R-spondin form a part of the niche for liver stem cells.23

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In this study, we observed the emergence of porcupine+ niche cells and Lgr5+ stem-like cells as KP lung adenomas progress to adenocarcinomas. This transition is also associated with amplification of the mutant KrasG12D locus and consequent increase in mitogen-activated protein kinase activity, as well as upregulation of tissue

Figure 4 | Porcupine inhibition improves survival by suppressing proliferative potential in mice with lung adenocarcinoma. a, Fold change in bioluminescence signal in autochthonous KP LUAD tumours with a Rosa26luciferase+ allele in mice treated with LGK974 or vehicle. n = 3. b, Survival of mice with autochthonous KP LUAD tumours treated with LGK974 or vehicle starting at 77 days following tumour initiation. P = 0.0088; n = 5 (LGK974), 8 (vehicle). c, Lung μCT (microcomputed tomography) images of mice treated with vehicle or LGK974 at 77 days after tumour initiation and after 40 days on therapy (day 117). H, heart; T, tumour. d, Quantification of proliferating (Ki67+) cells in autochthonous KP LUAD tumours two weeks after treatment with LGK974 or vehicle. n = 80 vehicle tumours, n = 59 LGK974 tumours. e, Quantification of the number of tdTomato+ surface tumours in recipient mice. Data are mean ± s.d.; *P < 0.05; ****P < 0.0001; Student’s two-sided t-test (a, b, d); or two-way ANOVA (e). f, The outcome of Wnt inhibition in LUAD. Porcupine+ niche cells provide Wnt to Lgr5+ cells with robust proliferative potential, which can be suppressed by Wnt inhibitors. g, The niche for Wnt responder cells in LUAD. Lgr5+ cells (green) reside next to porcupine+ cells (red). Wnt5a, Wnt7a and Wnt7b, provided by porcupine+ niche cells (red), bind frizzled on Wnt responder cells. Rspo1 and Rspo3, which bind Lgr4 and Lgr5, reinforce Wnt signalling by inhibiting Rnf43 and Znrf3 ubiquitin ligases that degrade frizzled-linked Wnt. Wnt is palmitoylated (seratted line) by porcupine, which is essential for Wnt secretion and binding to frizzled.1
repair pathways. Therefore increased proliferation and activation of regenerative pathways may contribute to activation of Wnt signalling in adenocarcinoma. Notably, heterogeneous Wnt pathway activation and Lgr5 expression in progenitor-like cells is also observed during repair of normal epithelial tissues, suggesting that lung adenocarcinomas may be co-opting a latent tissue regenerative program upon progression. Our results indicate that Wnt pathway activity in a subset of cancer cells is essential for the maintenance of proliferative potential in LUAD, which presents a therapeutic opportunity for the treatment of lung adenocarcinoma and other epithelial cancers.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions T.T. and T.J. designed and directed the study; T.T., K.W., Y.P. and R.A.W. performed all types of experiments reported in the study; F.J.S.-R. performed CRISPR gene activation experiments and analysed CRISPR-mutated loci; N.M.C. and K.H. performed gene expression analysis and N.M.C. performed ISH; N.S.J., L.S. and P.K. performed FACS; R.A. and N.R.K. performed bioinformatic analyses; W.X. generated shRNA reagents; A.B. conducted bioinformatic analyses; F.J.S.-R., N.S.J., Ö.H.Y., P.K. and A.B. provided conceptual advice; T.T., T.J. and T.J. designed and directed the study; T.T., K.W., Y.P. and R.A.W. performed all types of experiments reported in the study; F.J.S.-R. performed CRISPR gene activation experiments and analysed CRISPR-mutated loci; N.M.C. and K.H. performed gene expression analysis and N.M.C. performed ISH; N.S.J., L.S. and P.K. performed FACS; R.A. and N.R.K. performed molecular cloning and R.A. quantified Ki67+ nuclei; X.G. performed cell culture experiments; M.C.B. developed and used microcomputed tomography analysis methodology; W.X. generated shRNA reagents; A.B. conducted bioinformatic analyses; F.J.S.-R., N.S.J., Ö.H.Y., P.K. and A.B. provided conceptual advice; T.T. and T.J. wrote the manuscript with comments from all authors.

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METHODS

Mice. Previously published Kras<sup>SL-G12D</sup> (ref. 28), Trp53<sup>Flt3</sup> (ref. 29), Kras<sup>F595G12D</sup> (ref. 30), Trp53<sup>APC<sup>−/−</sup></sup> (ref. 31), Rosa26<sup>Lau</sup>-tdTomato (ref. 32), Aper<sup>−/−</sup> (ref. 33), Rosa26<sup>GFP</sup> (ref. 34), Rosa26<sup>GFP-CreER</sup> (ref. 35), Lgr5<sup>GFP-REER-CreER<sup>T2</sup></sup> (ref. 36) and Lgr5<sup>CreER<sup>T2</sup></sup> (ref. 8) gene-targeted mice were used in the study. All mice were maintained in a mixed sv129/C57BL/6 genetic background. Tumours were induced in KP mice with 2.5 × 10<sup>4</sup> plaque-forming units (PFU) of AdCMV-Cre (Iowa), 2 × 10<sup>4</sup> PFU of AdSPC-Cre<sup>−/−</sup>, 1 × 10<sup>4</sup> PFU of AdCMV-Flp(O) (Iowa) or 15–50,000 transforming ml−1 of ampicillin-resistant ml−1 in mice that were between 8–12 weeks of age. Approximately equal numbers of male and female mice were included in all experimental groups in all mouse experiments. Bearing lung tumours were treated with 10 μg per kg per day of LGK974 (ref. 20) resuspended in 0.5% carboxymethylcellulose (Sigma-Aldrich) and 0.5% Tween 80 (Sigma-Aldrich) or vehicle (0.5% carboxymethylcellulose and 0.5% Tween 80 only). Weights of mice were followed weekly. The growth of autochthonous Kras<sup>SL-G12D</sup>; Trp53<sup>APC<sup>−/−</sup></sup>; Rosa26<sup>Lau</sup> lung tumours was followed longitudinally by bioluminescence imaging, as previously described<sup>38</sup>. In brief, mice were anaesthetized by isoflurane inhalation, administered 100 mg kg<sup>−1</sup> intra-peritoneal thiopental (Perkin Elmer) by intraperitoneal injection and imaged after 10 min, using the IVIS imaging system (Perkin Elmer). Such longitudinal imaging experiments were repeated three times and representative data from one such experiment is shown in Fig. 4a. Survival experiments were repeated three times and representative data from one such experiment is shown in Fig. 4b. For survival experiments, mice were randomized based on their tumour burden as assessed by μCT. Mice were assigned a tumour burden score ranging from 0 (no tumours) to 10 (lungs completely full of tumours), and experimental groups were formed such that each group had approximately equal average tumour burdens. Mice with tumour burden scores under 2 were humanely euthanized. Animal studies were approved by the Massachusetts Institute of Technology (MIT) Committee for Animal Care (institutional animal welfare assurance no. A-3125-01). The maximal tumour dimensions permitted by the MIT Committee for Animal Care were 2 cm across the largest tumour diameter and this limit was not reached in any of the experiments.

Isolation of primary mouse lung adenocarcinoma cells. Mice bearing Kras<sup>G12D</sup>; Trp53<sup>APC<sup>−/−</sup></sup> (KPT) or Kras<sup>F595G12D</sup>; Trp53<sup>APC<sup>−/−</sup></sup>; Rosa26<sup>GFP-CreER</sup> (KPT;Lgr5<sup>GFP-REER-CreER<sup>T2</sup></sup>) lung tumours were euthanized 12–26 weeks after tumour induction and perfused with S-MEM (Gibco) through the right ventricle of the heart. Dissected lungs with tumours were dissociated in protease and DNase solution of the Lung Dissociation kit (Miltenyi Biotech) followed by mechanical dissociation using MACS C columns (Miltenyi Biotech) according to the manufacturer’s instructions. The dissociated cells were filtered using a 100-μm strainer and red blood cells were lysed using ACK (Thermo Scientific), followed by staining with APC-conjugated CD31 (Biolegend, 102510), CD45 (BD, 559864), CD11b (eBioscience, 17-0112-82) and TER119 (BD, 557909) antibodies and dead cells with DAPI (Sigma-Aldrich). The same approach using the Lung Dissociation kit (Miltenyi Biotech) was used to isolate tumour organoids from Aper<sup>−/−</sup> mice that were transplanted into NOD/SCID- γ<sup>−/−</sup> nu mice. All oligonucleotides used in this study are listed in Supplementary Table 4.

Low-density 3D organotypic cell culture. 150–1,000 primary mouse KP LUAD cells, cells from established KP LUAD cell lines, or primary mouse PDAC cells were mixed in 50% Matrigel (BD) and 50% advanced DMEM/F12 (Gibco) and plated on 10 μl of Matrigel. The gel was allowed to solidify at 37 °C, followed by addition of advanced DMEM/F12 (Thermo Scientific) supplemented with gentamicin (Thermo Scientific), penicillin–streptomycin (VWR), 10 mm HEPES (Thermo Scientific) and 2% heat-inactivated fetal bovine serum. For Wnt pathway manipulation, cultures were incubated with 1 μg ml<sup>−1</sup> recombinant mouse (rm)R-spondin 1 (Sino Biological), 100 ng ml<sup>−1</sup> IGF1R antibodies (R&D Systems), 500 ng ml<sup>−1</sup> or 1 μg ml<sup>−1</sup> rmmDKK1 (R&D Systems) or 100 nm LGK974 (Medchem Express) for 6–14 days. Medium was changed every two days. At the end of the experiment, proliferating cells were labelled with 10 μM Edu for 4 h, followed by paraformaldehyde fixation and fluorescent labelling of proliferating cells using the Click-IT Edu Alexa Fluor 488 Imaging kit (Thermo Scientific), according to the manufacturer’s protocol, in whole-mount preparations of tumour spheroids. Proliferating spheroids were quantified using a Nikon E800 microscope: a spheroid was classified as a cluster of at least 10 cells, and a proliferating spheroid contained at least one Edu<sup>−</sup> positive nucleus (proliferating cells were not observed in clusters of cells smaller than 10 cells). At least four replicate wells per condition were quantified in each experiment. Images were acquired using a Nikon A1B confocal microscope. Stimulation and inhibitor experiments were reproduced at least 10 times for each experimental condition.

Cell lines. Multiple cell lines were established from the mouse LUAD and PDAC KP GEMMs over the course of the study. The cell lines have not been authenticated. The cell lines were routinely tested for mycoplasma and found to be negative. At the time of conducting the experiments, no cell lines used were found to be listed in the ICLAC database of misidentified cell lines.

Immunohistochemistry. Tissues or tumour organoids were fixed in 10% formalin overnight and embedded in paraffin. Immunohistochemistry (IHC) was performed on a Thermo Autostainer 360 with or without heat-mo- xenolysis of tissue sections. Antibodies were used at 1:100 to 1:500 dilution and counterstaining with antibodies against β-catenin (BD, 610153), Ki67 (Vector Labs, VP-RM04), glutamine synthetase (BD, 610517), or porcupine (Abcam, ab105543). Lungs from at least three tumour-bearing mice were analysed for each antibody. Livers and small intestines collected from three normal, healthy mice were used for β-catenin, glutamine synthetase and porcupine IHC. 65 human LUAD tumours samples in two separate tissue microarrays were analysed by IHC for β-catenin and porcupine. 5 human colorectal adenocarcinoma samples were stained with porcupine antibodies. All human tissue material was obtained commercially from Janssen Pharmaceuticals.

Tissue immunofluorescence. Mice were anaesthetized and perfused through the right cardiac ventricle with 1% paraformaldehyde. Lungs with tumours were dissected, immersed in 4% PFA overnight and frozen in OCT medium (Sakura Finetek). 7 μm sections were stained with antibodies to EpCAM (eBioscience, 17-5971-82), β-catenin (BD, 610153), GFP (Cell Signaling Technologies, 2956S; or Aves Labs, GFP-1020), CD11b (eBioscience, 17-0112-82) or porcupine (Abcam, ab105543). Lungs from at least three tumour-bearing mice were analysed for each antibody.

Quantification of cell proliferation in tumours. Digitally scanned images of Ki67-stained slides were created with the Aperio ScanScope AT2 at 20 × magnification. Aperio’s WebScope software was used to assess Ki67<sup>−</sup> density per tumour area.

A built-in IHC nuclear image analysis algorithm was used to classify cells on the basis of the intensity of the nuclear Ki67<sup>−</sup> stain. Nuclei were classified from 0 to 3+: only nuclei with moderate nuclear staining (2+) or intense nuclear staining (3+) were considered Ki67<sup>−</sup>. Tumour regions were outlined on WebScope before running the IHC nuclear image analysis algorithm such that the number of 2+ and 3+ cells was normalized to tumour area.

Quantitative PCR (qPCR). Total RNA was isolated from tumours or cells using the RNeasy plus kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of RNA using the SuperScript VILO cDNA synthesis kit (Thermo Scientific) performed in triplicate in diluted cDNA (1:10) using PerCP/Cy5.5 SYBR Green FastMix (Thermo Scientific) on a Bio-Rad cycler RT–PCR detection system. Expression was normalized to Actb or Gapdh. All oligonucleotides used in this study are listed in Supplementary Table 4. All qPCR experiments were reproduced using at least three biological replicates.

Alternatively, a Mouse WNT Signalling Pathway RT<sup>2</sup> Profiler PCR Array (Qiagen) was used according to the manufacturer’s instructions. Raw expression values were thresholded to remove genes that were not detected or had low expression (maximum C<sub>T</sub> value set to 33; 0 values set to 33). Array position to gene-name mapping details were retrieved from the manufacturer’s website (www. agilent.com). Expression values for all genes per array were normalized to the expression of the housekeeping gene Gusb. Three replicates of stroma samples and three replicates of tumour samples were compared to calculate fold change and differential expression significance values (two-sided t-test).

Lentiviral shRNA-mediated gene silencing. shRNAs were cloned into lentiviral pLKO.1 vectors (Addgene, 10878) or into pTRIPZ (Dharmacon) vectors and...
lentivirus was produced as previously described46. KP mouse LUAD cell lines were infected with the lentiviral vectors, followed by puromycin selection and, in the case of cells infected with the TRIPZ virus, incubation in 1 μg ml⁻¹ doxycycline for four days and RNA extraction for testing target knockdown (Extended Data Fig. 2a and not shown). Lgr5 or Lgr3 silenced, cells expressing pLKO.1 driving Lgr4 or Lgr5 shRNAs were generated by puromycin selection, followed by infection with TRIPZ vectors driving miR303-based Lgr4 or Lgr5 shRNAs and turboRFP under the control of a TET-responsive promoter. Cells were incubated in 1 μg ml⁻¹ doxycycline for two days and red fluorescent cells were sorted to generate pure cell lines expressing combinations of Lgr4 and Lgr5 shRNAs. All shRNA experiments were reproduced using at least three independent cell lines.

**TOPFLASH assay.** 10,000 of KP LUAD cells were plated in 100 μl of medium containing 10% FBS per well of a white-walled 96-well plate (Perkin Elmer). After 24 h, mouse KP LUAD cell lines were transfected using Attractene transfection reagent (Qiagen) according to the manufacturer’s instructions with 150 ng of the TOPFLASH Firefly (MS5) reporter41 (Addgene, 12456) and 20 ng of pE主张 SV40 Renilla (Addgene, 27163) constructs. In initial experiments, the Wnt-insensitive FOPFLASH (negative control) Firefly (MS1) reporter41 (Addgene, 12457) was used to rule out signal background (not shown). Cells were stimulated for 16 h with recombinant Rspo1 (1 μg ml⁻¹, Sino Biological), recombinant Wnt3a (100 ng ml⁻¹, R&D Systems) or their combination (RW) in advanced DMEM/F12 (Gibco), with supplements listed above. After stimulation, Firefly and Renilla signals were detected using Dual-Glo luciferase detection reagents (Promega) according to the manufacturer’s instructions. A Tecan Infinite 200 Pro plate reader and automated injector system was used to detect lucinescence. To control for transfection efficiency, Firefly lucinescence values were normalized to Renilla lucinescence values to generate a measurement of relative lucinescence units. Experimental data are presented as mean ± s.d. from three independent wells. All TOPFLASH experiments were reproduced using up to three independent cell lines.

**Application of the synergistic activation mediator system to overexpress components of the R-spondin–Lgr5 axis.** Catalytically dead Cas9 (dCas9)-based systems have recently emerged as powerful tools for transcriptionally activating endogenous genes48. Notably, these systems allow for overexpression of genes in their endogenous genomic context. To overexpress Rspo2, Rspo3 or Lgr5 in 7TCF±/LUAD cell lines, we used the SAM system, which is a three-component system based on: (1) the fusion of dCas9 to the transcriptional activator VP64 (a tandem repeat of four DALDDFDLDML sequences from herpes simplex viral protein 16, VP16); (2) a modified gRNA scaffold containing two MS2 RNA aptamers; and (3) the MS2–p65–HSF1 tripartite synthetic transcriptional activator49. In this system, sgRNA-dependent recruitment of dCas9–VP64 and RNA aptamers; and (3) the MS2–p65–HSF1 tripartite synthetic transcriptional activator. Non-clonal KrasG12D/+;Rosa26gfp/+ or KrasG12D/++;Lgr5GFP/+;Lgr5CreER/T2 cell lines stably expressing dCas9–VP64–blast (Addgene, 61425) and MS2–p65–HSF1–hygro (Addgene, 61426) were generated using sequential lentiviral transduction and selection with blasticidin and hygromycin, respectively. To overexpress Rspo2 or Rspo3 we designed four independent sgRNA sequences targeting the Rspo2 or Rspo3 transcription start site; sgRNAs targeting the upstream region of the Lgr5 gene were provided by L. Gilbert, M. Hornbeck and J. Weissman43. The sgRNAs were cloned into a lentiviral vector (Lenti-sgRNA-EFS::Cre (pUSEC) and fused with a 0.25-kb EFS promoter part, a 1.0-kb part corresponding to Cre and U6::sgRNA-EFS::Cre (pUSEC) lentivirus vectors were generated by Gibson assembly49. The sgRNAs were cloned into pSCEC as previously described46. The most efficient Lgr4 and Lgr5 sgRNAs were cloned into the pSCEC vector together with a synthetic mouse/human U6 promoter (u6), as previously described50, to generate U6:sglgr4-u6:1-Lgr5-EFS::Cre (pUSEC).

**Measurement of Wnt signalling pathway activity in tumours in vivo.** A KPT LUAD cell line was engineered to express 7TCF:: Luciferase–PGK–Puro (7TTF) lentiviruses46, selected for puromycin resistance, and transplanted subcutaneously into flanks of immunodeficient athymic nu/nu mice. Three weeks after transplantation, tumour burden was measured by registering tdtomato fluorescence using an IVIS imaging system (Perkin Elmer), followed by administration of 100 mg kg⁻¹ d-Luciferin (Perkin Elmer) and detection of the luciferase signal (7TCF promoter activity). The luciferase signal was normalized to the tdtomato signal (Wnt pathway activity/total tumour burden). Quantification of Wnt pathway activity was performed every 24 h for a week in mice treated with 10 mg per kg per day of Lgr974 or vehicle. The maximal tumour dimensions permitted by the MIT IACUC were 2 cm across the largest tumour diameter and this limit was not reached in these experiments. This experiment was performed twice.

**Single-molecule mRNA in situ hybridization.** Single-molecule in situ hybridization was performed on formalin-fixed paraffin-embedded tissues using the Advanced Cell Diagnostics RNAscope 2.5 HD Detection kit (322360). Calibration tube numbers of the probes are 400331 (Axin2), 318321 (Lgr4), 312171 (Lgr5) and 40971 (Porcn), 40199 (Rpo1), 316791 (Wnt5a), 401121 (Wnt7a) and 401131 (Wnt7b). Lungs from three tumour-bearing mice were analysed.

**Lineage-tracing of Lgr5+ cells in KP tumours.** We generated KrasG12D/G12D;Rosa26mTmG/+ mice and induced tumours by intratracheal administration of AdCMV-FpO3. Lung tumours were collected, enzymatically dissociated and passed in vitro for 8–10 passages to erode stromal cells from the cultures. Such early-passage cell lines were transplanted subcutaneously into flanks of NSG mice. When mice developed palpable tumours, they were administered a single tamoxifen pulse (20 mg kg⁻¹) or corn-oil vehicle control. Tumours were collected at 2 days or 14 days after tamoxifen administration and prepared for cryosectioning. Three sections 50 μm apart were prepared from each tumour and imaged under a fluorescence microscope. The number of GFP+ cells per section was quantified in nine tumours per time point.

**Tumour burden and microcomputed tomography (μCT) imaging.** The previously published Wnt signalling geneset22 (24 genes upregulated in advanced DMEM/−/−DOX HU was determined to represent the range of normal lung parenchyma values. A custom analysis script was created using MATLAB (MathWorks) to identify a region of interest (ROI) including the soft tissue of the mouse thorax. Within this region the volume of tissue within the ‘healthy’ density range was measured. Within this volume subintensity windows (MinP) were created, both to confirm the accuracy of the ROI and to qualitatively assess lung pathology. For data visualization, the change in healthy lung volume was inverted to represent the change in tumour volume (Extended Data Fig. 10b).

**Human clinical data analyses.** RNA-seq gene expression profiles of primary tumours and relevant clinical data of 488 patients with lung adenocarcinoma were obtained from The Cancer Genome Atlas (TCGA LUAD; http://cancergenome.nih.gov/). The previously published Wnt signalling geneset22 (24 genes upregulated after stimulation with recombinant human WNT3A) was obtained from the Molecular Signatures Database (MSigDB)23 and used to score individual patient expression profiles using ssGSEA23. Patients were stratified according to their correlation score, into top (n = 115) and bottom (n = 114) 20th percentile sets. Kaplan–Meier survival analysis and log-rank test was used to assess significance. Subsequently, the Kaplan–Meier survival analysis methodology was extended to assess significant survival differences across 35 TCGA cancer types using a similar strategy.

Additionally, the Cox proportional hazards regression model was used to analyse the prognostic value of the published geneset22 across all patients within the TCGA LUAD cohort, in the context of additional clinical covariates. All univariate and multivariable analyses were conducted within a five-year survival timeframe. The following patient and tumour-stage clinical characteristics were used: signature (signature from ref. 22 strong versus weak correlation); gender © 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
of healthy lung volume by the quantification of 3D spheroids, proliferating (Ki67 between the groups that were compared. No animals were excluded from any of
Variation was estimated for each group of data, the variance was found to be similar
for each

+ using Annovar 57. The alignment and post-processing code was implemented in

reads were summarized within the extents of the sgRNA sequence and PAM site by

gramming alignment 56 was performed between the reduced state space of the
gene

lower quality 3 sequenced on Illumina MiSeq sequencers to generate 150-bp, paired-end reads.

Preparation kit (Illumina) according to the manufacturer’s instructions and

target sequences were amplified using Herculase II Fusion DNA polymerase and

versus normal, versus non-smoker, reformed

15 years versus non-smoker, reformed (ref 15 years versus non-smoker, current smoker versus non-smoker); mutational load (as defined by the number of non-silent mutations per 30 Mb of coding sequence, continuous); Union for International Cancer Control (UICC) TNM Stage specification (stage III/IV versus I/II); UICC T score specification (T2 versus T1, T3/T4 versus T1); UICC N score specification (N1/N2 versus N0). Hazard ratio proportionality assumptions for the Cox regression model were validated by testing for all interactions simultaneously (P = 0.703). Interactions between gene expression and Trp53 status were defined a priori as being significant if the Cox regression model with the maximum likelihood estimate of the regression coefficient (significant covariates in the model) were tested using a likelihood ratio test to contrast a model consisting of both covariates another model consisting of both covariates plus an interaction term. No statistically significant difference was found between the two models (TNM, P = 0.8751; T score, P = 0.8204; N score: P = 0.8625; likelihood ratio test). To test for statistically significant differences between the previously published 34–36 signature correlation scores across TCGA LUAD grade levels (T scores), the Kurskal–Wallis test was used to assess overall significance and the Mann–Whitney–Wilcoxon test was used to assess pairwise differences. All statistical analyses were conducted in R (http://www.R-project.org) and all survival analyses and were conducted using the survival package in R.

Finally, we analysed the expression of Wnt pathway genes present in the Mouse WNT Signalling Pathway RT2 Profiler PCR Array (Qiagen) in the human TCGA LUAD data (Supplementary Table 3). Expression levels between 57 LUAD tumour samples and corresponding matched normal samples were analysed using empirical cumulative distribution function plots. Significance of different expression levels was assessed using the Kolmogorov–Smirnov test. For a more comprehensive analysis covering human orthologues of all WNT pathway genes tested on the mouse qPCR array, pairwise differential expression analysis (tumour versus normal for each) was performed using EBSeq version 1.4.0 (ref. 34). Massively parallel sequenced CRISPR-Cas9-induced mutations were detected as before 35. Briefly, genomic regions containing the sgPorcn, sgIgr4 or sgIgr5 target sequences were amplified using Herculase II Fusion DNA polymerase and gel purified (primer sequences are shown in Supplementary Table 4). Sequencing libraries were prepared from 50 ng of PCR product using the Nextera DNA Sample Preparation kit (Illumina) according to the manufacturer’s instructions and sequenced on Illumina MiSeq sequencers to generate 150-bp, paired-end reads.

Bioinformatic analysis of target loci. CRISPR-Cas9-mutated loci were computationally analysed as before 35. Briefly, Illumina MiSeq reads (150 bp paired-end) were trimmed to 120 bp after reviewing base profile quality, in order to remove lower quality 3′ ends. Traces of Nextera adapters were clipped using the FASTX toolkit (Hannon Laboratory, CSHL) and pairs with each read greater than 15 bp in length were retained. Additionally, read pairs where either read had 50% or more bases below a base quality threshold of Q30 (Sanger) were removed from subsequent analyses. The reference sequence of the target locus was supplemented with 10bp genomic flanks and was indexed using an enhanced suffix array 36. Read ends were anchored in the reference sequence using 10bp terminal segments for a suffix array index lookup to search for exact matches. A sliding window of unit step size and a maximal soft-clipping limit of 10 bp were used to search for possible anchors at either end or both ends of each read. The RepeatMasker 55 and GenScan 343–345 (2009) programs known to be transcribed. A base alignment 56 was performed between the reduced state space of the read sequence and the corresponding reference sequence spanning the maximally distanced anchor locations. Scoring parameters were selected to allow sensitive detection of short and long insertions and deletions while allowing for up to four mismatches and the highest scoring alignment was selected. Read pairs with both reads aligned in the proper orientation were processed to summarize the number of wild-type reads and the location and size of each insertion and deletion event. Overlapping reads within pairs were both required to support the event if they overlapped across the event location. Additionally, mutation events and wild-type reads occurring in the extents of the subregions were identified by considering read alignments that had a minimum of 20 bp overlap with this region. Mutation calls were translated to genomic coordinates and subsequently annotated using Annovar 57. The alignment and post-processing code was implemented in C++ along with library functions from SeqAn 58 and SSV 59 and utility functions in Perl and R (http://www.R-project.org). Mutation calls were subjected to manual review using the Integrated Genomics Viewer (IGV) 60.

Statistics and reproducibility. Statistical analysis was carried out as indicated in the Figure legends, Extended Data Figure legends and in the Methods for each experiment. The data were found to meet the assumptions of the statistical tests. Variance was estimated for each group of data, the significance with differences between the groups that were compared. No animals were excluded from any of the studies. The investigator was blinded with respect to group assignment for the quantification of 3D spheroids, proliferating (Ki67+) cells and for the analysis of healthy lung volume by μCT. Power calculations were performed to estimate the sample size for experiments involving LGK974 treatment. In brief, to detect a difference of 30% in average survival between the two groups (effect size = 1.2 s.d. of survival based on Cohens’ d (ref. 61) using untreated sample baseline survival from ref 39) with 90% power, a minimum of five mice per group needed to be used.

Data availability. Massively parallel sequenced data are available in the NCBI/ SRA data repository under accession number PRJNA379539. Source code and all other data available from the authors upon reasonable request.
Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | Inhibiting Wnt synthesis with the porcupine inhibitor LGK974 suppresses Wnt pathway activation by the R-spondin–Lgr5 axis in primary lung adenocarcinoma cultures.

**a**, Percentage of EdU+ (proliferating) cells in 3D cultures of KPT lung adenocarcinoma (LUAD) cells followed by Wnt pathway stimulation with Rspo1 (1 μg ml⁻¹), Wnt3a (100 ng ml⁻¹) or both (1 μg ml⁻¹ Rspo1 and 100 ng ml⁻¹ Wnt3a), or Wnt pathway inhibition with LGK974 (100 nM) or DKK1 (500 ng ml⁻¹) for seven days (starting at seven days after plating). EdU labelling was performed 16 h before analysis of proliferating cells by flow cytometry. n = 6 wells per condition.

**b**, Percentage of spheroids proliferating in low-density 3D cultures of primary mouse KPT LUAD cells 14 days after plating. n = 8 wells per condition. Representative data from eight replicate experiments; TT5678 and TT5861 identify donor mice. **c**, qPCR of Porcn transcripts in sublines of a KP LUAD cell line expressing shRNAs targeting Porcn (shPorcn) or control shRNA (shLuc). Quantification of 3D tumour spheroids containing EdU+ cells of KP LUAD cells expressing the indicated shRNAs in response to 100 ng ml⁻¹ Wnt3a or control at six days after plating. Representative data of n = 3 technical replicates, the experiment was performed with three independent cell lines.

**d**, Quantitative real-time PCR (qRT–PCR) analysis of Axin2 and Lgr5 transcripts in 3D cultures of primary KP LUAD cells following Wnt pathway stimulation with Rspo1 (1 μg ml⁻¹), Wnt3a (100 ng ml⁻¹) or both (1 μg ml⁻¹ Rspo1 and 100 ng ml⁻¹ Wnt3a), or Wnt pathway inhibition with LGK974 (100 nM) or DKK1 (1 μg ml⁻¹) for six days (starting at 10 days after plating). Representative data of n = 3 technical replicates. The experiment was carried out three times, each time with cells isolated from a different tumour-bearing mouse.

**e**, Quantification of tumour spheroids containing EdU+ cells per 100 primary KPT LUAD cells 14 days after plating. Combined treatment (1 μg ml⁻¹ Rspo1 and 100 ng ml⁻¹ Wnt3a; LGK974 (100 nM). n = 8 wells per condition. **f**, Recipient mouse lungs eight weeks after orthotopic transplantation of 30,000 primary tdTomato+ (red) mouse KP LUAD cells. Arrowheads indicate tdTomato+ tumours. Recipient mice were treated with 10 mg per kg per day LGK974 or vehicle for eight weeks, starting from the day of transplantation. Scale bar, 2 mm. The experiment was performed three times, each time with cells isolated from a different (donor) tumour-bearing mouse. **h**, Wnt-pathway activity measured by detection of firefly luciferase driven by a Wnt-sensitive 7TCF promoter (Topflash assay) in mouse KP LUAD cells stimulated for 24 h with the indicated growth factors. n = 3 technical replicates per condition. Representative data from experiments that were performed with four different cell lines. **i–k**, qPCR analysis of Rspo2 (i), Rspo3 (j) or Lgr5 (k) transcripts in two independent mouse KP LUAD cell lines (TT5734 and TT5764) expressing SAM components driving expression of the indicated genes. **l**, Flow cytometry analysis of GFP fluorescence in a KP LUAD cell line containing the Lgr5GFP-CreER reporter expressing vector control (top) or an sgRNA targeting the transcription start site of Lgr5 (bottom). **m, n**, qRT–PCR analysis of the Wnt target genes Axin2 (m) and Lgr5 (n) in 3D cultures of sublines of a KP LUAD cell line (TT5764) expressing the CRISPR-activator system driving expression of Rspo2 (Rspo2-a), Rspo3 (Rspo3-a) or Lgr5 (Lgr5-a). Data are mean ± s.d.; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; n.s., not significant; two-way ANOVA (a, b, d, h, m, n) or Student’s two-sided t-test (c, f, i–k).
Extended Data Figure 2 | Lgr4 and Lgr5 are R-spondin receptors in lung adenocarcinoma. a, qRT–PCR analysis of Lgr4, Lgr5 and Lgr6 transcripts in sublines of a KP LUAD cell line stably expressing shLgr4, shLgr5, shLgr6 or shLuciferase (shLuc) control. Note minimal effects of the indicated shRNAs on other Lgr5 family members. n = 3 technical replicates per condition; representative data from three experiments carried out in different cell lines. b–e, Formation of 3D tumour spheroids of sublines of a KP LUAD cell line expressing the indicated shRNAs in response to 1 μg ml⁻¹ Rspo1 (b, c) or 100 ng ml⁻¹ Wnt3a (d, e) six days after plating. n = 8 wells per condition, representative data from three experiments carried out in different cell lines. No difference in cell morphology (f) or growth rate (g) in sublines of a KP LUAD cell line expressing shLgr4, shLgr5, shLgr6 or control shLuciferase (shLuc) over six days in two-dimensional cell culture. Scale bars, 500 μm (b, d) and 200 μm (f). Data are mean ± s.d.; *P < 0.05; **P < 0.01; ***P < 0.001; Student’s two-sided t-test (a) or two-way ANOVA (c, e, g). All experiments were performed three times, each time with a different KP LUAD cell line.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | Wnt ligands predominantly produced by cancer cells drive activation of the Wnt signalling pathway in lung adenocarcinoma. a, Schematic representation of the lentiviral vector used to transduce a KPT LUAD cell line, followed by puromycin selection. b, tdTomato and 7TCF::Luciferase signals at baseline (0 h) and 48 h after treatment with 10 mg per kg per day LGK974 or vehicle. Red arrows indicate two subcutaneous tumours with reduced 7TCF-dependent bioluminescence in response to 48 h of LGK974 treatment. c, Suppression of 7TCF-driven bioluminescence by LGK974 relative to the tdTomato signal in mice with subcutaneous transplants of the KPT LUAD cell line stably expressing the 7TCF::Luciferase-PGK-Puro lentivirus. n = 6 tumours, three mice per group; representative data from two independent experiments. Data are mean ± s.d.; Student’s two-sided t-test. d, Haematoxylin and eosin (HE) or β-catenin staining in KP adenomas or in adenocarcinomas. e, Immunofluorescence for β-catenin (red) and porcupine (green) in a KP LUAD. f, ISH for Axin2 mRNA (purple, arrowheads) in KP lung adenomas or adenocarcinomas. g, Immunofluorescence for porcupine (green, white arrowheads) and ISH for Axin2 (red, yellow arrowheads) in a KP LUAD. h, Immunofluorescence for porcupine (green) in an autochthonous grade 3 KPT adenocarcinoma. Arrowheads indicate peritumoural porcupine+ cells, which are tdTomato− (that is, not cancer cells). i, CD11b (green) and porcupine (red) immunofluorescence in a peritumoural region. White line delineates the tumour (T). j, Immunohistochemistry for β-catenin or porcupine in human LUAD. Arrowheads indicate cells with nuclear β-catenin. 65 human LUAD tumours in two tissue microarrays were analysed. Scale bars, 100 μm (d, e, h – j), 50 μm (f) and 10 μm (g). k, Comparison of PORCN gene expression in tumours versus normal tissue in the TCGA lung adenocarcinoma cohort: Empirical cumulative density function (CDF) plots of standardized gene expression values are shown. A right-shift indicates relatively higher expression, with P values indicated to assess statistical significance (Kolmogorov–Smirnov test).
Extended Data Figure 4 | Expression of porcupine in lung adenocarcinoma, the normal lung and in stem cell niches of the intestine and liver. a, ISH for Porcn in a grade 3 KP lung adenocarcinoma. b, c, Immunohistochemistry for porcupine (brown) (b), or Porcn ISH in the normal lung (c). Arrowheads indicate porcupine expression in bronchioles. d, Immunohistochemistry for β-catenin (brown) and porcupine (purple) in the small intestine. Note porcupine expression in intestinal crypts (black arrowheads), the location of Lgr5\(^+\) intestinal stem cells\(^{36}\), as well as in transit-amplifying cells (blue arrowheads) and stromal cells (dark green). e, Immunostaining for porcupine (brown) and glutamine synthetase (GS, purple) expression localizes to areas around the central vein of the liver (e), coinciding with the location of Lgr5\(^+\) Axin2\(^+\) liver stem cells\(^{8,62}\). Scale bars, 100\(\mu\)m.
Extended Data Figure 5 | Analysis of the Porcn locus following CRISPR–Cas9-mediated genome editing in vivo.

a, Haematoxylin and eosin staining of KP LUAD-bearing lungs generated with pSECC–sgTom or pSECC–sgPorcn and quantification of the proportion of adenomas versus adenocarcinomas at 12 weeks after tumour initiation. Scale bar, 2 mm. Data are mean ± s.d.; Student’s two-sided t-test; n = 5 mice per group. b, Massively parallel sequencing analysis of allelic fractions of the Porcn locus in lung lobes containing microscopic tumours (sgPorcn.2 L) or microdissected macroscopic tumours (sgPorcn.2 T) induced in KP mice using pSECC–sgPorcn.2, or in lung lobes or macroscopic tumours induced in KP mice using pSECC–sgTom.2 (sgTom.2 T/L). FS, frameshift mutation; low freq, low-frequency mutation event; NFS, non-frameshift mutation; WT, wild-type read. Note predominantly wild-type or non-frameshift reads in microdissected tumours, whereas mutations in tumours containing microscopic tumours have introduced frameshifts. The large contribution of wild-type reads in sgPorcn.2 L samples is owing to domination of the normal stroma in whole-lobe samples, whereas wild-type reads in sgPorcn.2 T indicate cancer cells where genome editing did not function, as in whole-tumour samples, tumour cells are expected to contribute at least 50% (ref. 49). c, Qualitative analysis of mutations introduced by sgPorcn.2 in vivo. bp, base pair (indicates the size of the insertion or deletion); INS, insertion; DEL, deletion. Ratio indicates frequency of event across the 15 samples analysed.
Extended Data Figure 6  See next page for caption.
Extended Data Figure 6 | Lgr5 and Lgr4 are expressed in lung adenocarcinoma and Lgr5 marks cells with increased tumour-forming ability. a, qPCR analysis of Lgr5 gene expression in KP LUAD tumours microdissected at 9 weeks (adenomas) or 20 weeks (adenocarcinomas) after initiation with adenoviral Cre. \( n = 6 \) tumours per group. b, ISH for Lgr5 or Lgr4 mRNA (purple) in grade 3 KP LUAD adenocarcinomas 12 weeks after tumour induction with AdCre. c, d, FACS sorting (d) of Lgr5\(^+\) (GFP\(^+\)) cells in a cultured KP LUAD cell line containing the Lgr5\(^{GFP-CreER}\)/+ reporter allele (KP;Lgr5\(^{GFP-CreER}\)/+), followed by qRT–PCR analysis (c) of Lgr4 expression in Lgr5\(^+\) cells in two independent cell lines (TT1937 and TT6280). This experiment was performed once. e, FACS of GFP\(^+\) and GFP\(^-\) cells isolated from KP;Lgr5\(^{GFP-CreER}\)/+ primary LUAD 14 weeks after tumour initiation with intratracheally administered AdCre. The FACS plot is gated on tdTomato\(^-\)CD11b\(^-\)CD31\(^-\)CD45\(^-\)TER119\(^-\) cells. Note bleeding of the tdTomato signal to the GFP channel in the panel on the right. Gates were drawn as shown to increase cell yield at the cost of purity to enrich for Lgr5\(^+\) cells. Such FACS sorting was performed on 21 KPT;Lgr5\(^{GFP-CreER}\)/+ mice. f, Recipient mouse lungs, 12 weeks after orthotopic transplantation of 15,000 primary Lgr5\(^+\)tdTomato\(^+\) or Lgr5\(^-\)tdTomato\(^+\) primary mouse LUAD cells. Arrowheads indicate tdTomato\(^+\) tumours (red). Representative data from three replicate experiments. g, Quantification of tumours per 1,000 cells in recipient mouse lungs 12 weeks after orthotopic transplantation of 15,000 primary Lgr5\(^+\)/tdTomato\(^+\) or Lgr5\(^-\)/tdTomato\(^+\) primary mouse LUAD cells. \( n = 3 \) recipient mice per group; representative data from three replicate experiments. h, Number of membrane-associated GFP\(^+\) (mGFP\(^+\)) clones following lineage-tracing in established subcutaneous KP;Lgr5\(^{CreER}\)/+;Rosa26\({LacZ-mTmG}\)/+ LUAD primary transplants (see Fig. 3c). \( n = 9 \) tumours/time point. Scale bars, 2 mm (f) and 100 \( \mu \)m (b). Data are mean ± s.d.; \(* P < 0.05; \text{n.s.}, \) not significant; Student’s two-sided \( t \)-test (a, c, g, h).
Extended Data Figure 7 | See next page for caption.
Extended Data Figure 7 | Phenotypic plasticity of Lgr5+ cells that reside in porcupine+ niches in lung, pancreatic and colon tumours. a, Immunofluorescence for GFP (green) and EpCAM (red) in a subcutaneous transplant established from a single-cell clone of a KPT;Lgr5CreER/cell line. b, Immunofluorescence for GFP (green) and porcupine (red) in a subcutaneous transplant established from a single-cell clone of a KPT;Lgr5CreER/cell line. a, b, Data are representative of four replicate experiments, each with a different KPT;Lgr5CreER/cell line. c, ISH for Lgr5 mRNA (purple) in KP;Pdx1::Cre PDAC. Representative of three PDAC tumours analysed. d, Immunofluorescence staining for GFP (green) in a tdTomato (red) autochthonous KP;Lgr5GFP-CreER/::.Pdx1::Cre PDAC. Note juxtaposition of Lgr5+ and porcupine+ cells in the tumours. d, f, Representative data from six KP;Lgr5GFP-CreER/::.Pdx1::Cre PDAC tumours analysed. g, Immunofluorescence staining for GFP (green) and porcupine (red) in an autochthonous ApcΔ/Δ;Lgr5GFP-CreER/::.intestinal adenoma. Again, note juxtaposition of Lgr5+ and porcupine+ cells in the tumours. n = 3 tumour samples. h, Immunohistochemistry for porcupine (brown) in human colorectal adenocarcinoma. Five human colorectal adenocarcinoma samples were analysed. Scale bars, 1 mm (a), 100 μm (b, c, d, f and h (top)), g) and 10 μm (c, d, f and h (bottom)).
Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | Wnt pathway activation correlates with poor survival in human lung adenocarcinoma, pancreatic ductal adenocarcinoma and mesothelioma, but not in human squamous cell lung cancer; analysis of the Lgr4 and Lgr5 loci following CRISPR–Cas9-mediated genome editing in vivo. a, Kaplan–Meier survival curve comparing the 20% strongest (red, n = 91) and weakest (blue, n = 92) correlations of the Wnt signature from ref. 22 and patient samples from the TCGA lung adenocarcinoma cohort. b, Empirical CDF plots of standardized gene expression values showing a correlation between the Wnt pathway activation gene expression signature correlation score and histological grade of primary tumours. A right-shift indicates relatively higher expression, with P values indicated to assess statistical significance (Kolmogorov–Smirnov test). c–e, Kaplan–Meier survival curves comparing the 20% strongest (red) and weakest (blue) correlations of the Wnt signature from ref. 22 and patients samples from the TCGA squamous cell lung cancer (c, most-correlated (red) n = 100, least-correlated (blue) n = 34), pancreatic ductal adenocarcinoma (d, most-correlated (red) n = 34, least-correlated (blue) n = 34) and mesothelioma (e, most-correlated (red) n = 17, least-correlated (blue) n = 18) cohorts. f, Massively parallel sequencing analysis of allelic fractions of the Lgr4 and Lgr5 loci in lung lobes containing microscopic tumours (lobe) or microdissected macroscopic tumours (tumour) induced in KrasLSL-G12D⁺/⁻;Trp53fl/fl;Rosa26LSL-Cas9²-CreERT²;hU6::sgLgr4-sU6::sgLgr5-EFS::Cre pU2SEC or hU6::sgTom-EFS::Cre pUSEC lentiviral vectors. FS, frameshift mutation; NFS, non-frameshift mutation; WT, wild-type read. Note predominantly wild-type or non-frameshift reads in microdissected tumours, whereas mutations in tumours containing microscopic tumours have introduced frameshifts. The large contribution of wild-type reads in lobe samples is owing to domination of the normal stroma in whole-lobe samples, whereas wild-type reads in Lgr4/Lgr5 co-targeted tumours indicate cancer cells where genome editing did not function, as in whole tumour samples, tumour cells are expected to contribute at least 50% (ref. 49). g, h, Qualitative analysis of mutations introduced by sgLgr4 or sgLgr5 in vivo. bp, base pair (indicates size of insertion/deletion); INS, insertion; DEL, deletion. Ratio indicates frequency of event across all samples analysed. P values are indicated (log-rank test).
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | Characterization of the niche for stem-like cells in lung adenocarcinoma. a, qPCR analysis of Rspo gene expression in 16 KP LUAD tumours, normalized to Actb expression. Tumours were collected at 16 weeks after initiation with adenoviral Cre. ISH for Rspo1 mRNA (purple, arrowheads) in a KP LUAD tumour. Note the Rspo1 transcripts in endothelial cells. b, qPCR for Rspo1 and Rspa3 in tdTomato+ tumour cells (tumour), CD31+ endothelial cells and the rest of the cells (stroma) in microdissected KPT LUAD tumours following sorting. The expression of Pecam1 (which encodes CD31) was found to be >400-fold enriched in the CD31+ fraction compared to the stroma (not shown). n = 3 mice, representative of two replicate experiments. c, Heatmap showing relative expression levels of Porcn and the 19 mouse Wnt genes on the basis of the qPCR analysis in sorted tdTomato+ KP LUAD cells (T) versus tdTomato− stromal cells (S) in microdissected tumours collected at 20 weeks after tumour initiation (a time point when most tumours are adenocarcinomas). d, Volcano plot of qPCR array gene expression analysis showing statistically significant differentially expressed genes (in red, Fzd receptors are circled). x axis is the log2 fold change (tumour/stroma) and y axis is the –log_{10} P value of the differential enrichment (two-sided t-test). e, qPCR analysis of Wnt5a, Wnt7a and Wnt7b gene expression in KP tumours microdissected at 9 weeks (adenomas) or 20 weeks (adenocarcinomas) after initiation with adenoviral Cre. n = 6 mice, representative of two replicate experiments. f, Comparison of WNT gene expression in tumours versus normal tissue in the TCGA lung adenocarcinoma cohort: Empirical CDF plots of standardized gene expression values for WNT5A and WNT7B are shown. A right-shift indicates relatively higher expression, with P values indicated to assess statistical significance (Kolmogorov–Smirnov test). g, Heatmap showing relative expression levels of Lrp5, Lrp6 and nine mouse Fzd genes on the basis of the qPCR analysis in sorted tdTomato+ KP LUAD cells (T) versus tdTomato− stromal cells (S) in microdissected tumours collected at 20 weeks after tumour initiation (a time point when most tumours are adenocarcinomas). h, qPCR analysis of eight Fzd receptors in KP tumours microdissected at 9 weeks (adenomas) or 20 weeks (adenocarcinomas) after initiation with adenoviral Cre. n = 3 mice. Data are mean ± s.d.; *P < 0.05; Student’s two-sided t-test (b, e, h).
Extended Data Figure 10 | Porcupine inhibition suppresses Wnt pathway activity, progression and proliferative potential in autochthonous mouse KP lung adenocarcinomas. a, qPCR analysis of Axin2 and Lgr5 transcripts in KP LUAD tumours two weeks after treatment with 10 mg per kg per day LGK974 or vehicle. Treatment was started at 11 weeks after tumour initiation. n = 6 tumours per group. b, Quantification of μCT data showing change in tumour volume compared to baseline (obtained at 76 days after tumour initiation, dashed line) after four weeks of 10 mg per kg per day LGK974 or vehicle control. c, Recipient mouse lungs four weeks after orthotopic GEMM-DA of 50,000 primary tdTomato⁺ (red) mouse LUAD cells. Arrowheads indicate tdTomato⁺ tumours. Donor mice bearing autochthonous KPT LUAD tumours were treated for two weeks with LGK974 or vehicle (starting at 84 days after tumour induction). The recipient mice were treated with LGK974 or vehicle for four weeks. d, tdTomato⁺ tumours in sections from lungs in c containing EdU⁺ (black) or EdU⁻ (grey) tumours per section through the lungs depicted in c. d, n = 5 (vehicle–vehicle), representative data from three replicate experiments. Data are mean ± s.d.; *P < 0.05; Student’s two-sided t-test (a, b) or two-way ANOVA (e).