A conserved YAP/Notch/REST network controls the neuroendocrine cell fate in the lungs

Yan Ting Shue1,2, Alexandros P. Drainas1,2, Nancy Yanzhe Li3, Sarah M. Pearsall4, Derrick Morgan4, Nasa Sinnott-Armstrong2, Susan Q. Hipkins1,2, Garry L. Coles1,2, Jing Shan Lim1,2, Anthony E. Oro3, Kathryn L. Simpson4, Caroline Dive4 & Julien Sage1,2

The Notch pathway is a conserved cell-cell communication pathway that controls cell fate decisions. Here we sought to determine how Notch pathway activation inhibits the neuroendocrine cell fate in the lungs, an archetypal process for cell fate decisions orchestrated by Notch signaling that has remained poorly understood at the molecular level. Using intratumoral heterogeneity in small-cell lung cancer as a tractable model system, we uncovered a role for the transcriptional regulators REST and YAP as promoters of the neuroendocrine to non-neuroendocrine transition. We further identified the specific neuroendocrine gene programs repressed by REST downstream of Notch in this process. Importantly, we validated the importance of REST and YAP in neuroendocrine to non-neuroendocrine cell fate switches in both developmental and tissue repair processes in the lungs. Altogether, these experiments identify conserved roles for REST and YAP in Notch-driven inhibition of the neuroendocrine cell fate in embryonic lungs, adult lungs, and lung cancer.
Cell fate decisions are critical to ensure proper organ development during embryogenesis and homeostasis in adult organisms. Accordingly, defects in signaling pathways regulating cell fate are associated with developmental disorders and diseases such as cancer. The Notch signaling pathway serves as a paradigm in studies of cell fate control (reviewed in refs. 1–5). Schematically, in this cell–cell communication pathway, ligand molecules expressed on the surface of cells activate receptors on neighboring cells, resulting in receptor cleavage and the nuclear translocation of the NOTCH intra-cellular domain (NICD), which acts as a transcriptional regulator with its DNA binding partner RBP-J. NICD targets include regulators of the cell cycle, cell survival, and cell differentiation (reviewed in refs. 6,7). Despite a large number of studies, however, the mechanisms by which Notch signaling is regulated and how it controls cell fate changes are still only partly understood.

The control of the neuroendocrine (NE) cell fate in the lung epithelium has provided a model to investigate Notch signaling in vivo (reviewed in refs. 8–11). During embryonic development, ligand-expressing epithelial cells accumulate expression of ASCL1 (Achaete-Scute Family BHLH Transcription Factor 1) while activation of NOTCH receptors and their transcriptional target HES1 (Hes Family BHLH Transcription Factor 1) in neighboring cells results in ASCL1 downregulation and inhibition12–14. ASCL1 is a key activator of NE gene programs, including genes such as Calca coding for the calcitonin gene-related peptide CGRP15–17. Thus, ligand-high Notch-inactive cells express ASCL1 and give rise to NE cells while receptor-high Notch-active cells lose ASCL1 expression and give rise to non-NE lung epithelial cells15,18–21. A similar process is at play in the adult lung following injury where Notch activation in a subset of NE cells promotes the generation of non-NE epithelial cells such as club cells22–24.

Remarkably, accumulating evidence indicates that developmental processes involving Notch signaling and decisions between NE and non-NE cell fates are also at play in small-cell lung cancer (SCLC), an aggressive and early disseminating form of lung cancer characterized by resistance to multiple therapeutic approaches and dismal survival rates (reviewed in ref. 25). In mouse models, ASCL1 is critical for the initiation of SCLC16,26. SCLC cells can switch from a NE state to non-less-neuroendocrine (non-NE) states in response to various signals, and these non-NE states can affect the metastatic ability of SCLC cells as well as their response to chemotherapy27–30. In particular, mouse and human SCLC cells can acquire non-NE features upon activation of Notch signaling29. Notch-active non-NE SCLC cells lose the expression of classical NE markers, and express high levels of NICD targets such as HES1 and REST (RE1-silencing transcription factor, NRFSF). These HES1-high non-NE SCLC cells are inherently more resistant to chemotherapy and also support the growth of NE SCLC cells in the tumor microenvironment29. Other transcription regulators associated with loss of classical NE features in SCLC include YAP, POU2F3, ATOH1, and MYC31–37. But how these factors control NE and non-NE cell states and how they possibly connect with Notch signaling remains poorly understood in SCLC and during development.

Here we seek to further investigate conserved molecular networks around Notch implicated in the transition from NE to non-NE cell states. We identify REST and YAP as key transcriptional regulators of this transition in multiple contexts. In SCLC, REST represses a set of neuroendocrine genes largely distinct from ASCL1 targets and is required for the complete repression of the NE program when SCLC cells transdifferentiate into a non-NE club cell-like fate. In non-cancer contexts, deletion of Rest results in a higher number of PNECs specified during early lung development and a delay in NE to non-NE transition following repair after lung injury with naphthalene. Similarly, YAP is also found to be antagonistic to the neuroendocrine fate. When YAP is deleted following lung injury, PNECs are unable to transdifferentiate to the non-NE fate to replenish the epithelium of the airways. These findings provide further insights into the molecular mechanisms by which Notch signaling controls cell fate transitions in vivo.

Results

HES1-high non-NE SCLC cells have features of club cells. To better characterize Notch-active HES1-high non-NE SCLC cells, we sought to compare their transcriptional profile to that of HES1low NE SCLC cells. To this end, we conducted RNA-seq on cancer cell populations isolated by flow cytometry from Rb/p53/p30 triple knockout (TKO) tumors growing in the lungs of Rbfl/fl; p53fl/fl; p130fl/fl;Hes1GFP+/ mice following intra-tracheal installation of an adenovirus expressing Cre (Ad-CMV-Cre). In these TKO/Hes1GFP+ tumors, a green fluorescent protein (GFP) reporter is expressed from the regulatory regions of the endogenous Hes1 gene (Fig. 1a and Supplementary Fig. 1). The transcriptomic analysis showed clear separation between GFPhigh and GFPlow SCLC cells by principal component analysis (PCA) (Supplementary Fig. 1b). We identified 2518 upregulated and 2017 downregulated genes with corrected p-value < 0.05 and log2-fold change of >1.0 and <-1.0 (Supplementary Fig. 1c and Supplementary Data 1). Gene set enrichment analysis (GSEA) confirmed Notch pathway enrichment in the GFPhigh cells (Supplementary Fig. 1d). Gene ontology (GO) term analysis of the differentially expressed genes showed downregulation of NE differentiation in GFPhigh cells, with upregulation of programs involved in cell adhesion, collagen-containing extracellular matrix, blood vessel morphogenesis and inflammation (Supplementary Fig. 1e, f and Supplementary Data 2). GFPhigh cells also expressed lower levels of genes typically associated with pulmonary neuroendocrine cells (PNECs) and higher levels of markers commonly expressed in non-NE epithelial cells such as ciliated cells, club cells, and alveolar type 1 (AT1) and type 2 (AT2) cells compared to GFPneg NE SCLC cells (Fig. 1b).

The expression of a variety of non-NE differentiation markers in GFPhigh cells from TKO/Hes1GFP+ tumors raised the possibility that this cell population was heterogeneous. However, using single-cell RT-qPCR, we found that GFPhigh SCLC cells had a relatively homogenous pattern of expression for the genes tested, including high levels of genes coding for club cell markers as well as lower levels of AT1 cell markers, which are also expressed at low levels in normal club cells24 (Fig. 1c and Supplementary Fig. 1g). The fold-change differences between the bulk RNA-seq and single-cell RT-qPCR data are likely due to differences in absolute transcript levels and the different platforms (Supplementary Data 1). Immunostaining only detected expression of the club cell marker CC10 (also known as SGCB1A1) and not the AT1 marker AGER on tumor sections (Fig. 1d). CC10 also frequently co-localized with HES1, indicating that HES1-high non-NE SCLC cells do not express detectable levels of this AT1 marker (Fig. 1e, f).

To investigate the role of Notch signaling in the generation of these non-NE club cell-like SCLC cells, we deleted Rbpj in TKO mice at the time of tumor initiation. Loss of RBP-J did not affect overall tumor burden but led to a significant reduction in HES1+ and CC10+ cells in the tumors (Fig. 1g–i and Supplementary Fig. 2a–c). The remaining few CC10+ cells were found to express detectable levels of RBP-J and hence, likely to be infiltrating normal club cells (Supplementary Fig. 2d). A similar requirement for RBP-J/Notch signaling in the NE to non-NE transdifferentiation process was recently described in a different mouse model of SCLC28.
Therefore, GFPhigh SCLC cells in TKO;Hes1GFp/+ tumors are a relatively homogeneous population of club cell-like cancer cells generated by activation of Notch signaling. These club cell-like phenotypes in SCLC tumors are reminiscent of both the transdifferentiation of wild-type NE epithelial cells into club cells in the adult lungs in response to injury via activation of Notch signaling\(^{23,24}\) and the generation of NE and non-NE cell during embryonic lung development\(^{11,19}\). The co-optation of these developmental and regeneration pathways in cancer suggested that SCLC may provide a useful model to further investigate the...
mechanisms of cell fate switch upon activation of Notch signaling in NE cells.

**REST is a regulator of the NE to non-NE transition in SCLC.** We previously identified REST as a candidate effector of Notch signaling in SCLC cells from microarray studies. From our RNA-seq dataset and single-cell RT-qPCR analysis, Rest was significantly upregulated in HES1<sup>high</sup>/GFPhigh non-NE SCLC cells (Supplementary Figs. 1c, 3a and Supplementary Data 1) and was again identified as the top candidate transcription factor repressing the downregulated genes during the cell fate transition (Supplementary Fig. 3b and Supplementary Data 1). In our previous study, REST overexpression in NE SCLC cells was sufficient to decrease the mRNA levels of the NE genes Chga, Chgb and Syp, but not of Ascl1 and the ASCL1 target Calca (coding for CGRP). ASCL1 downregulation and inhibition is viewed as a key event downstream of Notch in the inhibition of the NE cell fate<sup>15–17</sup>. Hence, we sought to determine the extent of overlap between REST and ASCL1 targets in SCLC.

ChIP-seq analysis of REST in GFPhigh SCLC cell lines in culture identified 1317 gene targets (FDR cutoff < 0.05 and more than 2-fold change) (Fig. 2a and Supplementary Data 3). About half of the binding sites were within 3 kb of transcription start sites and HOMER motif analysis identified REST binding motif as the top hit (Supplementary Fig. 3c, d). Upon ectopic expression of REST in NE SCLC cells in culture, 99 and 205 genes were downregulated by RNA-seq after 48 h and 5 days, respectively, with corrected p-value < 0.05 and log2 fold change of <-1.0 (Fig. 2a, Supplementary Fig. 3e, f and Supplementary Data 4). Combining the ChIP-seq and the 5-day REST overexpression RNA-seq datasets, we identified 141 high-confidence REST targets in NE SCLC cells (Fig. 2a and Supplementary Data 5). Ascl1 targets were obtained from a ChIP-seq dataset generated from primary TKO tumors, identifying 3008 candidate gene targets following our analysis pipeline (Fig. 2a and Supplementary Data 6). Comparing the genomic regions bound by ASCL1 and REST in the ChIP-seq datasets showed little overlap in their binding sites (Supplementary Fig. 3g). To generate high-confidence ASCL1 targets, we knocked down Ascl1 in NE SCLC cells for RNA-seq analysis after 3 days. In this context, ASCL1 knock-down has detrimental effects on population growth (Supplementary Fig. 4a, b). 180 and 493 genes were downregulated by shAscl1 #1 and shAscl1 #2, respectively, with corrected p-value < 0.05 and log2 fold change of <-1.0 (Supplementary Fig. 4c–e and Supplementary Data 7). Filtering the downregulated genes with no fold change cut-off and comparing with genes identified in the ASCL1 ChIP-seq identified 250 genes (Supplementary Fig. 4f and Supplementary Data 7). GO terms analysis showed enrichment in Notch signaling and nervous system development pathways (Supplementary Fig. 4g and Supplementary Data 8). Out of these, only 9 overlapped with the 141 high-confidence REST targets (Supplementary Fig. 4h). As the ASCL1 knockdown was detrimental to the cells, it was possible that genes with more subtle changes would be overlooked using this approach. Thus, we decided to instead narrow down the list of genes from the ASCL1 ChIP-seq to 559 by filtering out those that were not downregulated in GFPhigh cells (in which ASCL1 is expressed at very low levels) (Fig. 2a and Supplementary Data 5). By doing so, the overlap between ASCL1 targets and REST targets in SCLC cells was still only 43 genes (Fig. 2a and Supplementary Data 5). Examples of NE genes specifically induced by ASCL1 include Calca and Dil3, while genes specifically repressed by REST include Chga, Chgb and Resp18, and common genes include Kcn1l and Olfm1 (Fig. 2b, c). Notably, GO terms analysis for both lists of targets were similar but not identical (Fig. 2d and Supplementary Data 9). Even in the same categories, many genes were regulated by only one of the two transcriptional regulators (Fig. 2e).

Together, these analyses show that REST targets are largely distinct from ascl1 targets and support a model in which both inhibition of the pro-NE transcription factor ASCL1 and activation of the REST repressor contribute to the NE to non-NE cell fate change observed in SCLC downstream of Notch signaling activation. These results led us to test the functional role of REST in Notch-driven NE to non-NE transitions in vivo.

**REST is required for complete repression of SCLC NE programs.** To first investigate a possible requirement for REST in the NE to non-NE transition in vivo in SCLC, we crossed p53<sup>fl/fl</sup>/Rb<sup>fl/fl</sup>/p130<sup>fl/fl</sup> mice to a conditional allele of Rest (Rest<sup>fl</sup>) and analyzed tumors in triple and quadruple mutant mice upon Ad-CMV-Cre delivery to the lungs of these mice. In this model, the vast majority of tumors are initiated in non-NE lung epithelial cells<sup>40</sup>. Rest deletion was sufficient in this context, as assessed by in situ hybridization on lung sections (Supplementary Fig. 5a, b). We observed a reduction in tumor numbers in TKO;Rest quadruple mutant mice compared to TKO controls while TKO;Rest tumors tended to be slightly larger than TKO tumors (Fig. 3a–c). Expression of the individual markers HES1 and CC10 was similar on sections from TKO and TKO;Rest tumors (Supplementary Fig. 5c–e), suggesting that loss of REST is not sufficient to completely block the induction of these two non-NE markers. To gain a broader and unbiased view of the phenotypes related to REST loss, we isolated NE and non-NE cells from TKO and TKO;Rest<sup>fl/fl</sup> mutant mice (which were not crossed to the Hes1<sup>GFP</sup> allele) using the cell surface markers NCAM1 and ICAM1 based on the RNA-seq and FACS analysis of GFPhigh and GFP<sup>neg</sup> SCLC cells (Supplementary Data 1, Supplementary Fig. 6a,b and Fig. 3d). Cell populations isolated following this protocol grew in culture similar to NE and non-NE cells isolated based on HES1 levels<sup>29</sup>, with NCAM1<sup>high</sup> ICAM1<sup>low</sup> NE cells classically forming loosely adherent clusters and NCAM1<sup>low</sup> ICAM1<sup>high</sup> non-NE cells growing in 2D layers on the plate (Fig. 3e). These two cell populations clustered separately by RNA-seq analysis and in a manner similar to the Hes1<sup>low</sup>/GFPhigh and Hes1<sup>high</sup>/GFPhigh SCLC cell lines, respectively, with complete deletion of p53, Rb and p130 alleles confirmed by PCR on genomic DNA, further validating the isolation protocol (Supplementary Fig. 6c–e). Importantly, we observed separate clustering of the NCAM1<sup>low</sup> ICAM1<sup>high</sup> non-NE samples in the PCA analysis when comparing the TKO and TKO;Rest mutant cell lines (Fig. 3f). 240 genes were differentially expressed between the two groups, with the majority being upregulated (217 genes) with the loss of REST, in concordance with its role as a transcription repressor (Fig. 3g and Supplementary Data 10). Notably, the genes not repressed in REST mutant cells were significantly enriched in NE genes with 76 of them also identified as REST targets in our ChIP-seq/RNA-seq analysis (Fig. 3h, i and Supplementary Data 10). Of the 141 genes which did not overlap with our list of REST targets (possibly because cell lines and primary tumors may not be exactly the same), transcription factor analysis showed REST to be the top candidate transcriptional regulator binding in the vicinity of these genes, suggesting that a number of these genes are REST targets (Fig. 3i and Supplementary Data 11). Some of the genes (e.g., L1cam) were identified in the REST ChIP-seq experiment but did not meet the threshold for downregulation in the REST overexpression RNA-seq data. Ascl1, which is not a REST target, showed no differential expression in REST wild-type or mutant non-NE cells in contrast to the REST targets Chga and Syp (Supplementary Fig. 6f).
Together, these data indicate that REST is the major regulator of the derepressed genes when Rest is deleted in non-NE SCLC cells. These experiments also conclusively identify REST as a key regulator of the Notch-driven NE to non-NE transition in SCLC in vivo.

Loss of REST delays the NE to non-NE transition after injury. Treatment of mice with naphthalene induces cell death in most club cells, and part of the tissue repair following this injury is due to PNECs that proliferate and differentiate into club cells in response to Notch pathway activation24,41–43. Because of the similarities between SCLC tumors and lung repair after injury regarding the Notch-driven NE to non-NE transition, we next sought to examine a possible requirement for REST in this other in vivo context.

First, to determine Rest expression in the adult mouse lung, we performed single-cell RT-qPCR analysis of lung epithelial cells dissociated from the lungs of adult Chga-GFP mice, which have constitutive GFP expression driven by the promoter of the NE.
gene and REST target Chga44 (Supplementary Fig. 7a). As expected, based on the role of REST as a suppressor of the NE fate in SCLC, Rest expression was undetectable in GFP-expressing PNECs from Chga-GFP mice but present in other non-NE lung epithelial cell types (Fig. 4a). The analysis of a distinct single-cell RNA-seq dataset of mouse lung epithelial cells24 showed a similar result (Supplementary Fig. 7b).

To compare lung repair in wild-type and Rest mutant mice, we crossed Restfl/fl mice to Ascl1CreER/+;Rosa26LSL-Tom/LSL-Tom mice. In these mice, PNECs were first labeled with Tomato by injecting
tamoxifen to activate the Cre recombinase expressed from the Ascl1 locus. Mice carrying the floxed Rest alleles also had Rest deleted simultaneously without any observable effect on the PNEC since it is not expressed at this point (Supplementary Fig. 7c). This was followed by lung injury with naphthalene and a recovery period of three weeks (Fig. 4b). By quantifying outgrowths from labeled PNECs based on the size of Tomato-positive clusters, we found that loss of Rest resulted in the formation of much larger outgrowths with no change in the overall number or median area (Fig. 4c–e and Supplementary
REST regulates PNEC specification during lung development. Notch signaling also plays a central role in cell fate decisions between NE and non-NE cells during embryonic lung development. To test the possibility of REST also playing a role in this setting, we crossed Rest−/− mice to Shh−/− mice, thereby deleting Rest in early lung progenitor cells and the entire lung epithelium. The analysis of lungs one day before birth (E18.5) showed a significant increase in the number of CGRP+ PNECs and NEBs (neuroendocrine bodies) upon loss of REST (Fig. 5a–c). PNECs were also more often found in larger clusters in the mutant lungs as compared to controls (Fig. 5d, e). BrdU analysis showed that this increase was unlikely to be due to more proliferation (Supplementary Fig. 8a, b). RT-qPCR of entire lung lobes from Rest mutant mice found a 2-fold increase in non-REST targets, Ascl1 and Calca, in agreement with the doubled amount of PNECs. REST targets, Chga, Chgb and Syp, in contrast showed more than a 2-fold increase, with Chga being expressed a few hundred folds higher than control (Fig. 5f). In situ hybridization showed that Chga became expressed in non-NE lung epithelial cells, as confirmed by co-localization with E-cadherin, upon REST deletion in embryonic lungs (Fig. 5g–i). Thus, in the absence of REST, non-NE epithelial cells of the developing lung misexpress REST-repressed NE genes.

While Rest is a direct target of NICD, regulatory networks often involve positive and negative feedback loops. We were particularly intrigued by RNA-seq and ChIP-seq data in SCLC cells showing regulation by REST of the expression of the Dner gene coding for an atypical and poorly studied NOTCH ligand (DNER, Delta/Notch like epidermal growth factor related receptor) in SCLC tumors and in PNECs (Supplementary Fig. 8e, f). RT-qPCR of E18.5 embryonic lungs also showed an increase in Dner expression in Rest knockouts compared to controls (Supplementary Fig. 8g). These observations and the existence of genetic variants in DNER associated with the development of diabetes, suggesting a link with neuroendocrine phenotypes, led us to investigate the role of DNER in the NE fate in mice. We generated Dner knockout mice by CRISPR/Cas9 targeting in mouse oocytes and confirmed deletion (Supplementary Fig. 8f). However, we did not observe any difference in PNEC numbers in Dner mutant E18.5 embryonic lungs (Supplementary Fig. 8h, i). We also found no effect of Dner inactivation in the context of injury in adult mice (Supplementary Fig. 8j, k). Thus, feedback control of Notch signaling by REST remains an open question, but the atypical NOTCH ligand and REST target DNER does not control the NE fate in the contexts examined.

ATAC-seq identifies a TEAD signature in non-NE SCLC cells. Our data demonstrate that REST is a bona fide downstream mediator of Notch signaling in NE lung cells, and that REST targets are distinct from ASCL1 targets. But these data did not exclude the possibility of other regulators being involved in NE to non-NE transitions. We examined differences in chromatin accessibility between the NE and non-NE SCLC cells using ATAC-seq as a different unbiased way to investigate this cell fate transition (Fig. 6a). Principal component analysis (PCA) on the ATAC-seq data showed clear separation between NE and non-NE cells (Supplementary Fig. 9a). We found in total 80,367 differentially accessible peaks, with 39,539 more closed and 40,828 more open in the non-NE cells as compared to the NE cells with corrected p-value < 0.001 and log2 fold change of −1.0 or >1.0 (Fig. 6b). Motif analysis on the more accessible peaks ranked FOX family motifs and TEAD motifs at the top of the list of transcription factor motifs enriched in non-NE cells (Fig. 6c and Supplementary Data 12). However, as the FOX family motifs were also top hits in the analysis of the closed peaks (Supplementary Data 12), we decided to focus on the TEAD motifs. TEAD transcription factors are often (but not exclusively) associated with YAP1/TAZ factors downstream of the Hippo signaling pathway (reviewed in refs. 50,51). As noted above, YAP1 has been associated with non-NE phenotypes in SCLC cells. In agreement with the ATAC-seq findings, Yap1 and Wwtr1 (Taz) as well as their downstream targets were upregulated in GFP+ cells in the RNA-seq dataset (Fig. 6d). Immunostaining confirmed that a large proportion of YAP1-positive cells also co-express the non-NE club cell marker CC10 in mouse SCLC tumor sections (Supplementary Fig. 9b, c).

These observations raised the possibility of YAP1 being capable of activating the non-NE program in SCLC cells. We over-expressed Yap1-GFP or GFP in NE mouse SCLC cells and found that YAP1-expressing cells became adherent, a feature of non-NE cells, after 14 days (Fig. 6e–g). NOTC2H and HES1 were upregulated in Yap1-overexpressing cells while the NE markers ASCL1 and UCHL1 were downregulated (Fig. 6h). RNA-seq analysis 5 days after expression of YAP1 showed significant upregulation of Notch2 and the Notch pathway targets Hes1 and Rest (Fig. 6i and Supplementary Data 13). This observation was confirmed by RT-qPCR on independent samples (Supplementary Fig. 9d). GO term analysis of upregulated genes showed genes induced by Yap1 to be involved in blood vessel morphogenesis and cell adhesion, similar to Hes1+/-/GFP+ cells from Tko;Hes1+/- cells (Supplementary Fig. 9e and Supplementary Data 13). YAP1 has been shown to directly upregulate Notch2 in liver cells. We conducted ChIP-qPCR on GFP+ SCLC cell lines generated from Tko;Hes1+/- SCLC tumors and found YAP1 binding to the promoter region of Notch2 (Fig. 6j). Together, these observations suggest that increased activity of YAPI/TEAD can sensitize NE cells to Notch signaling by induction of NOTCH2 and is sufficient to promote the NE to non-NE cell fate switch.

With our data placing YAP1 upstream of Notch signaling, we expected to detect YAP1 expression when Notch signaling is blocked in tumor sections from Tko;Rbpj mutant mice. Instead, we found that YAP1 levels were reduced similarly to HES1 and CC10 upon loss of RBP-J (Supplementary Fig. 9f, g). Thus, it is possible that YAP1 expression is both upstream and downstream of Notch signaling, or that the two pathways enforce each other’s activity in this context, as suggested by a recent study.
Fig. 5 REST is antagonistic to pulmonary neuroendocrine cells specification in normal lung development. a Immunostaining for CGRP, a marker of pulmonary neuroendocrine cells (PNECs) on sections from E18.5 Shh<sup>Cre/+;Rest<sup>+/+</sup></sup>+, Rest<sup>+/+</sup>+, and Rest<sup>fl/fl</sup>+ lungs. DAPI stains DNA in blue. Scale bar, 50 μm. b–d Quantification of the average number of CGRP<sup>+</sup> PNECs (b) and neuroepithelial bodies (NEBs) (≥5 PNECs) (e) per lung section from each embryo (n = 5 embryos with 37 sections counted for Rest<sup>+/+</sup>+, 4 embryos with 30 sections counted for Rest<sup>+/fl</sup> and Rest<sup>fl/fl</sup>+) and neuroepithelial bodies (NEBs) (≥5 PNECs) (e) per lung section from each embryo (n = 5 embryos with 37 sections counted for Rest<sup>+/+</sup>+, 4 embryos with 30 sections counted for Rest<sup>+/fl</sup> and Rest<sup>fl/fl</sup>+). Unpaired t-test, data represented as mean ± s.d. In (d), a scatterplot of all solitary PNECs and clusters of each embryo (n = 438 for Rest<sup>+/+</sup>+, 688 for Rest<sup>fl/fl</sup>+) and 4 embryos for Rest<sup>fl/fl</sup>+. Unpaired t-test data represented as mean ± s.d. e Median number of PNECs per cluster for each embryo as in (d) (n = 5 embryos for Rest<sup>+/+</sup>+) and 4 embryos for Rest<sup>fl/fl</sup>+. Unpaired t-test data represented as mean ± s.d. f Relative mRNA expression levels of NE markers and Rest in E18.5 Shh<sup>Cre/+;Rest<sup>+/+</sup>+, Rest<sup>+/fl</sup>+, and Rest<sup>fl/fl</sup>+ lungs (n = 5 embryos for Rest<sup>+/+</sup>+) and 4 embryos for Rest<sup>fl/fl</sup>+). Unpaired t-test, data represented as mean ± s.d. g Representative brightfield images of Chga RNAscope in situ hybridization (ISH, red signal) in E18.5 Shh<sup>Cre/+;Rest<sup>+/+</sup>+</sup> and Rest<sup>fl/fl</sup> lungs (hematoxylin counterstain). Scale bar, 50 μm. h Quantification of the average number of RNAscope signal per μm<sup>2</sup> of lung section in (g) (n = 3 embryos for Rest<sup>+/+</sup>+ and 4 embryos for Rest<sup>fl/fl</sup>+) and 4 embryos for Rest<sup>fl/fl</sup>+. Unpaired t-test, data represented as mean ± s.d. i Representative images for Chga RNAscope ISH (orange) and E-cadherin immunofluorescence (red) in E18.5 Shh<sup>Cre/+;Rest<sup>+/+</sup>+</sup> and Rest<sup>fl/fl</sup> lungs. DAPI stains the DNA in blue. White arrows, NEBs. Scale bar, 100 μm. See also Supplementary Fig. 8. Source data are provided as a Source Data file.

Yap1 deletion prevents NE to non-NE transition after injury. In the adult lung, Yap1 and Wwtr1 are not expressed in PNECs except for a few rare cells (Supplementary Fig. 10a). In contrast, PNECs that transdifferentiate to a club cell fate during naphthalene-induced injury express detectable levels of Yap1 (Supplementary Fig. 10b). We therefore wondered if Yap1, like REST, can also modulate cell fate decisions in PNECs in non-cancer contexts. Yap1 deletion early in the embryonic lung epithelium in mice leads to a general failure in cell type specification<sup>33</sup>, making it impossible to investigate a specific role for YAP1 in PNEC specification. We thus tested the requirement for Yap1 in PNEC transdifferentiation during injury.

We crossed Yap1<sup>fl/fl</sup> mice to Ascl1<sup>CreER</sup>/Rosa26-Tom/LSL-Tom mice and labeled PNECs with Tomato by injecting tamoxifen followed by naphthalene to induce injury. We then allowed the mice a 3-week recovery period before harvesting the lungs for analysis. Immunofluorescence staining of lung sections from these mice identified Tomato-positive cells expressing CC10 in control
Fig. 6 YAP1 promotes the transdifferentiation of SCLC cells to a non-neuroendocrine fate. a Schematic representation of neuroendocrine (NE) and non-NE SCLC cells isolated from the TKO;Hes1GFP/+ mouse model for ATAC-seq. b Volcano plot of differentially accessible peaks from the ATAC-seq data comparing non-NE SCLC cells to NE SCLC cells (significant peaks with more than 2-fold change and p-value < 0.001 are in red). Wald test with Benjamini–Hochberg correction. c Scatterplot showing the enriched motifs identified by HOMER analysis on the more open peaks in non-NE SCLC cells (significant motifs with more than 1.5-fold change and p-value < 0.001 are in red). d Expression of Hippo pathway members and targets from RNA-seq data in GFP<sup>high</sup> cells compared to GFP<sup>neg</sup> cells (n = 4). Error bars, log2 fold change estimate ± SE. e-g Representative brightfield images of NE mouse KP1 SCLC cells 14 days after Ctrl-GFP (control) or YAP1-GFP overexpression. Scale bar, 100 μm. Quantification of the number of adherent cells counted per well of a 6-well plate (f) and the percentage of cells that became adherent (g) as in (e) (n = 3 independent experiments). Unpaired t-test, data represented as mean ± s.d. h Immunoassay of non-NE and NE markers in KP1 cells after overexpression of either Ctrl-GFP or YAP1-GFP (4 weeks). Bands of two molecular weights are detected with the antibody against YAP1 due to some incomplete cleavage of P2A. Samples from 2 independent experiments. I Volcano plot of differentially expressed genes from RNA-seq data of 5 days overexpression of YAP1 in KP1 cells. Notch2, Hes1, Rest and Hippo pathway members are highlighted (significant genes with more than 2-fold change and p-value < 0.05 are in red). Wald test with Benjamini–Hochberg correction. J ChIP-qPCR analysis of YAP1 binding at the promoters of Pou2f1 (negative ctrl), Ctgf (positive ctrl) and Notch2 in independently generated GFP<sup>high</sup> cell lines from TKO;Hes1GFP/+ mouse SCLC tumors (n = 3). Unpaired t-test, data represented as mean ± s.d. See also Supplementary Fig. 9 and Supplementary Data 12 and 13. Source data are provided as a Source Data file.
by the rarity of the cells involved in these transitions in vivo and the challenges associated with culturing these cells. We show here that SCLC hijacks normal developmental pathways and that findings relevant to Notch signaling in this cancer model where cells are not limiting can be extrapolated to normal tissue development and injury. Using SCLC, we identified REST and YAP1 as two additional important contributors to Notch-driven regulation of pulmonary neuroendocrine cell fate decisions. Both REST and YAP are antagonistic to the neuroendocrine fate and when lost, promotes NE specification or impedes transition towards the non-NE fate depending on the context (Fig. 8a, b).

Fig. 7 YAP1 is required for the transdifferentiation of pulmonary neuroendocrine cells after naphthalene injury. a Representative immunofluorescence images for Tomato expression (red) and either CC10 or YAP1 (cyan) on consecutive sections from an Ascl1CreER+;Rosa26LSL-Tom/LSL-Tom mouse 3 weeks after naphthalene-induced lung injury and tamoxifen injection (as in Fig. 4b). DAPI stains DNA in blue in the images on the right. Scale bar, 50 μm. b Quantification (%) of clusters that are Tom+ or Tom+, CC10+ after injury as in (a) (n = 27 clusters for Yap1+/+ and 32 clusters for Yap1fl/fl counted from 3 mice per genotype). Fisher’s exact test, two-tailed. c Whole mount fluorescence images of left lung lobes 3 weeks after injury. Images representative of n = 2 independent experiments. Scale bar, 5 mm. d Scatterplot of normal NEBs and PNEC outgrowths after injury as in (c) with each column in the scatterplot representing a single mouse and each data point a tomato-positive cluster in that mouse (n = 2 mice for corn oil controls, 4 mice for naphthalene-treated Yap1+/+, n = 8 mice for Yap1fl/fl). Unpaired t-test was conducted on the number of clusters from each mouse in naphthalene-treated groups that are above the threshold size of the non-injured controls (n = 4 mice for Yap1+/+, n = 8 mice for Yap1fl/fl), data represented as mean ± s.d. See also Supplementary Fig. 10. Source data are provided as a Source Data file.
Studies on PNEC specification in early development and transdifferentiation after injury have centered mainly around NOTCH receptors/ligands and ASCL1. The loss of NE signature when Notch signaling is activated has been attributed to the decrease in expression and activity of ASCL1, a well-known transcriptional activator of NE genes. However, our previous work showed that knock-down of ASCL1 in NE SCLC cells in culture is not sufficient to switch these cells to a non-NE fate and placed the transcriptional repressor of neuronal genes, REST, downstream of Notch. Our new data reveal that REST and ASCL1 govern largely independent transcriptional programs determining NE fate. In the absence of REST, a large number of NE genes cannot be repressed or show significantly delayed repression as cells switch fate towards a non-NE state. These experiments conclusively demonstrate that REST is an important mediator of Notch signaling to promote the non-NE fate. Recent work in the pancreas indicates that this role for REST in controlling neuroendocrine differentiation is conserved in other lineages.

Based on our findings, we place YAP1 upstream and REST downstream of Notch signaling. However, we cannot exclude the possibility of different sequences of events in different scenarios or the existence of feedback loops. For example, we observed upregulation of Notch2 after REST overexpression in SCLC cells.

Fig. 8 A conserved YAP/Notch/REST network controls the neuroendocrine cell fate in the lungs. a Schematics of the molecular pathway governing the activation/repression of the neuroendocrine programs. Active YAP1 upregulates the expression of NOTCH2, which in turn increases the expression of REST and HES1. HES1 represses the transcription of Ascl1, indirectly repressing the genes under ASCL1 transcriptional control, while REST directly represses the transcription of a distinct part of the neuroendocrine program. b Schematics of the in vivo experiments testing the effect of the loss of Rest/Rbpj/Yap1 on intratumoral heterogeneity, transdifferentiation during injury repair, and neuroendocrine fate specification during lung development. In the tumor model, loss of Rest prevents full transition to the non-neuroendocrine fate in contrast to Rbpj, which completely blocks the transition. In the injury model, Rest deletion delays terminal transdifferentiation resulting in a longer proliferation phase and larger outgrowths. Yap1 deletion blocks the ability of PNECs to respond to the injury, leading to the reduction in outgrowths/transdifferentiation. In the development model, the absence of REST promotes the neuroendocrine fate, causing an increase in the number of pulmonary neuroendocrine cells (PNECs) and neuroepithelial bodies (NEBs) in the lung.
in our RNA-seq data. Rest expression may also be induced by YAP1 in mechanisms independent of Notch signaling35. There is further evidence of crosstalk and bidirectional regulation between the Hippo/YAP and Notch pathways in other settings36,37. In an example directly relevant to our study, Yao and colleagues observed nuclear YAP1 in lineage-labeled PNECs expressing constitutively active NICD 3 days post-naphthalene injury24. Assuming that YAP1 is downstream of Notch signaling based on these data, the authors deleted Yap1 in NICD-expressing cells and observed no changes in the phenotypes analyzed, which led them to conclude that Hippo pathway was not required23. Yet it remains possible that YAP1 is upstream of Notch signaling initially, but that the two pathways work together in a positive feedback loop to drive the transdifferentiation process38.

By single-cell RNA-seq analysis, most of the PNECs do not express Yap1 except for a few rare cells24. Interestingly, only 20–30% of NEBs form “outgrowths” after naphthalene injury, and it has been postulated that these outgrowths arise from a rare type of “stem-cell-like” PNECs that express Notch224. Other than naphthalene-induced injury, another commonly studied lung injury is hypoxia. Studies in breast cancer have shown that hypoxia can inhibit Hippo signaling leading to YAP1 accumulation in the nucleus60. In the lungs, hypoxia induces proliferation in ~20% of the NEBs61 similar to the 20–30% noted in naphthalene-induced lung injury24. It is tempting to conjecture that the subset of NE cells induced to proliferate by hypoxia and naphthalene is the group of “stem-cell-like” NE cells that may express Yap1. A simple model would then be that Yap1 and Notch2 are co-expressed in the same “stem-cell-like” PNECs. The current single-cell RNA-seq data do not support this idea, although this may be due to detection limits of the technology. Dual lineage-tracing systems to label Ascl1 and Yap1 expressing PNECs will be needed to address this question.

SCLC is classified into four major subtypes based on expression of four key transcription regulators ASCL1, NEUROD1, POU2F3, and YAP124, although there has been some debate whether YAP1 expression marks a true subtype or is a re

Our identification of common transcriptional regulators and pathways in PNEC fate decisions and SCLC transdifferentiation underscores the relevance of PNEC biology to the evolution of SCLC intratumoral heterogeneity and vice versa. Discoveries in both fields will aid in development of better treatment options for not just SCLC but also other diseases linked to the activity of PNEC, including asthma and other immune-related activities in the lungs73,74.

Methods

Ethics statement. Mice were maintained according to practices prescribed by the NIH at Stanford’s Research Animal Facility (protocol #13565). Additional accreditation of Stanford animal research facilities was provided by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Animal studies. Mouse lines used were the triple-knockout (TKO) SCLC mouse model bearing deletions of floxed (fl) alleles of p53, Rb, and p130 as previously described25. GFP reporter expressed from the endogenous Hes1 promoter Hes1GFP allele78. Tomato reporter Rosa26Rosa26-TomatoTg77. GFP reporter under the control of Chga promoter Chga-GFP (inserted as multiple copies of BAC, also crossed to TKO but no Cre was introduced)44, floxed Yap1 allele Yap1fl78, floxed Rest allele Restfl (also named RestTg77), floxed Rbpj allele Rbpjfl79. Cre recombinase knocked into Ascl1 locus Ascl1'-Crefl (also named Ascl1fl)78. Constitutively active NICD (also named NICDfl)78 and Cre recombinase (Cre fused to GFP)71. Dner KO mice were generated by the Stanford Transgenic, Knockout, and Tumor Model Center (TKTC) via injection of C57BL/6 mouse zygotes with sgRNAs and Cas9 targeting sites in intron 1 and 4, resulting in a deletion of exon 2–4 containing the delta/notch-like EGF repeat region.

In vivo SCLC studies, tumors were induced in 8–12 weeks old mice of mixed gender by intratracheal instillation with 4 × 10⁶ PFU of Adeno-CMV-Cre (Baylor College of Medicine, Houston, TX)29. Mice were euthanized before or at the first sign of respiratory distress in accordance with Stanford APLAC guidelines. Mice were housed at 22 ± 2°C ambient temperature with 40% humidity and a light/dark cycle of 12 h (7 a.m.–7 p.m.).

Flow cytometry sorting of cells. Tumors isolated from the lungs of TKO;Hes1GFP+ mice approximately 6–7 months after tumor induction were pooled, finely chopped with a razor blade, and digested in 6 mL of PBS with 120 µl of 100 mg/ml collagenase/dispase (Roche) for 45 min in a 37°C shaker. The mixture was then cooled on ice before adding 15 µl of 1 mg/ml DNase (Sigma) for 5 min. Digested tissue was passed through a 40 µm filter, spun down, and resuspended in 1 mL of red blood cell lysis buffer (150 mM NH₄Cl, 10 mM KCl, 0.1 mM EDTA) for 1.5 min. Cells were spun down, washed once in DMEM, and resuspended in FACS buffer (10% BGS in PBS, 1 mL million cells per 100 µl). The resultant single-cell suspension was stained with the following FACS antibodies: CD45-PE-Cy7 (Biosciences 25-0451-82, clone 30-F11, 1:100), CD31-PE-Cy7 (Biosciences 25-0311-82, clone 390, 1:100), TER-119-PE-Cy7 (eBioscience 25-5921-82, clone TER-119, 1:100), CD24-APC (eBioscience 17-0242-82, clone M1/69, 1:200) or CD45-Pacific Blue (Biolegend 103126, clone 30-F11, 1:100), CD31-Pacific Blue (Biolegend 102422, clone 390, 1:100), TER-119-Pacific Blue (Biolegend 116232, clone TER-119, 1:100), CD24-Pacific Blue (Biolegend 101821, clone M1/69, 1:200), ICAM1-PE-Cy7 (Biolegend 17-5921-82, clone 30-F11, 1:100), ICAM1-PE (Biolegend 116232, clone YN1/1.7.4, 1:100), NCAM1/CAM5.2-Pacific Blue (Biolegend 116232, clone YN1/1.7.4, 1:100), NCAM1/CAM5.2-APC-R (R&D Systems FAB7820A, clone 809220, 1:50) and DAPI to label dead cells. Lungs from Chga-GFP mice were digested and stained as above with an additional lineage negative selection marker F4/80-PE-Cy7 (Biolegend 123114, clone BM8, 1:100) and EpCAM-APC (Biosciences 17-3791-82, clone GR8, 1:100) instead of CD24 as positive selection marker.

Tumors isolated from the lungs of TKO;Restfl or TKO;Yap1fl mice were digested in the same way but stained with the following FACS antibodies: CD45-Pacific Blue (Biolegend 103126, clone 30-F11, 1:100), CD31-Pacific Blue (Biolegend 102422, clone 390, 1:100), TER-119-Pacific Blue (Biolegend 116232, clone TER-119, 1:100), CD24-Pacific Blue (Biolegend 102422, clone 390, 1:100), TER-119-Pacific Blue (Biolegend 116232, clone TER-119, 1:100), CD24-Pacific Blue (Biolegend 101821, clone M1/69, 1:200), ICAM1/CAM5.2-PE-Cy7 (Biolegend 161623, clone YN1/1.7.4, 1:100), NCAM1/CAM5.2-APC-R (R&D Systems FAB7820A, clone 809220, 1:50) and ICAM1-PE-Cy7 (Biolegend 116232, clone YN1/1.7.4, 1:100). FACS was performed with a 100 µm nozzle on a BD FACSaria II using the FACSdiva software. Data were analyzed and plotted using FlowJo v10.

Genotyping. DNA was extracted from cells using the Allprep DNA/RNA microkit (QIAGEN 80284). 1 ng of DNA was used for each PCR reaction to detect uncombined (floxed) and recombined (delta, Δ) Rb, p53, and p130 alleles. Primer sequences are available in Supplementary Data 14.

RNA-seq library preparation, sequencing, and analysis. 2 × 10⁶ cells were sorted for each sample from tumors of TKO;He1tGFP+ mice, REST or YAP1 over-expressing cell lines in respective experiments. RNA was isolated using Allprep DNA/RNA microkit (QIAGEN 80284) according to manufacturer’s protocol and concentration determined by Agilent Bioanalyzer 2100. Libraries were prepared
using TRIO RNA-seq kit (Nugen 0507-32) according to manufacturer’s protocol with 5 ng of RNA for in-vivo tumor cells or 10 ng of RNA for in-vitro cell lines per sample as starting material. Concentration of libraries were determined using KAPA library quantification kit (KAPA Biosystems KK4835) and samples were pooled evenly for sequencing using Illumina Nextseq 500 or HiSeq 4000 to obtain approximately 30 million paired-end reads per sample. 5 × 10^6 cells were pelleted and snap frozen for each cell line generated from tumors of Tko or Tko/Red6® mice. RNA isolation, library preparation and sequencing were performed by Novogene using Illumina platforms to obtain approximately 30 million paired-end reads per sample. For the Ao1 knock-down experiments, RNA was isolated from shAo1-transduced cell lines using RNeasy Mini Kit (Qiagen, 74104) and sent to Novogene for library preparation and sequencing as described above.

Transcript count files for RNA-sequencing data were generated using Salmon82 (combine-lab.github.io/salmon/). Differential expression analysis was conducted using DESeq283 with default settings. Plots are generated using ggplot284 (https://ggplot2.tidyverse.org/) and EnhancedVolcano85 (github.com/kevinbhinge/EnhancedVolcano).

**Gene set enrichment analysis.** Normalized count files generated by DESeq2 were used as input for Gene set enrichment analysis.86,87 (GSEA). GSEA was performed using MSigDB Hallmark gene sets with the following settings (Number of permutations = 10,000, Permutation type = gene_set).

**Gene ontology enrichment analysis.** Gene ontology (GO) enrichment analysis was performed using Metascape88 with default settings in the “Express Analysis” or “Custom Analysis: Cellular components/Biological Processes” functions.

**Single-cell fluidid analysis.** Single cells were sorted into individual wells of a 96-well PCR plate containing 5 µL of 2× reaction mix (CellsDirect™ One-Step RT-qPCR kit, Invitrogen 11753) and 2 units of SUPERase-In RNase inhibitor (Ambion, AM2696). Plates are stored at −80 °C till processing. Primers were designed using Fluidigm DAS assay design system and pooled to a final concentration of 500 nM to be used at 10× dilution in the PCR reaction. One-step reverse transcription and pre-amplification were performed on the sorted cells with the pooled primers and SuperScript III RT/Platinum Taq Mix from the kit according to the following condition: 15 min 50 °C, 2 min 95 °C, 20 cycles of 15 s 95 °C and 4 min 60 °C, 15 min 4 °C. The cDNA products were treated with Exonuclease I (NEB M0293S) for 30 min at 37 °C, inactivation at 80 °C for 15 min to remove excess primers and diluted 5-fold with low EDTA TE (Affymetrix 75793) for the final reaction. 2.25 µL of diluted cDNA, 2.5 µL 2× Slow EvaGreen Supermix with low ROX (Bio-Rad #172-5211) and 0.25 µL 20X DNA binding dye sample loading reagent (Fluoronor #85000738) and then loaded onto 48.48 Dynamic Array™ fluidic integrated fluidic circuit (IFC) chip sample inlets. 0.25 µL of each 100 µM primer pair, 2.5 µL 2× Assay Loading reagent (Fluidigm #85000736) and 2.25 µL low EDTA TE were mixed and loaded into the IFC assay inlets. The chip was ran on a Biomark™ machine according to the manufacturer’s protocol for EvaGreen probes. Primer sequences are available in Supplementary Data 14.

**Single-cell RNA-seq analysis.** Single-cell RNA-seq data of PNECs and other lung epithelial cell types in a normal lung were downloaded from GEO:GSE136580.24.

**Immunostaining.** For immunofluorescence on paraffin sections, tissues were first fixed overnight in 10% neutral buffered formalin (NBF) and processed for paraffin embedding. Paraffin sections were deparaffinized with Histo-clear (National Diagnostics, Atlanta GA) and rehydrated in decreasing strengths of ethanol and finally in 100% ethanol. Antigen retrieval was carried out in citrate-based unmasking solution (Vector Laboratories H-3300) by heating in the microwave till boiling followed by cooling on the benchtop for 30 min. Sections were blocked in blocking solution (5% horse serum, 0.25% Triton X-100) for 10 min at room temperature before incubation with primary antibodies overnight at 4 °C. The following day, sections were washed three times for 5 min each in PBS followed by secondary antibody incubation for 1 h at room temperature and nuclei counterstain with DAPI (Invitrogen). Finally, sections were washed in PBST, blocked in 5% horse serum, 0.25% Triton X-100 for 10 min at room temperature before incubation with primary antibodies overnight at 4 °C. The following day, sections were washed three times for 5 min each in PBS followed by secondary antibody incubation for 1 h at room temperature and nuclei counterstain with DAPI. Finally, sections were washed in PBST, blocked in 5% horse serum, and incubated with primary antibodies overnight at 4 °C. CC10 was developed by using ImmPRESS® HRP Horse Anti-Rabbit IgG Polymer Detection Kit (Vector Laboratories MP-7402) for SYP, YAP1, C GRP, RBP-J or HES1 following the manufacturer’s protocol and color development with TSA Plus Fluorescein (Akoya Biosciences). Sections were then washed in PBST and incubated in fluorophore-conjugated secondary antibody and DAPI for 1 h at room temperature. Sections were then washed in PBST, blocked in 5% horse serum and incubated with primary antibodies overnight at 4 °C. SYP, YAP1, CGRP, RBP-J or HES1 was developed first by using ImmPRESS® HP Horse Anti-Mouse IgG Polymer Detection Kit (Vector Laboratories MP-7402) for SYP or ImmPRESS® HRP Horse Anti-Rabbit IgG Polymer Detection Kit (Vector Laboratories MP-7401) for CGRP, YAP1, RBP-J and HES1 following the manufacturer’s protocol and color development with TSA Plus Fluorescein (Akoya Biosciences). Sections were then washed in PBST and incubated in fluorophore-conjugated secondary antibody and DAPI for 1 h at room temperature. Sections were then washed in PBST, blocked in 5% horse serum and incubated with primary antibodies overnight at 4 °C. SYP, YAP1, C GRP, RBP-J or HES1 was developed first by using ImmPRESS® HP Horse Anti-Mouse IgG Polymer Detection Kit (Vector Laboratories MP-7402) for SYP or ImmPRESS® HRP Horse Anti-Rabbit IgG Polymer Detection Kit (Vector Laboratories MP-7401) for CGRP, YAP1, RBP-J and HES1 following the manufacturer’s protocol and color development with TSA Plus Fluorescein (Akoya Biosciences). Sections were then washed in PBST. 

**Naphthofluor-based induced lung injury.** Naphthalene induced lung injury were taken with 1.5 mg of tamoxifen (20 mg/mL in corn oil, Sigma-Aldrich T5648) i.p. in 8- to 10-week old mice of mixed gender were injected with 1.5 mg of tamoxifen (20 mg/mL in corn oil, Sigma-Aldrich T5648) i.p.
daily for three consecutive days. Cages were changed 72 h later to allow tamoxifen to wash out completely. 7 days after the last tamoxifen injection, mice were injected with an intraperitoneal injection of 200 µg of tamoxifen in 50 µL of PBS (Sigma-Aldrich 84500) to induce 3 weeks, 3 days after neutrophil injection, lungs were harvested and fixed in cold 4% paraformaldehyde (diluted with PBS from 32% paraformaldehyde, Electron Microscopy Sciences 15714) with rocking at 4 °C for 4–5 h, protected from light to preserve endogenous fluorescence. Fixed lungs were washed once in PBS for 2 h followed by 4°C and PBS wash overnight at 4 °C. After which, they were transferred into Cubic-L (Tokyo Chemical Industry T3740) for 4 days (with one change of solution after 2 days) for clearing at room temperature with slow rocking. The lungs were then washed twice in PBS, 2 h each at room temperature, before storing in Cubic-R (Tokyo Chemical Industry T3741) at 4 °C for the rest of the time (1–2 days later) till imaging. For whole mount imaging, cleared lungs were placed in inverted coverglass chambers (Nunc Lab Tek 155361) and imaged with 2x objectives using Keyence BX-Z700 all-in-one fluorescence microscope with BX-Z.Viewer program version 1.3.1.1 and BX-Z Analyst 1.4.0.1. Images were manually demarcated as region of interests (ROIs) and tumor number/area measured with Fiji ls Just ImageJ). Statistical analyses for these injury experiments were performed on clusters containing cells over a threshold (based on corn oil-treated mice) because only a small fraction of neuroendocrine cells is expected to respond to injury.15 Statistical analysis using median values showed no significance, as shown in the panels.

Cell cultures and cell lines. Mouse SCLC cell lines were grown in RPMI-1640 medium (Corning MT5040CV) and 293T in DMEM medium (Hyclone SH30430.1) supplemented with 10% bovine growth serum (Hyclone SH30430.1) and penicillin–streptomycin–glutamine ( Gibco 10378016). Mouse KPI SCLC cell line was generated in the lab and been described.

REST and YAP1 overexpression in vitro studies. Control-P2A-GFP, mREST-P2A-GFP, and YAP1-P2A-GFP were cloned into a lentiviral pCDH-MVC vector backbone (Addgene plasmid #72265) and packaged in 293T cells by co-transfection with delta.8 and VSVG using PEI. Virus supernatant was collected 48 h after transfection and added to KPI1 cells. KPI1 cells were then sorted 48 h or 5 days after virus transduction for RNA-seq. For YAP1 transdifferentiation assay, YAP1 was co-transfected in 293T cells. Virus supernatant was collected every 4 days. At the end of day 14, the number of floating and adherent cells were trypsinized and quantified.

Ascl1 knockdown in vitro studies. Short hairpin RNAs against Ascl1 (1:5'-CTTCAAGAGTCTTTGACTACAT-3'; 2: 5'-CCAGGGATTTCGTTGTCGTTT-3') from the MISSION shRNA library (Sigma-Aldrich) were packaged in 293T cells by co-transfection with delta.8 and VSVG using PEI-based transfection. Virus supernatant was collected 48 h after transfection to infect KPI1 cells. Purovycin (1 µg/mL) was added 24 h later for selection. The transduced cells were then harvested on Day 3 after transfection for RNA-seq analysis.

ATAC-seq library preparation and analysis. 5 × 10^6 cells were sorted for each Hes1-GFP high or negative sample from pooled tumors of each individual TKO/ Hes1(GFPl) mouse. ATAC-seq libraries were constructed as described in ref. 16. Briefly, cells were washed in cold PBS buffer and resuspended in 50 µL of cold lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.1% Igepal CA-630) and centrifuged immediately for 10 min at 500 × g, 4 °C to generate a crude nuclei preparation. Nuclei pellets were resuspended in transposon mixture (Illumina FC-121-1036: Nextera kit components—25 µL 2× TD buffer, 2.5 µL TDEI transposase and 22.5 µL nuclease-free water) and incubated at 37 °C for 30 min. The samples were then purified using MinElute PCR purification Kit (Qiagen 28004). Library fragments were amplified in 1× NENext High Fidelity PCR Master Mix (New England BioLabs M0541) and 1× of custom Nextera PCR primers 1 and 2. Number of amplification cycles for each sample was determined by qPCR to prevent saturation. Final libraries were once again purified using MinElute PCR purification kit (Qiagen 28004) and sequenced on Illumina HiSeq4000 to generate paired-end 50-bp reads.

Pair-ended reads were trimmed for Illumina adaptor sequences and transposase sequences using a customized script and mapped to mm10 using Bowtie2 (2.3.4.1)18 with parameters –p 4–very-sensitive. Duplicate samples were removed with Samtools94. Narrow peaks were called using MACS219 with parameters--nomodel–extsize 73–shift -3 ≥ 0.01. Peaks from all samples were used for random read coverage mapping to each peak (using bedtools multicrov (Quinlan laboratory, University of Utah, Salt Lake City, UT) for each individual sample were quantified.

Differentially accessible peaks from the merged union peak list were selected with the DESeq2 R package from Bioconductor. Cutoffs were set at log2 fold change > 1 or < -1 and adjusted p-value < 0.01. Motif analysis was performed using HOMER function (http://homer.ucsd.edu/homer/motif/) "findMotifsGenome.pl" with default parameters to calculate the occurrence of a TF motif in peak regions compared to that in background regions. We used −log10(p-value) to rank the enrichment level of TF motifs.

In situ hybridization. Chromogenic In situ hybridization (ISH) for mouse Rest (RNAseq) L5 2.5 probe–Mon- Rest, ACDBio #36258) or mouse Ctnna1 (RNA-seq) L5 2.5 probe–Mm-Ctnna1, ACDBio #447858) was performed on a Leica Bond RX with Bond Polymer Refine Red Detection kit D9390). For multiple ISH and E-cadherin immunofluorescence (IF), ISH was detected with Opal650 using the Opal automation IHC kit (Akoya Biosciences, SKU NELR21001KT), followed by E-cadherin (Cell Signaling Technology 3195, 1:400) on the Leica Bond Max with EnVision+ System-HRP Labeled Polymer Anti-Rabbit (DAKO, K4003) and Opal570 followed with DAPI. Whole sections were scanned using the Olympus VSI120 and staining was quantified using the ISH detection algorithms in HALO.

Immunosassays. Cells were lysed in RIPA buffer (Thermo Fisher Scientific 89890) supplemented with protease and phosphatase inhibitors (Roche Complete ULTRA ture Proteinase Inhibitor 0.02% v/v) and Proteinase K (New England Biolabs FS1075) for 2 h at 55°C. The immunoprecipitated DNA was then purified using the PCR Purification kit (Millipore 28106) and Quantifluor (29106) with 1 µg of water for downstream qPCR, 1 µL per qPCR reaction. Primer sequences are available in Supplementary Data 14.

ρPCR-seq and analysis. For REST ChIP-seq (two biological replicates), ρPCR was performed as above with REST antibody (Millipore 17-641) skipping the 2 µm disuccinimidyl glutarate fixation step. ρPCR-seq libraries were constructed using the Ovation Ultralow System V2 kit (Nugen 0344NB-32) according to manufacturer’s protocol. The library was generated on Illumina Hiseq4000 to obtain single-end reads of 75 bp. For ASCL1 ρPCR-seq, fastq data were downloaded from GEO:GSE69394 (one replicate)16. ρPCR data were aligned using BWA3 and Samtools94 to the mouse genome mm10 using the default settings. Aligned sequences were then processed using HOMER3 (http://homer.ucsd.edu/homer/) using the ρPCR tag direction finding function. Fold change is relative to input. Heatmap of binding sites were generated with default settings using deepTools96.
Technology 11988, 1(50), UCHL1 (Sigma-Aldrich HPA005993, 1:5000), and HSP90 (Cell Signaling Technology 4877, 1:5000).

Statistics and reproducibility. Statistical significance was assayed with GraphPad Prism software apart from Wilcoxon rank sum test which was carried out in R. The specific tests used are indicated in the figure legends. Data were presented as mean ± standard deviation unless stated otherwise. All experiments were independently replicated at least twice or findings verified using other orthogonal methods. No data were excluded except for 4 cell lines isolated from TKORestΔf tumors because Rest was not properly deleted as deduced from Rest mRNA levels. Mice for in vivo studies were randomized where applicable and sample sizes determined by pilot experiments or previous experiments using similar models. Investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All genomic sequencing datasets generated in this study are available at Gene Expression Omnibus (GEO) under Super Series GSE171919. Single-cell RNA-seq data of the lungs were downloaded from GSE136580. Source data are provided with this paper.

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