MAPK pathway activation selectively inhibits ASCL1-driven small cell lung cancer

Highlights
- MAPK activation causes cell-cycle arrest and senescence selectively in SCLC-A subtype
- MAPK-induced growth inhibition is independent of NOTCH signaling
- MAPK activation increases ERK negative feedback and activates STAT3 signaling
MAPK pathway activation selectively inhibits ASCL1-driven small cell lung cancer

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SUMMARY

Activation of mitogenic signaling pathways is a common oncogenic driver of many solid tumors including lung cancer. Although activating mutations in the mitogen-activated protein kinase (MAPK) pathway are prevalent in non-small cell lung cancers, MAPK pathway activity, counterintuitively, is relatively suppressed in the more aggressively proliferative small cell lung cancer (SCLC). Here, we elucidate the role of the MAPK pathway and how it interacts with other signaling pathways in SCLC. We find that the most common SCLC subtype, SCLC-A associated with high expression of ASCL1, is selectively sensitive to MAPK activation in vitro and in vivo through induction of cell-cycle arrest and senescence. We show strong upregulation of ERK negative feedback regulators and STAT signaling upon MAPK activation in SCLC-A lines. These findings provide insight into the complexity of signaling networks in SCLC and suggest subtype-specific mitogenic vulnerabilities.

INTRODUCTION

Small cell lung cancer (SCLC) is an aggressive malignancy, often demonstrating neuroendocrine features, that accounts for ~15% of all lung cancers in the United States (Bernhardt and Jalal, 2016; Hann et al., 2019). Metastasis is often found at first diagnosis, and metastatic disease is exceptionally lethal with a 2-year survival of <5% (Byers and Rudin, 2015). For decades, the primary treatment options for SCLC were limited to combination chemotherapy and radiotherapy; the emergence of resistant tumors commonly occurs within months for these treatment regimens. The therapeutic benefits of targeted therapies for SCLC have been generally disappointing, including inhibitors of PARP, CHK1, WEE1, and BCL2 (Poirier et al., 2020; Rudin et al., 2021). The addition of immune checkpoint blockade targeting the PD-1/PD-L1 axis has changed the first-line standard of care for metastatic SCLC but leads to durable response in only about 10% of patients (Horn et al., 2018; Paz-Ares et al., 2019; Rudin et al., 2020).

A major challenge to identifying effective treatments for SCLC is its association with the loss of key tumor suppressors, such as inactivating RB1 and TP53 alterations, but seems to lack targetable oncogenic driver mutations (George et al., 2015; Peifer et al., 2012; Rudin et al., 2012). Defining additional therapeutic options will depend on a deeper understanding of SCLC biology and the associated signaling pathways. Of note, SCLC has been recently subdivided into biologically distinct subtypes based on differential expression of key transcriptional regulators (ASCL1, NEUROD1, POU2F3, YAP1), defining subtypes SCLC-A, -N, -P, and -Y, respectively (Rudin et al., 2019). The role of YAP1 as a subtype-defining factor has subsequently been called into question (Baine et al., 2020; Pearsall et al., 2020). Emerging data suggest that these subtypes may respond differently to therapy (Gay et al., 2021; Owonikoko et al., 2021; Poirier et al., 2020).

The mitogen-activated protein kinase (MAPK) pathway is a central axis driving aberrant proliferation in a broad spectrum of cancers. Although somatic gain-of-function mutations in MAPK genes occur in about 30% of human cancers (Schubbert et al., 2007), including non-small cell lung cancer (NSCLC), genomic and proteomic analyses indicate suppression of MAPK pathway activity in SCLC (Byers et al., 2012; Cerami et al., 2012; Gao et al., 2013; Wagle et al., 2018). This striking difference is not well understood, and previous attempts to determine whether deficits in MAPK signaling might be therapeutically important in SCLC have subsequently been called into question (Baine et al., 2020; Pearsall et al., 2020). Emerging data suggest that these subtypes may respond differently to therapy (Gay et al., 2021; Owonikoko et al., 2021; Poirier et al., 2020).

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Figure 1. SCLC-A is selectively sensitive to MAPK activation in vitro and in vivo
(A) Bar graph showing absolute counts for Mutation, Fusion, Deep Deletion, Amplification, and Multiple Alterations found in MAPK pathway genes (KRAS, HRAS, BRAF, RAF1, MAP3K1, MAP2K1, MAP2K2, MAP2K4, MAPK1, DUSP4, and CCND1) comparing SCLC (n = 469) and NSCLC (n = 8,948) clinical samples. The dataset was derived from the MSK-IMPACT clinical sequencing cohort (Cerami et al., 2012; Gao et al., 2013) as of 24 March, 2021, with variants of unknown significance and germline mutation excluded. p Value derived from Pearson’s chi-squared test considering all alterations.

(B) Graph showing MAPK Pathway Activity Score (SPRY2, SPRY4, ETV4, ETV5, DUSP4, DUSP6, CCND1, EPHA2, and EPHA4) comparing SCLC (n = 53) and NSCLC (n = 133) cell lines (GSDM). MAPK pathway activity scores were calculated in Wagle et al. (2018) using Z scores for each gene/cell line from CCLE. Unpaired t test was performed between SCLC and NSCLC so that ****p < 0.0001.

(C) Overview of SCLC cell lines and their subtype used in this study. ASCL1-driven (SCLC-A, red), NEUROD1-driven (SCLC-N, blue), and POU2F3-driven (SCLC-P, green).

(D) Western blot analysis on ASCL1-driven (H510, H69, DMS79, H720, red), NEUROD1-driven (H82, blue), POU2F3-driven (H526, green) SCLC cell lines and NSCLC cell line A549 upon MEKDDS217D/S221D expression (0, 1, 2, 3 days). MEKDDS217D/S221D (HA-tag) and total and phosphorylated ERK1/2 are shown, and β-actin was used as loading control.

(E) Cell viability assay with SCLC cell lines following MEKDDS217D/S221D expression for 9 days. Illustrated are the average relative changes (±SEM) of cell viability. Unpaired t test was performed between ASCL1-driven (DMS79, H69, H720, H510) and non-ASCL1-driven (H82, H526, A549) SCLC cells so that ****p < 0.0001.

(F) ASCL1-driven SCLC cell line H510-MEKDD was injected subcutaneously into athymic mice (n = 6/cohort) and checked biweekly for palpable tumors. Mice in the “MEKDD ON” cohort were kept on a doxycycline chow diet throughout the experiment. Graph illustrates average tumor volume (mm3, ±SEM) over time. Unpaired t test was performed between the two cohorts at the indicated time point so that **p < 0.01, ***p < 0.001.

(G) Overall survival of the recipient mice from F is plotted as a Kaplan-Meier curve and denoted as the time from injection of cells to tumor volume of 1,500 mm3. p Value was calculated by log rank test.
RESULTS

SCLC-A is selectively sensitive to MAPK activation

In contrast to NSCLC, genomic and proteomic analyses have indicated suppression of MAPK pathway activity in SCLC. We first assessed the frequency of somatic mutations in MAPK pathway mutations in our patients with lung cancer, profiled by MSK-IMPACT targeted tumor sequencing (Cheng et al., 2015). In a total of 8,948 patients with NSCLC and 469 patients with SCLC, MAPK pathway mutations in KRAS, HRAS, BRAF, RARF1, MAP3K1, MAP2K1, MAP2K2, MAP2K4, MAPK1, DUSP4, and CCND1 were found in 3,021 (34%) of the NSCLC cohort in comparison with 51 (11%) of the SCLC cohort (Figures 1A and S1A) (Cerami et al., 2012; Gao et al., 2013). A breakdown of mutation frequency of individual genes revealed KRAS to be the most prominent mutation in the NSCLC and SCLC cohorts (29.1% and 3.2%, respectively) followed by BRAF in the NSCLC cohort (4.1%) and MAP2K4 in the SCLC cohort (2.5%) (Figure S1B). SCLC also has a significantly lower transcriptional MAPK Pathway Activity Score (MPAS; SPRY2, SPRY4, ETV4, ETV5, DUSP4, DUSP6, CCND1, EPHA2, and EPHA4) in comparison with NSCLC (Figure 1B). This finding is in keeping with prior studies that have identified differences in MAPK activity and targeting between SCLC and NSCLC. The striking differences in MAPK activity and targeting between SCLC and NSCLC are not well understood. We hypothesized that the molecular differences in recently recognized SCLC subtypes might explain the inconsistent results from prior studies examining the apparent MAPK pathway suppression in SCLC.

To initially assess this hypothesis, we examined the phenotypic differences that result from exogenous MAPK activation in a cohort of SCLC cell lines and an NSCLC control. We transduced cell lines from the three predominant SCLC subtypes (SCLC-A (ASCL1high): H510, H720, H69, DMS79, H209, and H128; SCLC-P (POU2F3high): H526; SCLC-N (NEUROD1high): H82 and H446) and the NSCLC cell line A549 with a doxycycline-inducible vector driving expression of MEKDDS217D/S221D (MEK1) (Figure 1C). Table S1 shows cell lines used in this study with annotation of transcriptional signatures and therapeutic vulnerabilities, may also differ in their mitogenic signaling dependencies.

Here, we sought to elucidate the distinctions between and potential therapeutic vulnerabilities of MAPK pathway activation in different subtypes of SCLC. We show that activation of the MAPK pathway is particularly disadvantageous to the predominant SCLC-A subtype, associated with high expression of ASCL1. We link this sensitivity to downstream effects on other signaling pathways such as the STAT3 pathway. This study sheds light on the complexity and diversity of oncogenic signaling networks in SCLC, supporting that SCLC subtypes have distinct mitogenic signaling that may result in differential therapeutic vulnerabilities.

We further tested the effects of exogenous MAPK activation on tumor growth in two SCLC-A lines and one SCLC-N line established as in vivo xenografts. H510 cells transduced with MEKDDS217D/S221D were injected into athymic mice (n = 6/cohort), which resulted in a significantly smaller tumor volume and longer survival in the cohort of mice receiving a doxycycline chow diet (MEKDD activated) relative to no-doxycycline controls (Figures 1F and 1G). H510 tumors in which MEKDD was activated demonstrated tumor growth inhibition of over 75% at 36 days relative to tumors without MAPK pathway activation (267 versus 1,076 mm3).
tumor volume, respectively). No significant difference between mean body weights was seen (Figure S1D). Similar results were observed in another SCLC-A cell line H720 (Figures S1E–S1G). Regardless of whether MEKDD expression was induced in H720 or not, histological examination revealed retention of SCLC characteristics, including solid sheets and clusters of round/oval blue cells with scant cytoplasm, nuclei with fine chromatin, high mitotic rate, no distinct nucleoli, occasional molding, and smudging (Figure S1H). H510 cells exhibited classical SCLC morphology with solid, nested, and trabecular architecture. H510 tumor cells without MEKDD expression exhibited somewhat more loosely packed nests and trabeculae with occasional solid foci, whereas upon MEKDD expression, tumor cells revealed predominantly solid sheets of tumor cells. Tumor cells from both models (H510 and H720) stained positive for the subtype marker ASCL1 and neuroendocrine markers INS1 and NCAM1, regardless of MEKDD expression (Figure S1H). H82 (SCLC-N) cells that were transduced with MEKDD217D/221D and injected into athymic mice demonstrated the opposite phenotype. H82 tumors in which MEKDD was activated resulted in faster tumor formation relative to no-doxycycline controls (1,305 versus 734 mm³, respectively) at day 24 and shorter survival (Figures 1H and 1I). No significant difference between mean body weights was seen (Figure S1I). Taken together these findings demonstrate a subtype-specific suppression of cell viability and tumor growth arrest in vitro and vivo models, respectively, upon MAPK signaling activation.

MAPK activation in SCLC-A leads to a G2/M cell-cycle arrest and senescence

We next sought to further interrogate the cell growth and proliferative implications of aberrant MAPK activation in SCLC-A. Previous reports indicate that activation of the MAPK pathway via NRASG12D/RASV12 alterations or an activatable form of c-raf-1 can lead to morphological changes in several different cancer types (Calbo et al., 2011; Ravi et al., 1998, 1999; Shieh et al., 2013; Yu and Garcia, 2020). Indeed, we observed that ERK activation in SCLC-A cell lines led cells that typically grow in suspension to adapt a more adherent phenotype. This was primarily seen in H69 and DMS79 SCLC-A cell lines (Figure 2A). Of interest, H510 cells that grow in culture as intermixed adherent and suspension populations fully change to suspension cells, increasing the number of floating clusters of cells as early as 24 h after MAPK activation and becoming a fully apparent shift by day 3 (Figure 2A). No change in morphology was observed in SCLC-A H720 and SCLC-N and -P lines H82 and H526, respectively.

SCLC-A cell lines are notable for high-level expression of both ASCL1 and INS1, two transcription factors implicated in driving neuroendocrine cell fate (Borges et al., 1997; Borromeo et al., 2016). Lim et al. observed that upon NOTCH activation, neuroendocrine cells adopt a non-neuroendocrine phenotype (Lim et al., 2017). Given that prior reports had correlated ERK activation with NOTCH induction ( Tremblay et al., 2013), we proceeded to check ASCL1 and INS1 expression levels upon MAPK pathway activation. We observed a decrease in ASCL1 and INS1 protein levels 24 h after MAPK activation in all SCLC-A cell lines, although to variable extents, with the most marked suppression of both in H510 (Figure 2B). SCLC-N, SCLC-P, and NSCLC cells had no detectable ASCL1 expression. A modest decrease in INS1 was observed in H526 (SCLC-P) after 48 h; levels of NEUROD1 and POU2F3 were unaffected (Figure 2B).

As the most notable morphological change was observed in H69 cells, we proceeded to culture these cells long term, separating the adherent and suspension cells. MAPK activation resulted in similar decreases in ASCL1 and INS1 expression in adherent and suspension H69 cells cultured separately over 4 weeks (Figure 2C). However, gene expression for the neuroendocrine factor synaptophysin (SYP) seemed to be selectively depleted in the adherent cell population. The morphological change and decrease in neuroendocrine marker expression are suggestive of an epithelial-to-mesenchymal transition (EMT) phenotype, which prompted us to analyze well-known EMT markers. Indeed, adherent cells exclusively induced expression of E-cadherin, N-cadherin, Vimentin, and NOTCH1; none of these factors were appreciably altered in suspension cells (Figure 2C).

We next investigated the mechanism of growth arrest in SCLC-A models upon MAPK activation. We explored two alternative possibilities leading to cell growth abrogation—apoptosis or cell-cycle arrest. There were no significant increases in apoptosis, as measured by annexin V staining, even after 5 days of MAPK activation in SCLC-A cells (Figure 2D). In addition, growth inhibition upon MAPK activation could not be reversed through treatment with z-VAD-FMK (20 μM, day 6), an irreversible pan-caspase inhibitor that blocks apoptosis (Figure S2A).

Cell cycle profiling revealed that MAPK activation in H510 cells resulted in a significant accumulation of cells in the G2/M phase (p < 0.0001) accompanied by a significant S phase depletion (p = 0.0014) by 2 days
Figure 2. MAPK activation in SCLC-A leads to a G2/M cell-cycle arrest and senescence

(A) Images were taken of SCLC cell lines with and without MEKDD217D/S221D expression at the indicated time points. Scale bar as indicated in every image (400 or 1,000 μM).

(B) Western blot analysis on SCLC cell lines as indicated upon MEKDD217D/S221D expression (0, 1, 2, 3 days). Total and phosphorylated ERK1/2, NEUROD1, POU2F3, ASCL1, and INSM1 are shown with β-actin used as loading control.

(C) Western blot analysis on ASCL1-driven SCLC cell line H69 cultured as adherent, suspension, and mixed upon MEKDD217D/S221D expression at day 28. Total and phosphorylated ERK1/2, INSM1, ASCL1, and SYP are shown with β-actin used as loading control (left). E- and N-Cadherin, Vimentin, NOTCH1, and NOTCH2 are shown with β-actin used as loading control (right).

(D) Cell survival following MEKDD217D/S221D expression in DMS79, H69, and H720 was monitored by annexin-V and DAPI staining and analyzed by flow cytometry at day 5. Bar chart illustrates annexin V-positive cells for the indicated cell line (n = 6, ±SEM).

(E) Cell cycle analysis following MEKDD217D/S221D expression in H510 was monitored by Click-iT Edu and DAPI and analyzed by flow cytometry at day 2. Bar chart illustrates percentage of cells in the indicated cycle phase (n = 9, ±SEM). Unpaired t test was performed so that **p < 0.01, ****p < 0.0001. (F) Senescence analysis following MEKDD217D/S221D expression in H510 was monitored by CellEvent Senescence and analyzed by flow cytometry at day 2. Bar chart illustrates fold change of mean fluorescence intensity (n = 12, ±SEM). Unpaired t test was performed so that ****p < 0.0001.

We also observed a 2-fold increase (p < 0.0001) in senescent cells, as indicated by β-galactosidase hydrolysis, upon MAPK activation in H510 (Figure 2F). As mentioned earlier, H510 without MAPK activation grows as intermixed adherent and suspension cells, and for the analysis of β-galactosidase hydrolysis via flow cytometry, the adherent and suspension cell populations were not separated but measurement was taken from the mixed population. Upon MAPK activation, the intermixed populations fully change to suspension cells and were used for flow cytometric quantification. A similar observation (mean 3-fold increase in senescent cells, p = 0.0002) was seen in H720 after 7 days (Figure S2B). The increase...
in senescent cells was accompanied by an increase in p27 protein expression in H510 and H720 cells (Figure S2C). Together these data suggest that MAPK activation does not cause apoptosis but instead induces G2 cell-cycle arrest and senescence in SCLC-A cells.

**ERK-induced growth inhibition is independent of NOTCH and its target genes, HES1 and JAG1**

To identify the transcriptomic changes induced by MAPK activation, we next performed bulk RNA sequencing of SCLC cell lines (n = 6) across three subtypes after 24 h of MEKDDS217D/S221D expression. As previously seen at the protein level, we observed that INSM1 expression in SCLC-A cells (H69, H720, H209) was significantly decreased by MAPK activation relative to changes seen in non-SCLC-A cells (H82, H446, H526; p = 0.0137, Figure S2D). No significant difference in ASCL1 expression was observed.

We observed an enrichment of gene expression in NOTCH family genes including JAG1, HES1, and REST (Figure 3A, p = 0.14, 0.05, and 0.0027, respectively) in SCLC-A cells compared with non-SCLC-A cells. This is in agreement with previous reports of NOTCH activation in SCLC, including an increase in HES1 expression associated with cell-cycle arrest (Lim et al., 2017; Sriuranpong et al., 2001; Tremblay et al., 2013). We next investigated whether deletion of NOTCH target genes would rescue growth inhibition upon MAPK activation in our model. We disrupted the HES1 and JAG1 genes via CRISPR-Cas9 using two gRNAs against HES1 and three gRNAs against JAG1. Knockout of HES1 did not rescue cell growth inhibition (Figure 3B) or protein re-expression of ASCL1 and INSM1 (Figure 3C). All gRNAs led to effective depletion of HES1 (Figure 3C). Similar results were observed with JAG1 knockdown (Figures 3D and 3E). Effective depletion of JAG1 was confirmed on protein level, and with NOTCH1 being downstream of JAG1, a decrease in cleaved NOTCH1 expression was also seen upon JAG1 knockdown (Figure 3E).

Genetic disruption of NOTCH signaling did not rescue ERK-mediated growth inhibition or loss of lineage oncogenes, suggesting NOTCH signaling does not regulate ASCL1 or growth downstream of ERK. This is in agreement with prior data showing that NOTCH activity as a tumor suppressor or protooncogene is highly context dependent (Lim et al., 2017).

**MAPK activation increases ERK negative feedback and activates STAT3**

The observation that SCLC-A cells are sensitive to MAPK signaling activation prompted us to examine the key molecular changes in these models. Previous work established that hyperactivation of BRAFV600E, RAS, and MYC can result in oncogene-induced senescence (Michaloglou et al., 2005; Serrano et al., 1997) in some solid tumors and pre-B acute lymphoblastic leukemia, associated with upregulation of negative feedback on MAPK signaling through factors such as SPRY2, DUSP6, and ETV5 (Courtois-Cox et al., 2006; Shojaee et al., 2015). Thus, we next investigated whether upregulation of ERK negative regulators could be observed in the SCLC models (Figure 4A). MAPK activation significantly upregulated the expression of DUSP6 and SPRY2, but not ETV5 proteins, in a time-dependent manner (24–72 h) in SCLC-A cell lines (Figure 4B); the response was blunted or absent in SCLC-N (H82), SCLC-P (H526), or NSCLC (A549) cells. Bulk RNA sequencing 24 h after MAPK activation was assessed for differential gene expression of DUSP and SPRY family members (Figure 4C); of these, DUSP6 was consistently differentially induced in the SCLC-A versus non-SCLC-A lines (1.7-fold increase; p = 0.0049). Of interest, MAPK activation appeared to selectively increase DUSP6 and SPRY2 protein expression in adherent H69 cells but not in H69 cells in suspension (Figure 4D). This suggests that the transition from neuroendocrine to non-neuroendocrine phenotype in this setting might be associated with ERK negative feedback.

To more broadly interrogate the signaling network induced by MAPK activation, we performed a phospho-kinase array in SCLC-A (H720 and H69) and SCLC-N (H82) cells. Surprisingly, signaling changes in protein kinase phosphorylation upon MAPK activation were limited. The most striking change, apart from the expected increase in ERK phosphorylation, was a significant increase in STAT3 phosphorylation on serine 727 (Figures 4E and S3A). This was only seen in SCLC-A cell lines (p = 0.0001) (Figure 4F). The increase in STAT3-pS727 was verified in additional SCLC-A cell lines (H510, DMS79) and was not observed in SCLC-N (H82), SCLC-P (H526), and NSCLC (A549) cells (Figure 4G). The observed increases in ERK negative feedback regulators DUSP6 and SPRY2, as well as the increase in STAT3-pS727, were also maintained at the protein level in tumors from in vivo models injected with H720 MEKDD cells (described in Figures S1E and S1F) with variability seen between mice (Figure S3B).
DISCUSSION

The striking difference in the prevalence of MAPK alterations between NSCLC and SCLC is intriguing, particularly in light of the highly aggressive nature of SCLC. Limited research has gone into studying this network of signaling pathways in SCLC. Here, we focused on disentangling the MAPK pathway and its highly interconnected network of signaling pathways in SCLC.

We identified a subset of SCLC, comprised exclusively of SCLC-A models, that was selectively sensitive to MAPK activation in vitro and in vivo and showed strong upregulation of ERK negative feedback regulators DUSP6 and SPRY2 and the STAT signaling pathway through STAT3 (S727) upon MAPK activation. It was previously shown that Raf-activated human medullary thyroid cancer cells produce the autocrine-paracrine leukemia inhibitory factor, which subsequently activates the JAK-STAT3 pathway via phosphorylation on STAT3 (tyrosine 705 and serine 727) (Park et al., 2003). This autocrine-paracrine pathway was also active.
in two small cell lung cancer cell lines, yet at the time no subtype classification existed (Park et al., 2003).

Here, we describe subtype specificity in regards to MAPK pathway tolerance and suggest that patients with SCLC-A-predominant tumors could benefit from tumor-specific MAPK activation.

Figure 4. MAPK activation increases ERK negative feedback and activates STAT3
(A) A simplified schematic showing the MAPK pathway and its negative feedback regulators DUSP6, SPRY2, and ETV5. Created with BioRender.com.
(B) Western blot analysis on SCLC cell lines as indicated upon MEKDD S217D/S221D expression (0, 1, 2, 3 days). MEKDD, total and phosphorylated ERK1/2, DUSP6, SPRY2, ETV5 are shown with β-actin used as loading control. MEKDD, total and phosphorylated ERK1/2 were taken from Figure 1D for presentation purposes.
(C) Heatmap of SCLC cell lines showing log2 fold change for relative change in gene expression after MEKDD S217D/S221D expression (24 h) for the indicated genes. Unpaired t test was performed between ASCL1- and non-ASCL1-SCLC cell lines with p value as indicated.
(D) Western blot analysis on ASCL1-driven SCLC cell line H69 cultured as adherent, suspension, and mixed upon MEKDD S217D/S221D expression. Total and phosphorylated ERK1/2, DUSP6, and SPRY2 are shown with β-actin used as loading control. Total and phosphorylated ERK1/2 were taken from Figure 2C for presentation purposes.
(E) Phosphokinase array in H720 and H82 upon MEKDD S217D/S221D expression after 24 h. Phosphorylated ERK1/2 (T202/Y204) and STAT3 (S727) are indicated.
(F) Mean pixel density derived from (D) for phosphorylated ERK1/2 (T202/Y204) and STAT3 (S727) after 24 h of MEKDD S217D/S221D expression in the indicated cell lines (± SEM). Unpaired t test was performed so that ***p < 0.001, ****p < 0.0001.
(G) Western blot analysis on SCLC cell lines as indicated upon MEKDD S217D/S221D expression (0, 1, 2, 3 days). Total and phosphorylated ERK1/2, total and phosphorylated STAT3, and total and phosphorylated AKT are shown with β-actin used as loading control. Total and phosphorylated ERK1/2 were taken from Figure 2B for presentation purposes.
Phenotypic changes were also distinct between SCLC subtypes. SCLC-A cell lines underwent macroscopic changes upon MAPK activation (apart from H720). This was most prominent in SCLC-A H69 that almost fully changed from suspension to adherent cells. This was accompanied by a decrease in NE markers and increase in EMT markers in the adherent cell population. This was not observed in the non-SCLC-A cell lines, suggesting a potential EMT phenotype specific to the ASCL1 subtype. Slight morphological changes were also seen in the H510 in vivo model (based on H&E), but this change was not as obvious as seen in vitro. We believe the overt microenvironmental differences between tissue culture and 3-dimensional tumor growth with stromal support can account for the distinction between in vitro and in vivo tumor morphologies.

Hyperactivation of oncogenes (e.g., RAS or BRAF) can cause oncogene-induced senescence, thereby limiting tumor development (Courtois-Cox et al., 2006). It is believed that the cause of oncogene-induced senescence is not hyperactive RAS signaling itself but rather upregulation of negative feedback pathways (e.g., RasGEFs, Sprouty proteins, RasGAPs, and DUSPs) (Courtois-Cox et al., 2006). Consistent with this model, we also observed an increase in ERK negative feedback regulators DUSP6 and SPRY2 upon MAPK activation in SCLC-A. It has previously been reported that primary samples from patients with pre-B ALL had higher protein expression of DUSP6, ET5V, and SPRY2 and were sensitive to DUSP6 inhibition (Shojaae et al., 2015). Similarly, small molecule inhibition of PTEN, negative regulator of the PI3K pathway, also proved to be toxic in pre-B ALL cells (Shojaae et al., 2016). It is thought that blocking negative feedback regulators pushes signaling above the maximum threshold resulting in cell death. Current treatment strategies focus on inhibiting oncogenic signaling so activation of oncogenic signaling might seem counterintuitive and also risks that patients potentially develop new cancers. Hence, we are cautious to propose the use of drugs that activate the MAPK pathway in patients with SCLC-A tumors.

To this date, limited research has gone into studying the MAPK pathway in SCLC (Cristea et al., 2020; Cristea and Sage, 2016; Falco et al., 1990; Park et al., 2003; Ravi et al., 1998, 1999). Although our study and others (Ravi et al., 1998, 1999) suggest MAPK pathway activity to be detrimental in SCLC tumors, others have argued that activation of the MAPK pathway may promote survival of some SCLC lines (Cristea et al., 2020; Cristea and Sage, 2016). These somewhat paradoxical observations suggest that MAPK signaling in SCLC is highly context dependent. MAPK pathway activation may occur through several mechanisms such as alterations in genes of the MAPK pathway as well as its negative regulators, signaling through receptor tyrosine kinases, or G-protein-coupled receptors. The resulting signal intensity may differ across mechanisms, resulting in toxicity to tumor cells in some contexts and oncogenesis in others. The signal intensity and downstream consequences also differ between SCLC subtypes, suggesting that the optimal therapy may be dependent on the underlying SCLC subtype.

This study has unveiled certain similarities in regards to signaling between SCLC-A and hematological malignancies. In fact, Balanis et al. (2019) report that small-cell neuroendocrine cancers and hematological cancers share gene and protein expression profiles: hematological cancers had protein profiles highly similar to SCLC, whereas SCLC and LUAD cell lines were well segregated. Of interest, small-cell neuroendocrine and hematological cancers also shared drug sensitivities (Balanis et al., 2019). These observations suggest that it might be beneficial to test therapeutic approaches successful in hematological malignancies also in SCLC and vice versa.

This study provides additional understanding of SCLC biology, in particular regarding its complex signaling networks including interactions between MAPK and STAT3 pathways. It presents the first experiments suggesting that a certain subtype of SCLC only tolerates low MAPK activity. These data may provide insight into SCLC dependencies and potential subtype-specific drug susceptibilities.

Limitations of the study

We would like to acknowledge the following caveats in this study. First, SCLC cell lines mostly grow in clusters in suspension, which made it technically difficult to use the conventional senescence assay with β-galactosidase Staining Kit. Although viewed as the “gold standard” to study senescence, it is not optimized for suspension cells. We therefore took advantage of using a fluorescent, and not colorimetric, substrate for β-gal for the detection of senescent cells that can easily be quantified on a flow cytometer.

Second, directly comparing the expression of signaling pathways in cells grown in vitro to cells grown in vivo may be misleading owing to the distinct microenvironment in these settings. The in vivo models in this
study were created by injecting cells resuspended in Matrigel, an extracellular matrix mixture. For cells to engraft in this in vivo setting, different pathways may have to be upregulated, which might not be needed when cells are grown in a flask. We may therefore observe slight differences in the signaling expression levels in vitro versus in vivo such as the phosphorylation of ERK.

Third, to determine whether deletion of NOTCH target genes would reverse the effects induced by MAPK activation such as growth inhibition, we chose a proliferation assay as the major readout owing to proliferation being integrally connected to tumor progression. This was based on our in vivo experiments where MAPK-induced decrease in cell viability resulted in longer survival and smaller tumor volumes. It would also be interesting to determine any cellular macroscopic changes upon deletion of NOTCH target genes following MAPK activation, as well as identify whether NOTCH target gene deletion results in a switch in morphology when these cells are injected in vivo, although this is outside the scope of this study.

Finally, the results presented here could be further confirmed in a larger cohort of in vivo models representing different SCLC subtypes and determining potential therapeutic susceptibilities for ASCL1-driven SCLC, although again this is outside the scope of this study.

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103224.

ACKNOWLEDGMENTS
Supported by NCI R01 CA258784 (T.S.), NCI R01 CA197936 and U24 CA213274 (C.M.R.), the SU2C/VAI Epigenetics Dream Team (C.M.R.), NCI R35 CA263816 (C.M.R.), the Druckenmiller Center for Cancer Immunotherapy grant (T.S.); International Association for the Study of Lung Cancer grant (T.S.). We acknowledge the use of the Integrated Genomics Operation Core, funded by the NCI Cancer Center Support Grant (CCSG, P30 CA08748), Cycle for Survival, and the Marie-Josée and Henry R. Kravis Center for Molecular Oncology and the National Cancer Institute Cancer Center Core Grant No. P30-CA008748. The PPBC Biobank and Precision Pathology Center are supported
by the NCI Cancer Center Support Grant P30-CA008748. We gratefully acknowledge the members of the Molecular Diagnostics Service in the Department of Pathology. We thank all members of the MSKCC Antitumor Assessment Core Facility (core grant: P30 CA008748 SS). We thank all members of the Flow Cytometry Core Facility, especially Kathleen Daniels, for training and assistance. We thank all members of the Rudin laboratory for their collaborative efforts.

**AUTHOR CONTRIBUTIONS**

Conceptualization: R.C., C.H., C.M.R., T.S.; methodology: R.C., C.H., E.C., V.D., N.S.S., M.L., X.C., S.S.C., S.E.T., M.C., Ed.S., J.T.P., M.A., F.E.K., C.M.R., T.S.; investigation: R.C., C.H., T.S., C.M.R.; validation: R.C.; formal analysis: R.C., C.H., E.C., S.S.C., C.M.R., T.S.; writing: R.C., T.S., C.M.R.; supervision: T.S. and C.M.R.; funding acquisition: T.S. and C.M.R. All authors read and approved the final version of the manuscript.

**DECLARATION OF INTERESTS**

C.M.R. has consulted regarding oncology drug development with Amgen, Daiichi Sankyo, Genentech/Roche, Ipsen, Jazz, Merck, Pfizer, Syros, and Vavotek. C.M.R. serves on the scientific advisory boards of Bridge Medicines, Earl, and Harpoon Therapeutics.

Received: April 9, 2021
Revised: August 2, 2021
Accepted: September 30, 2021
Published: November 19, 2021

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Beta-actin          | Cell Signalling | Cat#4967; RRID: AB_2198872 |
| DUSP6               | Abcam  | Cat#ab76310; RRID: AB_1523517 |
| ERK1/2              | Cell Signalling | Cat#9102; RRID: AB_330744 |
| Phospho-ERK1/2 (T202/Y204) | Cell Signalling | Cat#4370; RRID: AB_2315112 |
| ETV5                | Proteintech | Cat#13011-1-AP; RRID: AB_2278092 |
| SPRY2               | Cell Signalling | Cat#14954; RRID: AB_2798658 |
| HA-Tag for Phospho-MEK1/2 (Ser217/221) | Cell Signalling | Cat#3724; RRID: AB_1549585 |
| NEUROD1             | Santa Cruz | Cat#sc-46684; RRID: AB_671759 |
| POU2F3              | Santa Cruz | Cat#sc-293402; RRID: AB_2890011 |
| Anti-MASH1 (ASCL1)  | BD Pharmingen | Cat#556604; RRID: AB_396479 |
| INSM1               | Santa Cruz | Cat#sc-271408; RRID: AB_10607955 |
| Synaptophysin (SYP) | Neuromics | Cat#MO20000; RRID: AB_2198872 |
| E-cadherin          | Cell Signalling | Cat#3195; RRID: AB_2291471 |
| N-Cadherin          | Cell Signalling | Cat#13116; RRID: AB_2687616 |
| Vimentin            | Cell Signalling | CAT#5741; RRID: AB_10695459 |
| NOTCH2              | Cell Signalling | CAT#5732; RRID: AB_10693319 |
| NOTCH1              | Cell Signalling | CAT#4380; RRID: AB_10691684 |
| Vinculin            | Cell Signalling | CAT#13901; RRID: AB_2714181 |
| Phospho-Stat3 (Ser727) | Cell Signalling | CAT#9134; RRID: AB_331589 |
| Stat3               | Cell Signalling | CAT#4904; RRID: AB_331269 |
| Cleaved NOTCH1      | Cell Signalling | CAT#4147; RRID: AB_2153348 |
| P27                 | Cell Signalling | CAT#3686; RRID: AB_2077850 |
| HES1                | Cell Signalling | CAT#11988; RRID: AB_2728766 |
| Jagged1             | Cell Signalling | CAT#2620; RRID: AB_10693295 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Z-VAD-FMK           | Promega | CAT#G7231 |
| Doxycycline Hydrochloride | Thermo Fisher Scientific | CAT#BP26531 |
| **Critical commercial assays** | | |
| Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit | Thermo Fisher | CAT#C10425 |
| CellEvent™ Senescence Green Flow Cytometry Assay Kit | Thermo Fisher | CAT#C10840 |
| Proteome Profiler Human Phospho-Kinase Array Kit | R&D | CAT#ARY003C |
| **Deposited data**  | | |
| Raw RNAseq data     | This paper | ArrayExpress: E-MTAB-11016 |
| **Experimental models: Cell lines** | | |
| H720                | ATCC  | CAT#CRL-5838; RRID: CVCL_1583 |
| H69                 | ATCC  | CAT#HTB-119; RRID: CVCL_1579 |
| DMS79               | ATCC  | CAT#CRL-2049; RRID: CVCL_1178 |
| H510                | ATCC  | CAT#HTB-184; RRID: CVCL_1565 |
| H82                 | ATCC  | CAT#HTB-175; RRID: CVCL_1591 |
| H526                | ATCC  | CAT#CRL-5811; RRID: CVCL_1569 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Triparna Sen (sent@mskcc.org).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- RNA seq data have been deposited at ArrayExpress: E-MTAB-11016 and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and cell lines
The following cell lines were used in this study: H720 (ATCC CAT#CRL-5838; RRID: CVCL_1583), H69 (ATCC CAT#HTB-119; RRID: CVCL_1579), DMS79 (ATCC CAT#CRL-2049; RRID: CVCL_1178), H510 (ATCC CAT#HTB-184; RRID: CVCL_1565), H82 (ATCC CAT#HTB-17; RRID: CVCL_1591), H526 (ATCC CAT#CRL-5811; RRID: CVCL_1569), A549 (ATCC CAT#CCL-185; RRID: CVCL_0023), H209 (ATCC CAT#HTB-172; RRID: CVCL_1525), H128 (ATCC CAT#HTB-120; RRID: CVCL_1460), H446

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| A549                | ATCC   | CAT#CCL-185; RRID: CVCL_0023 |
| H209                | ATCC   | CAT#HTB-172; RRID: CVCL_1525 |
| H128                | ATCC   | CAT#HTB-120; RRID: CVCL_1460 |
| H446                | ATCC   | CAT#HTB-171; RRID: CVCL_1562 |
| H460                | ATCC   | CAT#HTB-177; RRID: CVCL_0459 |
| H292                | ATCC   | CAT#CRL-1848; RRID: CVCL_0455 |
| H661                | ATCC   | CAT#HTB-183; RRID: CVCL_1577 |
| H522                | ATCC   | CAT#CRL-5810; RRID: CVCL_1567 |

Continued
(ATCC CAT# HTB-171; RRID: CVCL_1562), H460 (ATCC CAT# HTB-177; RRID: CVCL_0459), H292 (ATCC CAT# CRL-1848; RRID: CVCL_0455), H661 (ATCC CAT# HTB-183; RRID: CVCL_1577), H522 (ATCC CAT# CRL-5810; RRID: CVCL_1567). All cell lines apart from H510 were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Invitrogen, Carlsbad, CA) containing 10% Tet-free FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin and kept at 37°C in a humidified incubator (5% CO2 and 95% atmosphere). H510 was cultured in Kaighn's Modification of Ham's F-12 Medium (F12-K, ATCC 20-2004) containing 10% Tet-free FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin and kept at 37°C in a humidified incubator (5% CO2 and 95% atmosphere). All cell lines used in this study are negative for mycoplasma contamination and were confirmed by STR profiling.

Lenti-X 293 T Cell Line (Clontech Laboratories, 632180) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) that contained 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin and kept as above.

Mouse models
All animal experiments were approved by the Memorial Sloan Kettering Cancer Center (MSKCC) Animal Care and Use Committee. Female nude mice (6-8 weeks old) were obtained from Charles Rivers Laboratories and housed in accredited facilities under pathogen-free conditions. Additional information on experimental methods in next section.

METHOD DETAILS

Plasmids
The CDS for human gene sequences MEKDDS217D/S221D was cloned into BsrGI-cut pInducer20 backbone for the Tet-inducible expression of MEKDDS217D/S221D. pInducer20 was a gift from Stephen Elledge (Addgene plasmid # 44012; http://n2t.net/addgene:44012; RRID:Addgene_44012 (Meerbrey et al., 2011). Capillary sequencing was used to verify plasmids.

Western Blot
Cell lysates were prepared from frozen cell pellets or flash frozen tumour samples as follows: CellLytic buffer (Sigma) supplemented with 1% protease inhibitor cocktail (Roche), 1% phosphatase inhibitor cocktail (Calbiochem Millipore) was used to lyse cells. The same amount of protein per sample was loaded on NuPAGE (Invitrogen) 4-12% Bis-Tris gradient gels and further transferred on PVDF membranes (Bio-Rad). To detect human proteins, the HRP immunodetection system (Millipore) and the iBright Western Blot Imaging System (ThermoFisher) were used.

Cell viability assay
Cells (H720, H82, H69, H526: 1500 cells/well; DMS79,H510: 2000 cells/well; A549: 1000 cells/well) were plated in a volume of 180ul in Falcon Clear 96-well plate (BD Biosciences). After >3h incubation at 37°C, doxycycline (0.5 μg/ml) was diluted in medium and added to the indicated wells and re-added every 2 days. After incubation for the indicated timepoint, CellTiter-Glo 2.0 Cell Viability Assay (Promega, G9241) was added to each well and luminescence measured according to manufacturer’s instructions. Baseline values of untreated cells were used as a reference to calculate fold changes (set to 1).

Chemicals
Doxycycline Hydrochloride (Cat# BP26531) was purchased from FisherScientific (Hampton, NH, USA). Z-VAD-FMK (Cat# G7231) was purchased from Promega (Madison, WI).

Mouse studies
Cultured human SCLC cell lines were injected subcutaneously into the right flank of athymic nude female mice (6-8 weeks old) (ENVIGO). Up to 2 x 10⁶ cells were washed and resuspended in Matrigel and PBS in a 1:1 ratio. Three days prior to injection until the end of experiment, mice in the doxycycline cohort were fed doxycycline chow. Tumor dimensions were analyzed using a caliper once to twice weekly and volume calculated using the equation V = π/6*L*W² (L length; W width). Tumors were harvested once tumor volume reached 1500 mm³ and weights subsequently measured using a scale. Tumors were processed immediately after harvest and flash-frozen and submerged in formalin for further analysis. All animal experiments
were approved by the Memorial Sloan Kettering Cancer Center (MSKCC) Animal Care and Use Committee. Mice were housed in accredited facilities under pathogen-free conditions.

**Annexin V/DAPI apoptosis assay**

APC-Annexin V/Dead Cell Apoptosis Assay was used for the detection of apoptotic cells according to the manufacturer’s instructions. Externalization of phosphatidylserine (Annexin V, APC Conjugate, Cat # 640919) and DNA content (DAPI, 4',6-diamidino-2-phenylindole, Cat# D9542) were measured on a Fortessa Flow Cytometer. Gating on all cells using FlowJo software was used for analysis.

**Click-iT Edu cell cycle analysis**

Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermo Fisher, C10425) was used for the analysis of DNA replication in proliferating cells according to manufacturer’s instructions. Click-iT EdU labeling (Alexa Fluor 488 Conjugate) and DNA content (DAPI, 4',6-diamidino-2-phenylindole) were measured on a Fortessa Flow Cytometer and further analyzed using FlowJo software.

**Cell senescence analysis**

CellEvent Senescence Green Flow Cytometry Assay Kit (Thermo Fisher, C10840) was used for the analysis of cellular senescence via β-galactosidase hydrolysis according to manufacturer’s instructions. Activation of β-galactosidase (emitting a fluorogenic signal detected on 488 laser) was measured on a Fortessa Flow Cytometer.

**Lentiviral production and transduction**

Lentiviral packaging plasmids psPAX2 (Addgene plasmid #12260, gift from Didier Trono http://n2t.net/addgene:12260; RRID:Addgene_12260) and pMD.2G (Addgene plasmid # 12259, gift from Didier Trono http://n2t.net/addgene:12259; RRID:Addgene_12259) were used as follows: 8.33 mg psPAX2 (gag-pol) and 4.12 mg pMD.2G (envelope) and 12.5 μg of lentiviral construct were used to transfect a 15cm dish of Lenti-X 293T cell line, after mixing with 1ml of Jet Buffer and 50 μl of JetPRIME reagent. Media was changed 16 hours post transfection and supernatant filtered through a 0.45 μM filter 72h post transfection. Supernatant was concentrated using Lenti-X Concentrator (Fisher Scientific, NC0448638) according to manufacturer’s instructions. Virus was resuspended in 500 μl DMEM media (20 x concentrated) and stored at −80°C. For lentiviral transduction, 0.5 × 10^6 cells were resuspended in 1ml media and 2 μl Polybrene (8 mg/ml) and mixed with 300 μl concentrated virus. Following incubation at 37°C for 10min, cells were infected by centrifugation (800 g, 30min) in a 12-well plate. Cells were selected for pInducer20/MEKD using Neomycin (G418) (200–800 μg/ml) every 2–3 days until control cells are dead.

**Generation of doxycycline-inducible MEKD^S217D/S221D (MEK1) cell lines**

Cell lines were transduced with lentiviral pInducer20/MEKD construct and selected with Neomycin (G418) (200–800 μg/ml) every 2–3 days for 10–14 days. MEKDD^S217D/S221D was induced by the addition of Doxycycline Hydrochloride (Cat# BP26531) (0.5 μg/ml) every 2 days and confirmed on protein level by blotting for phospho-ERK1/2 (T202/Y204).

**Phosphokinase array**

H720, H69 and H82 transduced with doxycycline-inducible MEKD were cultured and harvested after 24 h upon MAPK activation. Relative levels of human protein kinase phosphorylation (43 human kinases) were detected using the Proteome Profiler Human Phospho-Kinase Array Kit (R&D, ARY003C) according to manufacturer’s instructions. 400 μg protein were used per sample and image developed using the iBright Western Blot Imaging System (ThermoFisher). Pixel density was quantified using ImageStudioLite software.

**Cell images**

Pictures of suspension and adherent cells were taken with EVOS® FL Cell Imaging System (Thermo Fisher Scientific).

**RNA extraction, sequencing, and analysis**

RNA extraction, sequencing and analysis were performed as in Wohlhieter et al. (2020). Briefly, QIAGEN RNeasy Plus Universal mini kit (QIAGEN, Hilden, Germany) was used to extract total RNA from cell pellets.
according to manufacturer’s instructions and RNA sequencing libraries prepared using NEBNext Ultra RNA Library Prep Kit for Illumina following manufacturer’s instructions (NEB, Ipswich, MA, USA). RNA sequencing libraries were sequenced on the Illumina HiSeq instrument (4000 or equivalent; a 2x150bp Paired End (PE)) according to manufacturer’s instructions.

**Histology**

Formalin-fixed paraffin embedded tissues were used to obtain 4μm sections for H&E staining. All slides were scanned in Aperio AT2 digital whole slide scanner and reviewed with Aperio ImageScope v12.4.3.7005 by an experienced pathologist.

**CRISPR/Cas9**

Small guide RNAs (gRNAs) targeting HES1, JAG1 and non-targeting control (NTC) were designed using the CRISPR design tool GuideScan (Perez et al., 2017) (www.guidescan.com) and chosen based on high targeting scores and lowest probability of off-target effects. gRNAs were validated by protein expression. gRNAs were cloned into lentiCRISPRv2 using Zhang lab protocol (genome-engineering.org, rev20140509). lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961; http://n2t.net/addgene:52961; RRID:Addgene_52961) (Sanjana et al., 2014).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

GraphPad (GraphPad, Software, San Diego, CA, USA) was used to analyze data. Data represent the mean ± SEM as indicated in figure legends. In all experiments where two conditions are being compared (Figures 1B, 1E, 1H, 2E, 2F, 3A, 2B, 2D, 4C, 4F, S1E, S2B, and S2D) significance was established using unpaired t test where ns > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 or as otherwise indicated in the Figure. For Figures 1A and S1A, p value was derived from Pearson’s Chi-squared test and is shown in the Figure. For Figures 1G, 1I, and S1F, p value was calculated by log-rank test and is shown in the Figure.