Intratumoural heterogeneity generated by Notch signalling promotes small-cell lung cancer

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The Notch signalling pathway mediates cell fate decisions1,2 and is tumour suppressive or oncogenic depending on the context2,3. During lung development, Notch pathway activation inhibits the differentiation of precursor cells to a neuroendocrine fate4–6. In small-cell lung cancer, an aggressive neuroendocrine lung cancer7, loss-of-function mutations in NOTCH genes and the inhibitory effects of ectopic Notch activation indicate that Notch signalling is tumour suppressive8–9. Here we show that Notch signalling can be both tumour suppressive and pro-tumorigenic in small-cell lung cancer. Endogenous activation of the Notch pathway results in a neuroendocrine to non-neuroendocrine fate switch in 10–50% of tumour cells in a mouse model of small-cell lung cancer and in human tumours. This switch is mediated in part by Rest (also known as Nrsf), a transcriptional repressor that inhibits neuroendocrine gene expression. Non-neuroendocrine Notch-active small-cell lung cancer cells are slow growing, consistent with a tumour-suppressive role for Notch, but these cells are also relatively chemoresistant and provide trophic support to neuroendocrine tumour cells, consistent with a pro-tumorigenic role. Importantly, Notch blockade in combination with chemotherapy suppresses tumour growth and delays relapse in pre-clinical models. Thus, small-cell lung cancer tumours generate their own microenvironment via activation of Notch signalling in a subset of tumour cells, and the presence of these cells may serve as a biomarker for the use of Notch pathway inhibitors in combination with chemotherapy in select patients with small-cell lung cancer.

We examined Notch pathway activity in small-cell lung cancer (SCLC) by immunostaining for Hes1, a transcriptional target of the pathway9. Almost all tumours in a p53−/−/flox;Rb−/−/flox;p130−/−/flox conditional triple knockout (TKO) SCLC mouse model10 and a majority of human SCLC tumours expressed detectable levels of Hes1 (Fig. 1a–d and Extended Data Fig. 1a, b). In TKO Hes1GFP+ mice, in which green fluorescent protein (GFP) was expressed from the endogenous Hes1 promoter11 (Fig. 1e and Extended Data Fig. 1c, d), both GFP+ and GFP− cells within tumours underwent Cre-mediated recombination (Extended Data Fig. 1e–g). HES1-positive (HES1pos) cells within human tumours had histopathological features of SCLC tumour cells (analysed by a board-certified pathologist, C.K.), further supporting their tumoural origin. Relative to GFP− cells, GFP+ cells sorted from TKO Hes1GFP+ tumours expressed higher levels of Hes1, Narp (a Notch target12), and Notch1/2/3 (Fig. 1f). Conversely, GFP− cells expressed higher levels of most Notch ligands, including the atypical ligand Dll3, a known target of Ascl1, itself a key regulator of neuroendocrine (NE) differentiation and SCLC growth3,13 (Fig. 1f). Immunostaining for Hes1 and Notch2 in mouse tumours showed similar staining patterns (Extended Data Fig. 2a). Notch pathway ligands, receptors, and targets were also expressed in human SCLC cell lines15 and tumours (Extended Data Fig. 2b). Treatment with the γ-secretase inhibitor dibenzazepine (DBZ)16 led to the accumulation of the inactive form of Notch and decreased GFP, Hes1 and Narp expression in TKO Hes1GFP+ tumours (Fig. 1g and Extended Data Fig. 2c–g). GFP+ SCLC cells grown without the Notch ligand Dll4 showed decreased expression of GFP, Hes1, and the transcriptionally active Notch1 intracellular domain (N1ICD) (Fig. 1h and Extended Data Fig. 2h, i). Thus, a significant fraction of SCLC cells activate endogenous Notch signalling.

Consistent with the inhibition of NE differentiation by Notch during development4–6, GFPhigh cells expressed lower levels of NE genes relative to GFPGFP cells in TKO Hes1GFP+ tumours (Fig. 1i and Extended Data Fig. 2j–l). Non-NE SCLC cells marked by high expression of CD44 and mesenchymal markers (for example, vimentin) were previously described17, but the majority of GFP+ cells expressed the epithelial marker EpCam, had no detectable CD44 on their surface, and did not upregulate vimentin (Extended Data Fig. 2m, n), indicating that GFP+ cells and CD44+ cell populations within primary TKO tumours are largely distinct. Cell lines of GFP+ cells grew as floating clusters typical of NE SCLC while GFP+ cells grew adherently, further suggestive of a change in differentiation (Fig. 1j). Microarray gene expression analysis of GFP+ and GFPGFP cells (Extended Data Fig. 3a, b and Supplementary Table 1) supported an enrichment for genes and the inhibitory NOTCH ligands, receptors, and targets were also expressed in human SCLC cell lines15 and tumours (Extended Data Fig. 2a). Notch pathway ligands, receptors, and targets were also expressed in human SCLC cell lines15 and tumours (Extended Data Fig. 2b). Treatment with the γ-secretase inhibitor dibenzazepine (DBZ)16 led to the accumulation of the inactive form of Notch and decreased GFP, Hes1 and Narp expression in TKO Hes1GFP+ tumours (Fig. 1g and Extended Data Fig. 2c–g). GFP+ SCLC cells grown without the Notch ligand Dll4 showed decreased expression of GFP, Hes1, and the transcriptionally active Notch1 intracellular domain (N1ICD) (Fig. 1h and Extended Data Fig. 2h, i). Thus, a significant fraction of SCLC cells activate endogenous Notch signalling.

On the basis of cell cycle and cell death analyses (Fig. 1k and Extended Data Fig. 5a), GFP+ cells should rapidly outcompete GFP− cells in tumours (Extended Data Fig. 5b), which is inconsistent with the observed ratio of approximately three GFP− cells to one GFP+ cell (Fig. 1e) and the similar frequencies of Hes1pos cells in early- and late-stage TKO tumours (Extended Data Fig. 1a). Tumours initiated by expressing Cre from the NE-specific CGRP promoter18 contained Hes1pos cells (Extended Data Fig. 5c–e), indicating that both non-NE Hes1pos and NE Hes1pos cells can arise from an NE cell of origin. In the analysis of TKO tumour cells by single-cell quantitative PCR with reverse transcription (qRT–PCR), all non-NE Hes1pos cells expressed at least one Notch receptor and all NE Hes1pos cells expressed at least...
one Notch ligand. However, a fraction of NE Hes1neg SCLC cells also expressed at least one receptor (Fig. 2a), suggesting that these cells may be responsive to Notch ligands. Indeed, stimulation of GFPpos cells with a high-affinity form of Dll4 (ref. 19) induced GFP expression and non-NE phenotypes in ~50% of cells (Fig. 2b, c and Extended Data Fig. 5f). Isolation and re-plating of the cells that remained GFPneg on Dll4-coated plates showed that a subset could again be induced to express GFP (Extended Data Fig. 5g, h), suggestive of a cyclic or

**Figure 1** | SCLC tumours contain slow-growing, Notch-active non-neuroendocrine tumour cells. a, b, Representative Hes1 immunohistochemistry (IHC) (a) and frequency of Hes1pos cells (b) in mouse SCLC (n = 5 mice, 83 tumours). c, d, As in a, b, for human SCLC (n = 172 sections). e, Flow cytometry analysis showing the percentage of GFPpos cells from pooled TKO Hes1GFPflox/flox tumours (n = 18 mice). f, qRT-PCR of Notch pathway genes in GFPpos relative to GFPneg tumour cells (n = 3 mice). g, As in e, with mice treated with dimethylsulfoxide (DMSO) (n = 5 mice) or DBZ (n = 4 mice). h, Immunoblots of GFPpos cell lines grown with Dll4 or 72 h after removal from Dll4. i, GFP and Uchl1 (NE marker) immunofluorescence in TKO Hes1GFPflox/flox tumours (representative of n = 3 biological replicates). j, Images of cell cultures established from GFPpos and GFPneg tumour cells (representative of n = 5 biological replicates). Inset: NE KP1 SCLC cell line. k, EdU incorporation in cells sorted from TKO Hes1GFPflox/flox tumours (n = 3 mice). *P < 0.05; **P < 0.01; ***P < 0.001. Two-tailed paired (f, k) or unpaired (g) Student’s t-test. Data are mean ± s.d. Scale bars, 50 μm.

**Figure 2** | Notch activation generates non-neuroendocrine cells from neuroendocrine SCLC cells. a, Unsupervised clustering of qRT–PCR (n = 46 cells) from one TKO Hes1GFPflox/flox tumour (representative of n = 2 experiments). Dark blue, undetectable expression; arrowheads, NE cells expressing at least one Notch receptor but have undetectable Hes1; C1, threshold cycle. b, Flow cytometry of freshly isolated GFPneg cells grown on Dll4 or PBS control (representative of n = 3 biological replicates). c, Immunoblot of b. GFPneg cells grown on Dll4 were re-sorted for GFPpos and GFPhigh populations. d, Relative number of GFPhigh cells generated from freshly isolated GFPneg cells grown on Dll4 after DBZ treatment (n = 3 biological replicates). e, Flow cytometry of a GFPhigh cell line treated with DMSO or DBZ while grown with or without Dll4 or co-cultured with three NE cell lines without Dll4 (n = 3 biological replicates). Median GFP intensity normalized to ‘DMSO; − Dll4. f, Freshly isolated GFPpos cells that became GFPhigh after Dll4 exposure were re-plated with or without Dll4. Flow cytometry and images are shown (representative of n = 2 biological replicates). *P < 0.05; **P < 0.01; ***P < 0.001. Two-tailed paired Student’s t-test. Data are mean ± s.d. Scale bars, 50 μm.
Rest is a Notch target that inhibits neuroendocrine differentiation in SCLC. a, Top ten candidate factors from Enrichr analysis for the regulation of genes downregulated in GFP<sup>high</sup> cells. b, qRT–PCR for Rest in cells sorted from TKO Hes<sup>1<sup>Cre<sup>ERT</sup></sup></sup> tumours (n = 4 mice). c, d, qRT–PCR for Rest (c) and Notch1 chromatin immunoprecipitation (ChIP)–qPCR (d) in KP1 cells overexpressing N1ICD (n = 3 biological replicates with n = 3 technical replicates). mSVE, mNC6R: negative control genomic regions. IgG, immunoglobulin-G.

Figure 3 | Rest is a Notch target that inhibits neuroendocrine differentiation in SCLC. a, Top ten candidate factors from Enrichr analysis for the regulation of genes downregulated in GFP<sup>high</sup> cells. b, qRT–PCR for Rest in cells sorted from TKO Hes<sup>1<sup>Cre<sup>ERT</sup></sup></sup> tumours (n = 4 mice). c, d, qRT–PCR for Rest (c) and Notch1 chromatin immunoprecipitation (ChIP)–qPCR (d) in KP1 cells overexpressing N1ICD (n = 3 biological replicates with n = 3 technical replicates). mSVE, mNC6R: negative control genomic regions. IgG, immunoglobulin-G.

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stochastic expression of Notch receptors on NE SCLC cells.Dll4 effects on GFP<sup>neg</sup> cells were suppressed by DBZ and the Notch2/3-blocking antibody tarextumab<sup>23</sup> (Fig. 2d and Extended Data Fig. 5i). Cells that express both Notch ligands and receptors are also present in human SCLC cell lines, and Dll4 induced HES1 in these lines (Extended Data Fig. 5j,k). Although GFP expression in GFP<sup>high</sup> cell lines depended on exogenous Dll4 (Fig. 1h and Extended Data Fig. 2h,i), this dependence was partly rescued by co-culture with NE cell lines and the rescue was blocked by DBZ (Fig. 2e and Extended Data Fig. 5l,m), indicating that NE SCLC cells can provide Notch ligands to SCLC cells expressing Notch receptors. Thus, a subset of NE SCLC cells express levels of Notch that may be sufficient for signalling induction by ligands on neighbouring cells, contributing to their transition towards a non-NE fate.

After establishment of the non-NE state, however, we observed no reversal to an NE state upon Notch pathway silencing (Fig. 2f and Extended Data Fig. 5n–p). SCLC cells with intermediate levels of Notch pathway activation results in a loss of neuronal phenotype (Fig. 3g, h and Extended Data Fig. 7l). Thus, induction of Rest is a critical step by which the Notch pathway inhibits NE differentiation in SCLC. Epigenetic remodelling involving repressor complexes containing Rest (reviewed in ref. 25) may contribute to the irreversibility of the Notch-driven NE to non-NE switch in SCLC cells.

Gene expression profiles suggested that non-NE Hes<sup>1pos</sup> SCLC cells might be involved in extracellular matrix organization and regulation of cell adhesion (Extended Data Fig. 3g), reminiscent of tumour–stroma interactions in other cancers<sup>24</sup>. Furthermore, GFP<sup>high</sup> and GFP<sup>neg</sup> cells resembled astroglial cells and neurons, respectively (Extended Data Fig. 8a), and astroglial cells can provide trophic support for neurons<sup>25</sup>. Therefore, non-NE tumour cells might act as a tumour-derived microenvironment and contribute to SCLC development through non-cell-autonomous effects on NE tumour cells. In a three-dimensional culture, bulk TKO Hes<sup>1<sup>Cre<sup>ERT</sup></sup></sup> tumour cells grew better than GFP<sup>high</sup> or GFP<sup>neg</sup> cells alone (Fig. 4a and Extended Data Fig. 8b–d). Co-culture with non-NE GFP<sup>high</sup> cell lines or with conditioned medium from GFP<sup>high</sup> cells promoted the growth of NE SCLC cell lines (Fig. 4b–e and Extended Data Fig. 8e–o). Of seven candidate secreted factors selected on the basis of fold expression changes from the microarray data (Supplementary Table 1) and biological features (for example, known role in neural/NE growth), only one showed a significant growth-promoting effect (Extended Data Fig. 8p,q), suggesting that factors not tested are implicated and/or that multiple factors act in concert to stimulate NE SCLC cell growth. Interestingly, however, the factor that promoted the growth of NE SCLC cells was midkine, which is secreted by glial cells to promote neuronal growth<sup>26</sup>. We detected midkine in the supernatant of GFPhigh SCLC cells and is induced after Dll4 stimulation of GFP<sup>neg</sup> cells (Fig. 3b and Extended Data Fig. 7f, g). Rest is also a direct target of N1ICD (Fig. 3c, d). Rest downregulation did not affect the expression of NE genes (Extended Data Fig. 7h, i) but reduced the ability of N1ICD to induce an NE to non-NE transition in SCLC cells (Fig. 3e and Extended Data Fig. 7j, k). Rest bound to the promoters of NE genes (Fig. 3f) and its overexpression in NE SCLC cells was sufficient to downregulate the expression of NE genes and convert these cells to a non-NE phenotype (Fig. 3g, h and Extended Data Fig. 7l). Thus, induction of Rest is a critical step by which the Notch pathway inhibits NE differentiation in SCLC. Epigenetic remodelling involving repressor complexes containing Rest (reviewed in ref. 25) may contribute to the irreversibility of the Notch-driven NE to non-NE switch in SCLC cells.

Figure 4 | Midkine induces growth of NE tumour cells. a, Adherent cell numbers in Rest wild-type (WT) or knockout (KO) clones after N1ICD transduction (see Extended Data Fig. 7j) (n = 3 biological replicates with n = 3 technical replicates each). b, Rest ChIP–qPCR in KP1 cells (n = 3 biological replicates with n = 3 technical replicates each). c, d, Images (g) and immunoblot (h) of KP1 cells ~1 month after Rest transduction (representative of n = 2 biological replicates). *P < 0.05; **P < 0.01. Two-tailed paired (b–d, f) or unpaired (e) Student’s t-test. Data are mean ± s.d. Scale bars, 50 μm.

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affected by Notch inhibition in co-culture assays (Extended Data Fig. 8t), indicating that the tumour-promoting effects of non-NE tumour cells are due to the differentiation state triggered by Notch activity, and not to ongoing Notch signalling.

A major factor in the dismal survival rates for SCLC is rapid relapse following standard-of-care chemotherapy (often cisplatin or carboplatin with etoposide).7 GFP<sup>high</sup> tumour cell lines survived better than GFP<sup>low</sup> tumour cell lines in response to cisplatin and etoposide (Extended Data Fig. 9a, b). Acute treatment of TKO mice with cisplatin and etoposide increased the number of cleaved caspase-3-positive (CC3<sup><sup>pos</sup></sup>) apoptotic tumour cells (Extended Data Fig. 9c), which correlated with an increase in the frequency of Hes1<sup><sup>pos</sup></sup> cells (Extended Data Fig. 9d); most of the CC3<sup><sup>pos</sup></sup> cells were Hes1<sup><sup>pos</sup></sup> GFP<sup><sup>low</sup></sup> cells in these experiments (Fig. 4f and Extended Data Fig. 9e). The frequency of Hes1<sup><sup>pos</sup></sup> SCLC cells was also higher in mice treated with a longer-term chemotherapy protocol (Extended Data Fig. 9f), suggesting that these cells might play a role in chemoresistance. In a cohort of patients with SCLC, we found a trend for HES1 positivity in tumours as a negative prognostic factor for overall survival and progression-free survival (Extended Data Fig. 9g–i and Supplementary Table 5). In an SCLC allograft model (Extended Data Fig. 10a, b), a combination of the Notch2/3 antagonist tarextumab and combined carboplatin and irinotecan (hereafter carboplatin/irinotecan) achieved greater tumour inhibition than tarextumab or carboplatin/irinotecan alone (Fig. 4g and Extended Data Fig. 10c), despite relatively low numbers of Hes1<sup><sup>pos</sup></sup> cells in this specific model (Fig. 4h and Extended Data Fig. 10d, e). We observed similar effects in a patient-derived xenograft model, OMP-LU66 treated with control antibody (n = 10 tumours), tarextumab (n = 9), carboplatin/irinotecan (n = 20), or tarextumab + carboplatin/irinotecan (n = 24) (one experiment). These results were generally accompanied by decreased proliferation in non-NE Hes1<sup><sup>pos</sup></sup> cells and increased apoptosis in NE Ascl1<sup><sup>pos</sup></sup> cells (Extended Data Fig. 10f–m), as well as a reduction in Hes1<sup><sup>pos</sup></sup> cells (Fig. 4i and Extended Data Fig. 10d–e, n–o). Thus, tarextumab treatment can block the generation of non-NE Hes1<sup><sup>pos</sup></sup> cells and limit their proliferation during tumour development. In combination with chemotherapy, tarextumab treatment can also affect the survival and possibly the proliferation of NE tumour cells.

SCLC tumours, which have few infiltrating stromal cells, can make their own diverse microenvironment<sup>17,29</sup>. Here we show that activation of Notch signalling during SCLC progression generates an unexpected level of intratumoural heterogeneity. The amount (duration and strength) of Notch signalling required for the NE to non-NE fate switch is unknown, but a threshold is probably required on the basis of analysis of GFP<sup><sup>ant</sup></sup> cells. Furthermore, although Notch1 is also expressed and active in SCLC, blockade of just Notch2/3 was sufficient to suppress tumour growth and the generation of non-NE cells. Notch activation is likely to be regulated at multiple levels in SCLC. First, our ex vivo data indicate that ligands expressed on neighbouring NE SCLC cells are sufficient to maintain Notch signalling in non-NE cells, but may not be sufficient for the generation of new non-NE cells. In primary tumours, Notch activation could be enhanced by signals from the lung microenvironment, as liver metastases (Extended Data Fig. 1a) and subcutaneous
allografts grown from Hes1pos/GFPpos cells (Extended Data Fig. 4d) contained fewer Hes1pos/GFPpos cells than primary tumours. The initial generation of non-NE SCLC cells may also promote the generation of more Notch-activated cells via unknown mechanisms. Second, although most of the loss-of-function NOTCH mutations in human SCLC tumours are heterozygous a, they might be sufficient to lower signalling from Notch receptors in NE SCLC cells and impair the NE to non-NE switch in response to ligands.

Notch signalling can be both tumour suppressive (intrinsically to NE cells) and pro-tumorigenic (through the generation of non-NE cells that are more chemoresistant and can promote NE cell growth) in SCLC. Our data provide a rationale for combining chemotherapy and Notch inhibition as a therapy for patients with SCLC whose tumours contain Notch-active tumour cells (Fig. 4j); expression of HES1 or other Notch targets in tumour biopsies, or even detection in the serum of patients of factors secreted by Notch-active SCLC cells (for example, midkine), may serve as biomarkers for this personalized therapy. While tarextumab treatment showed a trend towards an improvement in overall and progression-free survival in patients with extensive-stage SCLC whose tumours expressed elevated levels of Notch target genes, including HES1, in a phase 1b clinical trial (identifier NCT01859741); the same effect was not observed in a larger cohort of patients in a phase 2 study (unpublished data). It is possible that combination therapy with Notch inhibitors might be more beneficial to early-stage SCLC patients or as a second-line therapy following chemotherapy. In contrast, relapsed late-stage tumours with fast-growing NE tumour cells might be inhibited by acute activation of the Notch pathway. The dual role of Notch signalling in SCLC illustrates the complex functions of developmental pathways in cancer and should guide the development of novel therapeutic approaches.

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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Author Contributions J.S.L., M.W., K.C.G., T.H., C.L.M., and J.S. conceptualized the study. J.S.L. designed and performed cell culture assays and experiments using the mouse model. A.J. contributed to immunoblotting and immunostaining. M.M.F., B.C., G.O., J.C., A.M.K., and C.L.M. performed tarextumab and chemotherapy treatments and analysis. S.C.C. generated TKO allografts. V.C.L. and K.C.G. provided recombinant DI4. D.Y. and N.S.J. contributed reagents and to data interpretation. G.H., M.A., M.W., and C.P. performed and analysed HES1 immunostaining on patient samples. Y.-W.L. contributed to Lumexin assays. J.S.L., T.H., C.L.M., and J.S. wrote the manuscript with input from all authors. J.S. supervised the study, data interpretation, and manuscript preparation.

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METHODS

Ethics statement. Mice were maintained and animal experiments performed according to practices prescribed by the National Institutes of Health at Stanford’s Research Animal Facility (protocol 13566) and by the Institutional Animal Care and Use Committee at OncoMed Pharmaceuticals. Additional accreditation of Stanford and OncoMed Pharmaceuticals animal research facilities was provided by the Association for Assessment and Accreditation of Laboratory Animal Care.

Mouse models and treatment studies. Animal experiments were performed blinded except for allograft and patient-derived xenograft tumour growth measurements which were performed blinded. Immunostaining of sections from animal experiments were performed blinded.

Mouse SCLC model and adenoviral infections. The TKO SCLC mouse model bearing deletions in p53, Rb, and p103 has been described26. Mice were maintained on a mixed genetic background composed of C57BL/6, 129/Sv) and 129/SvOla. Endogenous Notch activity in TKO tumours was assessed through a GFP reporter expressed from the endogenous Hes1 promoter (Hes1GFP−/− allele)27. We also bred the Rosa26tm1020-opl-kar-gfp-lox-stop-flum (refs 31, 32) Cre-reporter alleles to the TKO model to label tumour cells with tdTomato and luciferase, respectively. SCLC tumours were induced in 7- to 10-week-old mice (with no discrimination by sex of mice) by intratracheal instillation with 4 × 107 plaque-forming units of Adeno-CMV-Cre (Baylor College of Medicine, Houston, Texas, USA) or Adeno-CGRP-Cre (University of Iowa). Tumours were collected for analysis after around 5–7 months for Ad-CMV-Cre or 7–8 months for Ad-CGRP-Cre, unless otherwise stated. In accordance with our animal protocol, mice were euthanized when they showed difficulty breathing, regardless of time point.

DBZ injections. TKO Hes1GFP−/− mice were treated with the γ-secretose inhibitor DBZ (Selleckchem, S2771) as previously described28. Mice were randomized and injected intraperitoneally with 100 μl of saline or 20 μl/mg (body weight) of DBZ (or DMSO control) for 5 days, and tumours were collected on day 6 for flow cytometry or fixed for histological analyses.

Chemotherapy treatments in TKO mice. TKO or TKO Hes1GFP−/− mice bearing tumours were randomized for treatment. For acute responses, mice were treated with cisplatin (7.5 mg per kg (body weight), Teva) on day 1, and a combination of cisplatin and etoposide (15 mg per kg (body weight), Novaplus) on days 2 and 4. Lungs were fixed for histological analyses a few hours after the last injection. For longer-term chemotherapy experiments, as we observed high toxicity with etoposide administration, TKO Rosa26tm1020-lox-flum Mice were treated weekly for 3 weeks with saline or 5 mg per kg (body weight) cisplatin only.

Subcutaneous tumour implantation. For subcutaneous tumour growth of GFPneg or GFPhigh cells, 2,000 cells were FACS-sorted and implanted subcutaneously on the lower left and right quadrants of 8- to 10-week-old immunocompromised NOD. Cg-Pkd1−/−L2rtm1020-Wt(Sv) (NSG) mice (no selection for sex of mice). Mice were euthanized and tumours were collected after approximately 2 months. The tumours did not exceed the 1.75 cm diameter limit permitted by our animal protocol.

For the human patient-derived xenograft and TKO allograft tumour growth models, NOD/SCID17-Pkd1−/−NcrCrl (NOD/SCID, Charles River Laboratories) mice were maintained under pathogen-free conditions and provided with sterile water and food and water ad libitum. Patient-derived xenograft models were established from patient biopsies provided by Molecular Response (San Diego, California, USA). OMP-LU66 was established at OncoMed Pharmaceuticals. For the subcutaneous xenograft studies, 100,000 OMP-LU66 cells in 100 μl 50% Matrigel (BD Biosciences)/50% Hank’s balanced salt solution supplemented with 2% heat-inactivated fetal bovine serum and 20 mM HEPES (Life Technologies) were implanted into the left flank region of 7- to 8-week-old NOD/SCID mice (no selection for sex of mice) with a 25-gauge needle.

Phage display selection and screening for tarextumab29. Using a human Fab phage library from Truncal, GOE (from days AG+) functional anti-Notch antibodies were discovered from selections against recombinant Notch2 extracellular domain (EGF1-12) containing the ligand-binding site.

Tarextumab treatments. NOD/SCID mice implanted with OMP-LU66 or TKO allografts were randomized and treated with a control antibody or tarextumab (OMP-59RS, 40 mg per kg (body weight), once every 2 weeks) as a single agent or in combination with the chemotherapy agents carboplatin (25 mg per kg (body weight), once-weekly, Teva) and irinotecan (25 mg per kg (body weight), once-weekly, Pfizer). We used carboplatin and irinotecan (instead of cisplatin and etoposide) for these longer-term studies as they are less toxic, better tolerated by the mice, and have been shown to have similar efficacies as cisplatin and etoposide35,36. To avoid the side effects of total Notch pathway inhibition in vivo37,38, we sought to reduce Notch signalling with the Notch2/3 antagonist tarextumab. After approximately four cycles, chemotherapy was discontinued and tarextumab dosing was continued until study completion. Mice with tumour volumes at or exceeding the 2,500 mm3 limit permitted by the Institutional Animal Care and Use Committee were euthanized regardless of time point.

Preparation of single-cell suspensions from SCLC tumours for flow cytometry. Tumours were dissected from the lungs of TKO Hes1GFP−/− mice approximately 5–7 months after tumour induction and digested as previously described29. The antibodies used were CD45-PE-Cy7 ( Bioscience, clone 30-F11, 1:100), CD31-PE-Cy7 ( Bioscience, clone 590, 1:100), TER-119-PE-Cy7 ( Bioscience, clone TER-119, 1:100), CD24-APC ( Bioscience, clone M1/69, 1:200), Ncam1 (Cedarlane, clone 3.12.3.1, 1:100), Hes1GFP ( Trichoplusia ni was expressed using baculovirus by infecting 1 l of Hi-Five cells (Invitrogen) from 100 mg per kg (body weight) EdU (5-ethynyl-2’-deoxyuridine; Life Technologies) 8 h before euthanasia. GFPneg and GFPhigh tumour cells were sorted by FACS before being fixed and subject to EdU staining using the Click-iT Plus EdU Pacific Blue flow cytometry assay kit (Life Technologies). Propidium iodide was used to stain for total DNA content and percentage EdU incorporation of GFPneg and GFPhigh cells was analysed using a BD FACSaria II.

Purification of soluble DLL4 ligand. The extracellular domain of rat DLL4 containing affinity-enhancing G285, F107L, 12606 H118I, 1143F, H194Y, and K215E mutations (named DLL4Δ2 for DLL4 in the manuscript) was cloned into the pAcGp67A vector and modified with a carboxy (C)-terminal 8 × His tag39. DLL4Δ2 was expressed using baculovirus by infecting 11 of Hi-Five cells (Invitrogen) from Trichoplusia ni at a density of 1 × 106 cells per millilitre and harvesting cultures after 72 h. The cultures were centrifuged to remove the cells, and proteins were purified from supernatants by nickel and size-exclusion chromatography.

Plasmids. The MigR1-ires-GFP (Ctrl) and MigR1-N1ICD-ires-GFP retroviral vectors were gifts from W. H. S. Pennington (University of Pennsylvania, Philadelphia). For doxycycline-inducible expression, we cloned N1ICD into the pLIX-403 vector (a gift from D. Root, Addgene 41395). For Rest overexpression, we cloned the Nrsf(Rest) fragment from pHRE-NEK7-RS-CITE-GFP (a gift from J. Nadeau, Addgene 21310 (ref. 40)) into the MigR1-ires-GFP or pLIX-403 vectors. Ascl1 promoter ([CATCATCTGCACGTACACGA] was designed using the sgRNA Designer (Broad Institute) and cloned into the lentiviral vector (a gift from P. Zhang, Addgene 52961 (ref. 41)).

Cell lines and cell culture assays. Except for 293T cells that were grown in DMEM, all cell lines were grown in RPMI-1640 medium supplemented with 10% bovine growth serum (BGS) (Fisher Scientific) and penicillin-streptomycin-glutamine (Gibco). Mouse KP1, KP2, and KP3 and human NIH29 SCLC cell lines were generated in the laboratory and have been described33,42. GFPneg and GFPhigh cell lines were isolated by FACS from patient derived 72h cultures. The cultures were centrifuged to remove the cells, and proteins were purified from supernatants by nickel and size-exclusion chromatography. Only strong signals were subjected to EdU analysis.
Antibodies were detected with UltraMap HRP kit and ChromoMap DAB, then counterstained with haematoxylin. Antibodies used were the same as listed above except Ascl1 (eBioscience 1405794) and Ki67 (Abcam ab16667).

For immunofluorescence, paraffin sections were deparaffinized, rehydrated, and unmasked by boiling in Trisodium phosphate (Cell Marque 920P-1) for 15 min, then blocked and stained with primary antibodies overnight, or subject to EdU staining (Life Technologies) before blocking and antibody staining. Nuclei were stained with DAPI (Sigma). The following primary antibodies were used: GFP (Rockland 600-101-215, 1:500), Ucd1 (Millipore HPA005993, 1:500), CGRP (Sigma C8188, 1:2000), synaptophysin (Sy, NeuroMics 020000, 1:100), RFP/Tomato (Rockland 600-401-379, 1:500), phospho-histone H3 (EMD Millipore 06-570, 1:500), and cleaved caspase-3 (CST 9664, 1:100).

Quantification of immunostaining. Quantification of all immunostaining was performed blinded. Hes1 TKO cells in TKO lung or sections or in human tissue microarrays were scored on the basis of the frequency and intensity of Hes1 staining and assigned scores of 0 (no staining), 1 (staining in ~1–20% of cells), 2 (staining in ~20–60% of cells or strong intense staining in <20% of cells), or 3 (>60% staining). Human SCLC tissue microarrays were purchased from US Biomax (LC245, LC802A, LC818), containing a total of 172 cores from 189 patients. H scores were calculated as the summation of (1 + pi) where i is the intensity score and pi is the percentage of the cells with that intensity.

The frequency of Hes1 TKO cells in TKO sections after chemotherapy was quantified from IHC staining using the ImageJ plugin, ImmunRatio44. The percentage of CCCI™ cells in GFPneg or GFPhigh cells after acute chemotherapy of TKO Hes1GFP+ -mice was quantified from immunofluorescence images by ImageJ. For studies with human patient-derived xenograft and allograft tumour models performed at OncoMed Pharmaceuticals, slides were scanned using an Aperio AT scanner, then analysed using Definiens Tissue Studio image analysis software. Positively stained cells within tumours were identified and quantitated for staining intensity and frequency. For quantification in Extended Data Fig. 10–f, some samples were excluded because the paraffin blocks did not have any tissue samples left to be cut (since the tumours were harvested at or close to minimum residual disease, the amount of tissue obtained was small). This exclusion due to unforeseen experimental limitations was not pre-established.

Patient survival data. The study was approved by the institutional review board of the East Paris University Hospitals Tumour Bio-bank, AP-HP, Tenon Hospital, Paris, France (AP-FP – GH-HEUP Tumourothérapie Bio-bank platform). Seventy-three patients diagnosed with SCLC at Hôpital Tenon, Assistance Publique-Hôpitaux de Paris, France, from January 2010 to January 2013 were first identified. Tumour samples were obtained after getting written informed consent. We performed HES1 IHC for 68 of the patients from whom formaldehyde-fixed and paraffin-embedded tumour tissue was available. The tumour samples were first reviewed by at least two independent expert pathologists and the diagnosis of SCLC was histomorphologically confirmed by haematoxylin and eosin staining and IHC for chromogranin A, synaptophysin, NCAM and TTF1. Clinical and biological characteristics of the patients are provided in the Supplementary Methods. For survival analysis, the patients were separated into two groups on the basis of the expression of the (Hes1-negative) or presence of (Hes1-positive) of HES1 immunostaining in their tumours.

Lumienx assay for midkine protein in human plasma. Human plasma samples from cancer-free normal donors were purchased from BioreclamationIVT. SCLC donor plasma was sourced from Conversant Biologics (Conversant Bio). The samples were collected, processed, and distributed in accordance with institutional review board approval following informed patient consent. Plasma samples were assayed by following the Lumienx assay protocol with adaption of the Drop Array system (Curiox Biosystems, Lumienx, Austin, Texas, USA). In brief, wells in the DropArray assay plate were blocked with 10μl 1% BSA/PBS for 30 min at room temperature before being blocked and probed according to manufacturer’s instructions. Bead mix (5μl) was added to all wells. Five-microlitre standards or diluted samples were then added to the plate; all standard and human plasma samples were tested in duplicate wells. The plate was shaken for 10s at 1,000rpm. Then placed on a magnetic stand in a humidified chamber and shaken overnight at 4°C. The plate was washed three times with a DropArray LT washing station MX96 (Curiox Biosystems). The detection antibody was added at 5μl per well and the plate was incubated for 60 min. Five microlitres per well of the streptavidin–PE substrate was added to each well and incubated for 30 min with shaking. The plate was washed three times before reading by Lumienx 200 instrument. Data were analysed using EMD Millipore’s Milliplex Analyst software. The standard curve readings were back-calculated and evaluated for accuracy (80–120%) and precision (percentage coefficient of variation of duplicates <30%).
deoxycylate, 30 mM NaCl; 20 mM Na2HPO4, 1 mM Na3VO4, 1 mM DTT, 60 mM β-glycerophosphate) supplemented with protease inhibitors aprotinin (10 μg ml−1), leupeptin (10 μg ml−1), and PMSF (1 mM). Protein concentration was measured with a Pierce BCA protein assay kit (Thermo Scientific). The antibodies used were Notch1 (Cell Signaling Technology (CST) 4380), cleaved Notch1 (CST4147), Notch2 (CST5732), Hes1 (CST11988), GFP (Invitrogen A-11122), Rest (Abcam 21635), alpha-tubulin (Sigma T9026), and HSP90 (CST 4677).

DNA and RNA extraction and gene expression analyses. For analysis of primary tumour cells, cells were sorted from pooled tumours from individual TKO Hes1(GFP)−/+ mice by FACS. DNA and RNA were isolated using a Qiagen Allprep DNA/RNA micro kit or an RNeasy mini kit according to the manufacturer’s protocol. qRT–PCR analysis was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using PerfeCTa SYBR Green FastMix (Quanta BioSciences 95073). Genes having Cq values that were high (>34) or undetermined (for example, Notch4) were removed from the graphical analyses. Data were normalized to RpL9 as a housekeeping gene, unless otherwise stated. Primer sequences are available in Supplementary Methods.

Microarray analysis. RNA from cells isolated from FACS from three TKO Hes1(GFP)−/+ mice (independent of the samples used for qRT–PCR) was subjected to quality assessment and microarray analysis by the Stanford Protein and Nucleic Acid (PAN) facility as previously described4. The microarray was performed using a GeneChip Mouse Gene 2.0 ST Array (Affymetrix), and the Robust Multichip Average (RMA) Express 1.1.0 program was used for background adjustment and quantile RNA normalization of the 41,345 probe sets encoding mouse genome transcripts. Linear models for microarray data (Limma) was used to compare GFP+/+ and GFP−/− cells on RNA normalized signal intensities. The command procmp in R was used for principal component analysis. Probe identifiers were annotated with gene symbols from the mouse gene 2.0 ST transcript cluster database (mogene20transcriptclusterdb). Of the 41,345 probe sets, 25,349 were annotated to genes, which were then used for gene set enrichment analysis45,46. Default parameters were used except that we performed gene set permutation instead of phenotype permutation because there were fewer than seven samples per phenotype. Probes with an adjusted P value of 0.05 or less were considered as significantly differentially expressed. Seven thousand and ninety-six probe sets annotated to 5,437 genes (5,289 unique) were significant, and a heatmap for these genes was generated using the heatmap2 function in R.

Significantly differentially expressed genes were also analysed by Enrichr47,48. To identify candidate transcription factors that might mediate the NE to non-NE switch, we used genes significantly downregulated in GFP+/− cells to search for enriched ENCODE and CHEA consensus transcription factors from the ChIP-X database. To identify a list of secreted factors, we first looked at genes that were classified in the ‘extracellular space’ gene signature and, by literature search, picked out the genes known to be secreted. We also input all significant genes into the Stanford Cancer Genomics characterization system for the whole mouse brain. Nature Neurosci. 13, 134–140 (2010).

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Image analysis and statistics. Sample sizes were chosen on the basis of our experience with similar experiments (a minimum of three to five mice for animal studies, or two to four biological replicates for in vitro/ex vivo assays, usually ensured statistical significance if the phenotypes were robust). Statistical significance was assessed by Student’s t-test with GraphPad Prism (two-tailed unpaired or paired t-test, depending on the experiment). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant. Variance was examined by an F-test. Data are represented as mean ± s.d. primary data are available from the corresponding author upon reasonable request.

Doxycycline-containing primary data are available at the NCBI Gene Expression Omnibus under accession number GSE81170. Normalized values for significantly differentially expressed genes are provided in Supplementary Table 1; gene set enrichment analyses are in Supplementary Tables 2–4. HES1 immunostaining and survival data of patients with SCLC are provided in Supplementary Table 5. For immunoblot Source Data, see Supplementary Fig. 1. Source Data are provided for Fig. 1b, d, 2a and 4g–i and Extended Data Figs 4c, 5j, 6c, 8c–d, and 10c, f–m. All other data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | SCLC tumours contain a population of Hes1-positive tumour cells. a, Quantification of the frequency (freq.) of Hes1pos cells in TKO tumours at different stages of tumour development. Scores: 0 (0%), 1 (1–20%), 2 (20–60%), 3 (>60%). Hyperplasias (n = 26, from five mice, defined by area less than 16,600 μm²) and early tumours (n = 69, from five mice) were analysed 3 months after Ad-CMV-Cre; late tumours (n = 83, from five mice) and liver metastases (n = 54, from five mice) were analysed 6–7 months after Ad-CMV-Cre (data for the late tumours are shown for comparison and are the same as in Fig. 1b). b, As in a, for human SCLC tumour microarray sections and segregated by clinical stage. H scores: stage I (123.9; n = 71 sections), stage II (135.3; n = 68 sections), stages III + IV (148.5; n = 33 sections). c, Representative immunofluorescence for GFP in TKO Hes1GFP/ + SCLC tumours. d, Representative flow cytometry plots of cells isolated from pooled tumours from a TKO Hes1GFP/ + mouse. Arrows depict the sequential gating strategy for enriching for single, live (by exclusion of 7-aminoactinomycin D (7-AAD)), and lineage (CD45, CD31, TER-119)-negative cells. CD24 labels more than 98% of Cre-recombined cells and thus further enriches for tumour cells39. c, Immunofluorescence of a TKO Rosa26lox-stop-lox-tdTomato;Hes1GFP/ + tumour section showing co-localization of GFP and Tomato signals. f, Flow cytometry shows that GFPhigh cells in pooled tumours from a TKO Rosa26lox-stop-lox-tdTomato;Hes1GFP/ + mouse infected with Ad-CMV-Cre are positive for Tomato expression (representative of n = 2 mice). g, Genotyping PCR analysis for recombination (delta, Δ) or the unrecombined (floxed) alleles at the Rb, p53, and p130 loci in GFPneg and GFPhigh tumour cells sorted from three TKO Hes1GFP/ + mice. DNA from fl/fl and/or Δ/Δ cells served as controls. Scale bars, 50 μm.
Extended Data Figure 2 | Hes1-positive SCLC cells are Notch-pathway-active, epithelial, and do not express neuroendocrine markers.

a, Representative IHC for Hes1 and Notch2 in serial TKO tumour sections. b, RNA-sequencing data of human SCLC tumours (from ref. 8), with positive (ASCL1 and SOX2) and negative (CDX2, MYOD1) controls for expression in SCLC (n = 81 tumours). c–g, TKO Hes1\textsuperscript{GFP+} mice were treated with DMSO control (n = 5 mice) or DBZ (n = 4 mice). c, Representative flow cytometry of GFP in pooled tumours from one mouse per condition. Dotted lines delineate the GFP\textsuperscript{high} population. d, Quantification of relative median GFP intensity of GFP\textsuperscript{high} cells. e, qRT–PCR of sorted GFP\textsuperscript{neg} and GFP\textsuperscript{high} cells. f, Immunoblot analysis of sorted GFP\textsuperscript{neg} and GFP\textsuperscript{high} cells. At these exposure levels, GFP is detected but not Hes1 in DBZ-treated GFP\textsuperscript{high} cells, possibly owing to different half-lives of the proteins (>24 h for GFP\textsuperscript{53} compared with <1 h for Hes1 (refs 54, 55)). For Notch, an antibody that detected Notch1 cleaved specifically before V1744 (Notch1 ICD), or antibodies that detected the transmembrane + intracellular (excluding the extracellular region, ΔECD) regions of Notch1 or Notch2 were used. g, Representative IHC in treated tumours. h, Flow cytometry of two GFP\textsuperscript{high} cell lines (n = 2 biological replicates) grown on dishes coated with Dll4 or 72 h after removal from Dll4. Negative control: GFP\textsuperscript{neg} cell line. i, Quantification of relative median GFP intensity from h (n = 2 biological replicates). j, Immunofluorescence for GFP and NE markers (CGRP and Syp) in TKO Hes1\textsuperscript{GFP+} tumours. k, Flow cytometry of the NE marker Ncam1 and GFP in a TKO Hes1\textsuperscript{GFP+} tumour (representative of n = 3 biological replicates). The quadrants delineate the negative gates for the GFP and Ncam1 signals and show that GFP\textsuperscript{high} cells are mostly negative for Ncam1 expression. l, qRT–PCR analysis of NE genes in GFPh\textsuperscript{high} cells relative to GFP\textsuperscript{neg} cells (sorted from pooled tumours from n = 3 mice, with n = 3 technical replicates each). m, Flow cytometry analysis of the epithelial marker EpCam and GFP (left) or CD44 and GFP (right) in a TKO Hes1\textsuperscript{GFP+} tumour (representative of n = 3 biological replicates). n, qRT–PCR of vimentin (Vim) in GFP\textsuperscript{neg} and GFP\textsuperscript{high} cells (n = 4 mice). *P < 0.05; **P < 0.01. Statistical significance was determined by two-tailed unpaired (d, e) or paired (i, l, n) Student’s t-test. Data are represented as mean ± s.d. Scale bars, 50 μm.
Extended Data Figure 3 | Notch-active SCLC cells have switched to a non-neuroendocrine fate.  

**a.** Principal component analysis of normalized microarray gene expression values of GFP<sup>neg</sup> and GFP<sup>high</sup> tumour cells sorted from three TKO Hes1<sup>GFP<sup>+/+</sup></sup> mice. The first two principal components (PCs) accounting for 77.6% of the total variance are shown.

**b.** Heatmap for differentially expressed genes in GFP<sup>neg</sup> and GFP<sup>high</sup> tumour cells by microarray.

**c.** Gene set enrichment analysis for a Notch pathway signature enriched in GFP<sup>high</sup> cells.

**d.** Gene set enrichment analysis for a neuronal gene set (from the MSigDB C2 curated gene sets collection) enriched in GFP<sup>neg</sup> cells.

**e.** Gene set enrichment analysis of differentially expressed genes in GFP<sup>neg</sup> and GFP<sup>high</sup> cells. Gene sets enriched at FDR <i>q</i> value &lt; 0.25 in the MSigDB C5 cellular component ontology are plotted as a function of normalized enrichment score against the <i>q</i> value. Left: gene sets enriched in GFP<sup>neg</sup> cells; gene sets related to neuronal signatures are highlighted in purple. Right: gene sets enriched in GFP<sup>high</sup> cells; gene sets related to the extracellular region are highlighted in green.

**f.** Enrichr analysis for cellular component (f) or biological process (g) Gene Ontology terms significantly enriched in GFP<sup>neg</sup> or GFP<sup>high</sup> cells. The top ten Gene Ontology terms ranked by combined score are shown.

**h.** Enrichr analysis for cell lines in the Cancer Cell Line Encyclopedia that closely resemble GFP<sup>neg</sup> or GFP<sup>high</sup> cells. The top ten Gene Ontology terms ranked by combined score are shown.
Extended Data Figure 4 | Non-neuroendocrine, Notch-active cells in SCLC tumours are slower-growing than the neuroendocrine cells.

a, Co-immunofluorescence for GFP and EdU (a) or phospho-histone H3 (b) in TKO Hes1<sup>GFP<sup>+</sup></sup> tumours. c, Weight of tumours formed from freshly sorted GFP<sup>neg</sup> and GFP<sup>high</sup> tumour cells implanted subcutaneously in immunocompromised NSG mice (n = 6 tumours each; two-tailed unpaired Student's t-test). d, Flow cytometry of GFP in a tumour formed from freshly sorted GFP<sup>neg</sup> cells implanted subcutaneously in immunocompromised NSG mice (representative of n = 6 biological replicates). *P < 0.05. Data are represented as mean ± s.d. Scale bars, 50 μm.
Extended Data Figure 5 | See next page for caption.
Non-neuroendocrine, Notch-active cells in SCLC tumours are generated from neuroendocrine tumour cells. 

a. Representative IHC for Hes1 and cleaved caspase-3 (CC3) in serial TKO tumour sections. Inset: higher magnification of a positive control for CC3 (tumour from a mouse treated with chemotherapy). 
b. Predicted numbers and ratios of GFPneg and GFPhigh tumour cells if the two populations divide independently of each other, and GFPhigh cells cycle approximately three times faster than GFPneg cells. 
c. Representative IHC in serial sections from TKO Hes1GFP+ tumours initiated by Adeno-CGRP-Cre. 
d. Quantification of the frequency (Freq.) of Hes1pos cells in TKO Hes1GFP+ mice (n = 23, from five mice) and tumours (n = 50, from seven mice) induced by Adeno-CGRP-Cre. Scores: 0 (0%), 1 (1–20%), 2 (20–60%), 3 (>60%). 
e. Flow cytometry analysis showing the percentage of GFPhigh cells in pooled tumours from TKO Hes1GFP+ mice (n = 4) infected with Ad-CGRP-Cre. 
f. Images of freshly isolated GFPneg cells grown on dishes coated with Dll4 or PBS control (representative of n = 3 biological replicates). 
g. h. Freshly isolated GFPneg cells that remained GFPneg after culture on Dll4-coated dishes were re-plated on dishes coated with Dll4 ligand (+Dll4) or PBS control (−Dll4). 
g. Flow cytometry and images (representative of n = 2 biological replicates). 
h. GFPneg and GFPhigh cells that formed after this second round of Dll4 stimulation were sorted and analysed by immunoblot. Control: GFPhigh cell line. 
i. Relative number of GFPhigh cells formed from freshly isolated GFPneg cells grown on Dll4 after 2 weeks of tarextumab treatment (n = 3 biological replicates each). 
j. Single-cell qRT–PCR (n = 45 each) of H29, H82, and H889 human SCLC cell lines. Heatmap was generated by unsupervised clustering of each cell line. Dark blue regions indicate undetectable expression. 
k. qRT–PCR for HES1 after 72 h of culture with or without Dll4. Data are normalized to GAPDH (n = 3 biological replicates with n = 3 technical replicates each). 
l. m. GFPhigh cell lines were treated with DMSO or DBZ while grown with or without Dll4 or co-cultured with three individual NE cell lines in the absence of Dll4. GFP expression was analysed by flow cytometry after 72 h. 
l. Flow cytometry of GFPhigh cell line 1 (representative of n = 3 biological replicates; GFP intensity quantified in Fig. 2e). 
m. Quantification of GFP intensity in GFPhigh cell line 2; relative median GFP intensity normalized to the ‘DMSO−Dll4’ condition (n = 3 biological replicates). 
n. o. Representative images (n) and qRT–PCR (o) of GFPhigh cell lines cultured in the presence of absence of Dll4 for more than a month (n = 3 biological replicates with n = 3 technical replicates). 
p. Freshly isolated GFPneg cells that became GFPhigh after culture on Dll4 were re-plated on dishes coated with Dll4 ligand or PBS control and analysed by immunoblot after a month. 
q. Freshly isolated GFPneg cells that became GFPhigh after culture on Dll4 were re-plated on dishes coated with Dll4 ligand or PBS control and analysed by immunoblot after a month. GFPneg cell lysate: positive control for Ascl1. *P < 0.05; **P < 0.01. Statistical significance was determined by two-tailed paired Student’s t-test. Data are represented as mean ± s.d. Scale bars, 50 μm.
Extended Data Figure 6 | Analysis of SCLC cells with intermediate levels of activation of the Notch pathway. a, Flow cytometry showing gates used to sort for tumour cells with negative (neg), intermediate (int), and high levels of GFP from a TKO Hes1\textsuperscript{GFP\textsuperscript{+}} mouse (representative of \(n = 4\) biological replicates). b, qRT–PCR for Notch pathway genes and NE genes in sorted cells (\(n = 4\) biological replicates with \(n = 3\) technical replicates each). c–e, Single-cell qRT–PCR of GFP\textsuperscript{neg}, GFP\textsuperscript{int}, and GFP\textsuperscript{high} cells sorted from one TKO Hes1\textsuperscript{GFP\textsuperscript{+}} tumour (\(n = 32\) cells sorted per group; 20 cells with low expression of housekeeping genes were excluded from the heatmaps). c, Unsupervised clustering segregates the cells into two main groups. d, e, Supervised clustering (d) and analysis (e) of Hes1 mRNA (normalized to Gapdh) shows that the number of GFP\textsuperscript{int} cells with detectable Hes1 levels is intermediate between GFP\textsuperscript{neg} and GFP\textsuperscript{high} cells. f, Representative images of freshly isolated cells in culture (without Dll4). Scale bars, 50 \(\mu\)m. \(*P < 0.05; \quad **P < 0.01; \quad ***P < 0.001; \quad ****P < 0.0001\). Statistical significance was determined by two-tailed paired Student's \(t\)-test. Data are represented as mean ± s.d.
Extended Data Figure 7 | Upregulation of Rest is required for the NE to non-NE transition. a, b, Images (a) and immunoblot analysis (b) of a mouse NE SCLC cell line (KP1) ~1 month after N1ICD transduction (representative of n = 2 biological replicates). c, Immunoblot of KP1 cells transduced with Ascl1 shRNAs or a shGFP control. Note that the level of Ascl1 downregulation is similar to that achieved by N1ICD overexpression at a similar time point (not shown). d, qRT–PCR of NE genes in KP1 cells transduced with Ascl1 shRNAs (n = 3 biological replicates each). e, Representative images of KP1 cells approximately one month after Ascl1 knockdown. f, qRT–PCR for Rest in GFP<sup>low</sup> (n = 3) and GFP<sup>high</sup> (n = 4) cell lines (n = 3 technical replicates each). g, qRT–PCR of freshly isolated GFP<sup>high</sup> cells 2 weeks after culture in the absence or presence of Dll4 (n = 2 biological replicates with n = 3 technical replicates each). h, Sanger sequencing verification of n = 4 Rest knockout (Rest KO) KP1 clones, with the sequence of both alleles shown. i, qRT–PCR for NE genes in Rest wild-type (n = 3) and Rest knockout (n = 4) clones (n = 3 technical replicates each). j, Schematic for assay to assess the ability of N1ICD to induce adherent non-NE cells from NE SCLC cells. NE SCLC cells (KP1) were transduced with a N1ICD retrovirus or an empty vector control. Seventy-two hours later, 1,200 cells were seeded per well of a 96-well plate. The number of adherent cells per well was counted after 2 weeks. k, KP1 cells stably integrated with Rest shRNAs or an shGFP control were transducted with an N1ICD retrovirus and the number of adherent cells counted after 2 weeks (see schematic in j) (n = 3 biological replicates with n = 3 technical replicates each). l, qRT–PCR for NE genes in KP1 cells 48 h after Rest overexpression (n = 3 biological replicates with n = 3 technical replicates each). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Statistical significance was determined by two-tailed paired (d, k, l) or unpaired (f) Student’s t-test. Data are represented as mean ± s.d. Scale bars, 50 μm.

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Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | Notch-active SCLC cells promote the survival of neuroendocrine SCLC cells. a, Gene set enrichment analysis signatures enriched in GFP^{high} (astroglial signature) and GFP^{low} (neuronal signature) tumour cells on the basis of microarray data of tumours from n = 3 mice. b, Images of GFP^{low}, GFP^{high}, or bulk tumour cells (mixture of GFP^{low} and GFP^{high}) 7 days after isolation from TKO Hes1^{GFP+/+} mice and seeded at equal numbers in 50% Matrigel (representative of n = 3 biological replicates). c, Immunofluorescence for Uchl1 (NE marker) and GFP 10 days after the cells were sorted from TKO Hes1^{GFP+/+} tumours and in 50% Matrigel. Note: most of the spheroids in the bulk culture were composed of only one type of cell (Uchl1^{pos}GFP^{neg} or Uchl1^{neg}GFP^{pos}); the mixed spheroids shown here are the minority. d, Quantification of the number of each type of spheroids in each culture condition from c (n = 3 mice; two sections each for immunostaining). e–g, Relative luciferase activity of a luciferase-labelled mouse SCLC NE cell line seeded alone or co-cultured with GFP^{high} cell lines (n = 2 (e) or n = 3 (f, g) biological replicates with n = 3 technical replicates each). e, KP2 NE cell line, 2% serum. f, g, KP1 (f) and KP2 (g) cell lines, 10% serum. h–j, As in e–g, but the cells were fixed and collected for EdU analysis by flow cytometry (n = 2 (h) or n = 3 (i, j) biological replicates). k, Representative images of KP1 cells seeded in conditioned medium (CM) (2% serum) from KP1 cells or from GFP^{high} cell lines. l, Relative luciferase activity of luciferase-labelled KP2 cells 72 h after seeding in CM (2% serum) from KP2 cells or from GFP^{high} cell lines (n = 3 biological replicates with n = 3 technical replicates each). m, As in l, but the cells were fixed and collected for EdU analysis by flow cytometry (n = 3 biological replicates). n, o, As in l, but with KP1 (n) or KP2 cells (o) in 10% serum (n = 3 biological replicates with n = 3 technical replicates each). p, q, AlamarBlue cell viability assay for NE SCLC cell lines 72 h after culture with the indicated recombinant proteins (n = 3 biological replicates with n = 3 technical replicates each). r, ELISA assay for midkine in supernatant from NE (n = 4) and GFP^{high} (n = 4) cell lines (n = 2 biological replicates each). s, Luminex assay for midkine in serum plasma from control (n = 11) and SCLC (n = 15) patients. t, Relative luciferase activity of luciferase-labelled mouse SCLC NE cell lines (n = 4) 72 h after seeding alone or co-cultured with GFP^{high} cell lines and treated with 10 μM DBZ or DMSO control. Data are normalized to NE-monoculture with DMSO (n = 3 biological replicates with n = 3 technical replicates each). *P < 0.05; **P < 0.01; ***P < 0.001. Statistical significance was determined by two-tailed paired Student’s t-test (except for r, s: two-tailed unpaired Student’s t-test). Data are represented as mean ± s.d. Scale bars, 50 μm.
Extended Data Figure 9 | Notch-active SCLC cells are more resistant to chemotherapy than neuroendocrine SCLC cells. **a**, b, Average cell viability (MTT assay) of NE (n = 4) and GFP^high (n = 3) cell lines 48 h after cisplatin (**a**) or etoposide (**b**) treatment (n = 3 biological replicates with n = 3 technical replicates each). **c**, d, Quantification of cells expressing cleaved caspase-3 (CC3) (**c**) and Hes1 (**d**) in tumours in TKO mice acutely treated with cisplatin and etoposide (CC3: n = 48; Hes1: n = 41 tumours) or a vehicle control (CC3: n = 49; Hes1: n = 41 tumours) (data from two groups of three mice previously described in Figure 4a, b in ref. 39 but analysed as number of CC3^pos per tumour, not per mouse). **e**, Representative immunofluorescence of tumours from TKO Hes1^GFP^-mice acutely treated with cisplatin and etoposide. Scale bars, 50 μm. **f**, Quantification of Hes1^pos cells in the tumours of TKO Rosa26lox-stop-lox-Luciferase mice after 3 weeks of weekly saline (n = 190 tumours, from seven mice) or cisplatin (n = 217 tumours, from eight mice) treatments. **g**–**i**, Overall survival of all (g; n = 65, 49 HES1^neg, 16 HES1^pos), stages I–III (h; n = 26, 19 HES1^neg, 7 HES1^pos), or stage IV (i; n = 39, 30 HES1^neg, 9 HES1^pos) patients with SCLC with HES1-negative or HES1-positive tumours. **j**–**l**, Progression-free survival of all (j; n = 67, 51 HES1^neg, 16 HES1^pos), stages I–III (k; n = 28, 21 HES1^neg, 7 HES1^pos), or stage IV (l; n = 39, 30 HES1^neg, 9 HES1^pos) patients with SCLC with HES1-negative or HES1-positive tumours. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Statistical significance was determined by two-tailed paired (a, b) or unpaired (c, d, f) Student’s t-test. For **g**–**l**, probability was calculated using a weighted log-rank test with emphasis on later time points (see Methods). Data are represented as mean ± s.d.
Extended Data Figure 10 | See next page for caption.
**Extended Data Figure 10** Notch2/3 inhibition in vivo reduces the Notch-active cell population in SCLC and improves anti-tumour response in combination with chemotherapy.  

a, Schematic for the generation of TKO allografts. A TKO tumour was isolated, digested to form single cells, and implanted subcutaneously in NSG mice.  

b, Representative haematoxylin and eosin (H&E) staining and IHC for Ascl1 in allograft tumour sections.  

c, Tumour volumes of TKO allografts treated with the indicated drugs (n = 16 tumours per group, one experiment). The experiment was stopped at day 18 and samples were collected for analysis.  

d, e, Representative IHC for Hes1 (d) or double IHC for Hes1 (brown) and Ascl1 (pink) (e) in TKO allograft tumours collected 18 days after the start of treatment.  

f, g, IHC staining quantification for the frequency of Hes1posCC3pos (f) and Hes1posKi67pos (g) cells in mouse TKO allografts after 18 days of treatment (n = 8 tumours per group, except for carboplatin/irinotecan and tarextumab groups in f (n = 7 tumours each)).  

h, i, IHC staining quantification for the frequency of Ascl1posCC3pos (h) and Ascl1posKi67pos (i) cells in xenograft tumours after 31 days of treatment (for h, control/tarextumab groups: n = 4 tumours each; carboplatin/irinotecan: n = 3 tumours; tarextumab + carboplatin/irinotecan: n = 2 tumours; for i, control/tarextumab groups: n = 4 tumours each; carboplatin/irinotecan and tarextumab + carboplatin/irinotecan groups: n = 5 tumours each).  

j, k, IHC staining quantification for the frequency of Ascl1posCC3pos (j) and Ascl1posKi67pos (k) cells in mouse TKO allografts after 18 days of treatment (n = 8 tumours per group, except for the carboplatin/irinotecan group in j (n = 7 tumours).  

l, m, IHC staining quantification for the frequency (%) of ASCL1posCC3pos (l) and ASCL1posKi67pos (m) cells in xenograft tumours after 31 days of treatment (n = 4 tumours per group, except for control and tarextumab groups in l (n = 2 tumours) and the tarextumab + carboplatin/irinotecan group in m (n = 3 tumours)).  

n, o, Representative IHC (n) and quantification of HES1pos (o) cells in LU66 patient-derived xenograft tumours collected 56 days after the start of treatment (control: n = 3 tumours; tarextumab: n = 4 tumours; carboplatin/irinotecan and tarextumab + carboplatin/irinotecan: n = 5 tumours per group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Statistical significance was determined by two-tailed unpaired Student’s t-test. Data are represented as mean ± s.e.m. Scale bars, 50 μm.