Unbiased Proteomic Profiling Uncovers a Targetable GNAS/PKA/PP2A Axis in Small Cell Lung Cancer Stem Cells

Highlights
- PKA inhibition or PP2A activation inhibits SCLC growth
- The GNAS/PKA axis promotes SCLC growth
- Proteomics studies uncover widespread PKA targets and downstream signaling networks
- PKA activation promotes a cancer stem cell state

In Brief
Using a combination of pre-clinical models and phosphoproteomics, Coles et al. identify protein kinase A (PKA) and molecules in the PKA signaling pathway as key regulators of small cell lung cancer initiation and progression.
Unbiased Proteomic Profiling Uncovers a Targetable GNAS/PKA/PP2A Axis in Small Cell Lung Cancer Stem Cells

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SUMMARY

Using unbiased kinase profiling, we identified protein kinase A (PKA) as an active kinase in small cell lung cancer (SCLC). Inhibition of PKA activity genetically, or pharmacologically by activation of the PP2A phosphatase, suppresses SCLC expansion in culture and in vivo. Conversely, GNAS (G-protein α subunit), a PKA activator that is genetically activated in a small subset of human SCLC, promotes SCLC development. Phosphoproteomic analyses identified many PKA substrates and mechanisms of action. In particular, PKA activity is required for the propagation of SCLC stem cells in transplantation studies. Broad proteomic analysis of recalcitrant cancers has the potential to uncover targetable signaling networks, such as the GNAS/PKA/PP2A axis in SCLC.

Significance

Rare human genetic syndromes in which mutations activate protein kinase A (PKA) signaling are associated with the development of a spectrum of neuroendocrine tumors. Here, we show that the PKA signaling pathway is also active and critical for the growth of small cell lung cancer (SCLC), a frequent and fatal form of neuroendocrine cancer. Furthermore, through a comprehensive proteomic analysis of SCLC tumors and cell lines, we identified proteins in the PKA holoenzyme network as well as a large number of potential targets of PKA. These data provide one of the largest and most diverse proteomic datasets exploring PKA signaling, and this resource should yield insight into the mechanisms driving SCLC progression as well as other PKA-dependent and G protein-coupled receptor-activated neuroendocrine cancers.
INTRODUCTION

Among all lung cancers, ~15% of cases show properties of neuroendocrine cells and are classified as small cell lung cancer (SCLC). SCLC development is usually associated with heavy smoking, and SCLC incidence is expected to increase in the next decades due to the increasing numbers of smokers. Worldwide, more than 200,000 people die from SCLC every year. SCLC tumors have a unique biology with a high proliferative and metastatic potential, resulting in very poor prognosis. The overall 5-year survival rate of SCLC patients is only 5%–10%. Treatment options for SCLC patients have remained similar for the past 30 years with little improvement in these overall survival rates (reviewed in Pietanza et al., 2015; Sabari et al., 2017). Even immunotherapies successful in other cancer types have only limited efficacy in the vast majority of SCLC patients (Horn et al., 2018; Ready et al., 2019).

As with other cancers, a better understanding of the signaling pathways that drive the growth of SCLC tumors may eventually lead to the identification of improved therapeutic strategies. Unbiased genomic and transcriptomic studies often help identify such key drivers and key targets in cancer. In the case of SCLC, the sequencing of human tumors has begun to reveal such key drivers and key targets in cancer. In the case of SCLC, the sequencing of human tumors has begun to reveal some opportunities (George et al., 2015; Peifer et al., 2012; Rudin et al., 2012). First, SCLC tumors have nearly ubiquitous inactivation of genes coding for the RB and p53 tumor suppressors. This dual inactivation results in a dysregulated G1/S transition of the cell cycle, which has led to the idea that these tumors may display enhanced sensitivity to the disruption of the G2/M transition. Indeed, inhibitors of G2/M kinases such as CHK1 and WEE1 may help inhibit the growth of SCLC (Doerr et al., 2017; Laloo et al., 2018; Sen et al., 2017a, 2017b). Second, RB loss and c-MYC upregulation have been associated with increased sensitivity to specific therapies such as inhibitors of Aurora kinase (AURK) (Gay et al., 2019; Gong et al., 2019; Mollaoglu et al., 2017; Oser et al., 2019; Sos et al., 2012). Third, inactivating mutations in the acetyltransferase CREBBP (CAMP Response Binding Element factor 1 [CREB]-binding protein) may specifically increase sensitivity to inhibition of histone deacetylases (Jia et al., 2018).

Despite these encouraging examples, most recurrent genetic events in SCLC are not directly linked to obvious mechanisms of action, and these events do not readily identify therapeutic opportunities. It is possible that proteins encoded by genes not recurrently mutated could still play important roles in the proliferation and/or survival of SCLC cells. In particular, kinases and phosphatases are key signaling enzymes and are often effective therapeutic targets (Cohen, 2002; Vintonyak et al., 2009; Zhang et al., 2009). However, few recurrent genetic events seem to directly activate kinases in SCLC, with rare cases of amplification of the gene coding for the FGFR1 (fibroblast growth factor receptor 1) tyrosine kinase receptor and rare mutations in kinases such as PI3K (phosphoinositide 3-kinase) or c-KIT (George et al., 2015). Whether these rare events contribute to SCLC development or response to therapy remains poorly understood (Ferone et al., 2020). Little is also known about phosphatases in SCLC beyond inactivation of the gene coding for PTEN (phosphatase and tensin homolog) (Cui et al., 2014; McFadden et al., 2014).

Here, we surmised that even in the absence of recurrent mutations in genes coding for kinases, SCLC cells may still have active kinases that contribute significantly to the proliferation, survival, and tumorigenic characteristics of SCLC.

RESULTS

A Kinome Profiling Strategy Identifies Active Kinases in SCLC

We used multiplexed inhibitor beads (MIBs) columns to enrich for active kinases in SCLC. In brief, protein lysates were placed on columns made of Sepharose beads bound to ATP-mimic kinase inhibitors. Kinases in an active conformation are bound by the specific inhibitors at the top of the column or by pan-kinase inhibitors at the bottom while inactive kinases flow through (Duncan et al., 2012) (Figure 1A) (see STAR Methods). We compared three SCLC patient-derived xenografts (PDXs) and eight allografts (isolated from an autochthonous mouse model, see below) to non-small cell lung cancer (NSCLC) samples (two adenocarcinoma PDXs, one squamous lung cancer PDX, and four single-tumor-derived allografts from a lung adenocarcinoma mouse model driven by oncogenic K-RasG12D) to avoid enriching solely for kinases activated during the cell cycle. Normal murine lungs were used as an additional control. Unsupervised hierarchical clustering analysis of the mass spectrometry data showed that SCLC and NSCLC samples clustered according to their biological identity (Figures S1A and S1B). At the intersection of these comparisons, we identified 20 kinases that were more active in SCLC than in normal lungs or NSCLC (Figures 1B and 1C; Table S1). The 20 kinases have a wide range of mRNA expression levels in human SCLC tumors (Table S1), indicating that the MIBs approach was not biased toward highly expressed proteins. Among the top 20 candidates, we identified kinases such as WEE1 and AURKB (Aurora kinase B), which are known to play a role in SCLC (Gay et al., 2019; Gong et al., 2019; Laloo et al., 2018; Mollaoglu et al., 2017; Oser et al., 2019; Sos et al., 2012). We note that the ATP-binding sites of other kinases that may play a role in SCLC, such as CHK1, CHK2, ATM, and ATR, are not expected to be bound by the inhibitors on the columns (Manning et al., 2002). EPHA5 has been found to be upregulated in SCLC metastases (Wu et al., 2016), but its role in SCLC is not known. Overall, the MIBs approach successfully identified active kinases in SCLC.

PKA Is Expressed and Active in Human SCLC

Among the top kinases identified, we decided to focus on protein kinase A (PKA) because of observations in rare human genetic syndromes in which mutations in the PKA pathway lead to the development of neuroendocrine tumors, including pituitary tumors in people with Carney complex and adrenal tumors in people with Cushing’s syndrome (see Discussion). Based on our data and these observations, we surmised that PKA might have a broader role than previously suspected in neuroendocrine cancers, especially SCLC.

PKA in its inactive form is a tetramer of two catalytic and two regulatory subunits; dissociation of the regulatory subunits upon binding to cyclic AMP (cAMP) results in the activation of the kinase. PRKACA, which codes for the catalytic subunit a of PKA (PKA-a2) identified in the MIBs assays and is the dominant
catalytic subunit expressed in SCLC (Figures 1D and S1C; Table S1). When we analyzed SCLC human tissue microarrays by immunostaining with an antibody recognizing a phosphorylated PKA (pPKA) substrate consensus peptide, we found that 37.5% of the human tumors examined showed positive staining in this assay (Figure 1E). Tissue microarrays probed with an antibody recognizing phosphorylated CREB (pCREB), a well-known target of PKA, suggested activation of the PKA/CREB pathway in 17.5% of tumors (Figure S1D).

Finally, \( Rb1^{flox/flox};Trp53^{flox/flox};Rbl2^{flox/flox} \) conditional mutant mice develop \( Rb/Trp53/Rbl2 \) triple-knockout (TKO) SCLC tumors in their lungs upon infection with an adenovirus expressing the Cre recombinase (Ad-Cre) (Gazdar et al., 2015; Schaffer et al., 2010). Immunoblot analysis of TKO SCLC tumors also revealed elevated PKA relative to normal mouse lung (Figure 1F). Thus, PKA is expressed and active in SCLC.

**PKA Activity Is Required for the Development and the Maintenance of SCLC**

To investigate the role of PKA activity in SCLC in vivo, we crossed TKO mice to a conditional allele of \( Prkaca \), whereby expression of Cre results in a switch from wild-type to a mutant form in which a methionine has been replaced by an alanine in the ATP-binding site (analog-sensitive [AS], see below; PKA-C\(^{\alpha M120A} \)). PKA-C\(^{\alpha M120A} \) does not utilize endogenous ATP as efficiently as the wild-type enzyme (PKA-C\(^{\alpha WT} \)) and is thus less catalytically active (Morgan et al., 2008). Switching from PKA-C\(^{\alpha WT} \) to PKA-C\(^{\alpha M120A} \) at the time of cancer initiation in TKO;\( Prkaca^{M120A/M120A} \) mice (Figures S2A–S2C) led to a significant inhibition of SCLC development compared with control TKO mice (Figures 2A–C). PKA-C\(^{\alpha WT} \) and PKA-C\(^{\alpha M120A} \) TKO tumors had a similar histology and expressed neuroendocrine markers such as synaptophysin (SYP) (Figure 2A). We were unable to accurately compare cell proliferation and death...
in the two groups, as the PKA-Cα<sup>WT</sup> tumors were much more advanced than PKA-Cα<sup>M120A</sup> tumors. Expression of PKA-Cα was similar in both models but PKA activity was decreased by ~50% in TKO PKA-Cα<sup>M120A</sup>, as would be expected for this hypomorphic mutation (Figures 2D–2F). Thus, a reduction of PKA-Cα activity by half is sufficient to potently inhibit SCLC development in this mouse model.

SCLC cell lines derived from PKA-Cα<sup>M120A</sup> TKO tumors showed decreased survival in culture compared with TKO cells (Figure S2D). Knockdown of PKA-Cα in KP1 (Rb1/Trip53 mutant) and KP11 (Rb1/Trip53/Rbl2 mutant) cell lines also led to decreased growth and increased apoptosis (Figures S2E–S2H). Similar phenotypes were observed with allografts from KP1 cells (Figures S2I and S2J). In the Cancer Dependency

Figure 2. PKA Is Required for the Growth of SCLC

(A–C) (A) Representative images of sections counterstained with H&E from the lungs of TKO (control) and TKO;Prkaca<sup>M120A/M120A</sup> (Prkaca<sup>M120A</sup>) mice 5 months after Ad-Cre. Scale bar, 5 mm. Insets show representative Synaptophysin (SYP) immunostaining (brown signal). Scale bar, 100 μm. (B and C) Quantification of tumor numbers (B) and tumor area (C) (n = 8–9 mice/group). **p = 0.0016 and *p = 0.0142.

(D) Immunoblot (IB) for PKA-Cα levels in TKO (control) and TKO;Prkaca<sup>M120A</sup> SCLC cell cultures (from independent tumors). HSP90 is a loading control.

(E) Immunoblot for phospho-PKA substrates (pPKA) following in vitro kinase reactions using immunoprecipitated (IP) PKA-Cα from TKO (control) or TKO;Prkaca<sup>M120A</sup> SCLC cell cultures. A native tumor extract was used as the kinase substrate. HSP90 is a loading control. A substrate-only lane with IgG control kinase reaction is shown.

(F) Quantification of PKA kinase activity in a control TKO and two TKO;Prkaca<sup>M120A</sup> SCLC cell cultures (n = 3). M120A-1, **p = 0.0054; M120A-2, **p < 0.0001.

(G) DepMap analysis of the requirement for PRKACA in human SCLC cells.

(H) Immunoblot for PKA-Cα levels in control (shControl) and PRKACA knockdown (shPRKACA) human NCI-H526 SCLC cells. HSP90 is a loading control.

(I) Growth of shControl and shPRKACA NCI-H526 xenografts in NSG mice (n = 12 tumors/group). Day 10, *p = 0.036; day 21, *p = 0.041; day 24, *p = 0.040; day 26, *p = 0.032; day 28, *p = 0.024.

(J) Quantification of expression of PKA-Cα and cleaved PARP (Cl-PARP) by immunoblot (n = 2 tumors closest to the mean) (see Figure S2K). PKA-Cα, **p = 0.002; Cl-PARP, **p = 0.002.

Data are presented as mean ± SEM. See also Figure S2.
Map project (DepMap, www.depmap.org), knockdown of PKA-Cα also led to decreased growth in 25 human SCLC cell lines (Figure 2G). Notably, NCI-H526 cells were identified in this analysis as the most dependent on PKA-Cα. Indeed, knockdown of PKA-Cα in these cells led to an increase in apoptosis and a significant inhibition of tumor growth in mice (Figures 2H–2J and S2K).

**Protein Phosphatase 2A Counteracts the Promalignant Effects of PKA in SCLC**

Previous studies have indicated possible functional interactions between PKA, a serine/threonine protein kinase, and the serine/threonine protein phosphatase PP2A (Ahn et al., 2007; Dodge-Kafka et al., 2010; Musante et al., 2017; Zakany et al., 2002). We found that treatment with the PP2A inhibitor (PP2Ai) cantharidin inhibited PP2A activity (Figure S3A) and enhanced the phosphorylation of PKA substrates in SCLC cells, including VASP (vasodilator-stimulated phosphoprotein) and CREB (Figures 3A and 3B). This was prevented by pretreatment with a PKA inhibitor (PKAi) (Figure 3B), supporting a model in which PP2A activity and phosphorylation of PKA substrates in SCLC cells contribute to cell survival and cell division (see below). These experiments demonstrate that amplification and activating mutations of PP2A in SCLC cells are dependent on PP2R1A expression for their growth in the DepMap dataset (Figures S3B and S3C). Treatment of SCLC cells with two independent small-molecule activators of PP2A (SMAPs), which act via binding to PP2R1A (Grossman et al., 2017; Kastrinsky et al., 2015; Sangodkar et al., 2017), suppressed PKA substrate phosphorylation and induced apoptosis in SCLC cells (Figures 3C, S3D, and S3E). PP2A has multiple targets in cells, and we found that SMAP treatment also had potent effects on c-MYC expression and mTOR (mammalian target of rapamycin) activity in cells (Figure S3F), indicating that PKA signaling inhibition may represent only a fraction of the effects of PP2A activation in SCLC cells. Nevertheless, treatment with the SMAP-1154 compound as a single agent led to suppression of tumor growth in NCI-H69 SCLC xenografts (Figure S3G), with decreased phosphorylation of PKA substrates (Figure S3H). Treatment with a more bioavailable compound (SMAP-DT-061) (Sangodkar et al., 2017) had a potent inhibitory effect with induction of apoptosis in a different SCLC xenograft model (NCI-H69 cells) in combination with chemotherapy treatment (cisplatin) (Figures 3D–3F, S3I, and S3J). Thus, inhibition of PKA itself or manipulation of other enzymes in the PKA signaling network may have anticancer effects in SCLC.

**GNAS Is Genetically Activated in Human SCLC and Promotes PKA Activity and SCLC Growth in Mouse Models**

PKA is activated in cells in response to increased concentrations of cAMP following conversion of ATP by adenylate cyclase (AC) enzymes. AC and PKA are key mediators of signaling downstream of G-protein-coupled receptors (GPCRs). We found Gxs (encoded by GNAS) to be one of the most highly expressed G-protein α subunit (GNAS) in human SCLC (Figure S4A). Gxs mediates GPCR signaling by activating AC, leading to downstream activation of PKA (Iglesias-Bartolome et al., 2015). In a clinical sequencing cohort of over 300 patients at the Memorial Sloan Kettering Cancer Center, five tumors had amplification of GNAS and one tumor had a known oncogenic R201C mutation in Gxs (Landis et al., 1989) (Figure S4B). These observations suggested that the PKA pathway may be activated in human SCLC via genetic events leading to increased Gxs activity.

To test the role of Gxs in SCLC, we crossed Gnas<sup>lox/lox</sup> mice to TKO mice (Figure S4C). Gnas deletion in this context (Figure S4D) led to decreased tumor development compared with TKO mice (Figures 4A–4D), indicating that Gxs is required for optimal cancer initiation in this model. To determine whether Gxs activation could promote SCLC development, we crossed TKO mice to transgenic mice expressing a constitutive active form of Gxs (Gnas<sup>R201C</sup> allele) following Cre expression and doxycycline (Dox) treatment (Iglesias-Bartolome et al., 2015) (Figure S4E). TKO;Gnas<sup>R201C</sup> mice developed both SCLC tumors and non-SCLC tumors 3.5 months after Ad-Cre instillation (Figure S4F), indicating that Gxs<sup>R201C</sup> strongly promotes lung cancer development in this genetic context. Nevertheless, TKO;Gnas<sup>R201C</sup> mice still developed more and larger SCLC tumors compared with TKO controls, as determined by immunostaining for the neuroendocrine marker synaptophysin (SYP) (Figures S4G–S4I). Induction of Gxs<sup>R201C</sup> for 6 weeks (with Dox), 14 weeks after Ad-Cre injection, led to a significant increase in SCLC development, with no growth of large NSCLC tumors (Figures 4E–4H), demonstrating an oncogenic role for Gxs<sup>R201C</sup> in SCLC progression. In primary cultures derived from TKO;Gnas<sup>R201C</sup> tumors, expression of Gxs<sup>R201C</sup> led to an increase in PKA kinase activity and in the phosphorylation of PKA substrates (Figures 4I and 4J). When we analyzed tumor sections from TKO and TKO;Gnas<sup>R201C</sup> SCLC tumors, as well as TKO;Gnas<sup>lox</sup> SCLC tumors, we found no differences in cell death or proliferation associated with changes in Gxs activity (Figures S4J–S4M). While these counts may be confounded by differences in tumor sizes, these observations suggested that the mechanisms by which activation of PKA potently promotes the development of SCLC in vivo may not be as simple as regulation of cell survival and cell division (see below). These experiments demonstrate that amplification and activating mutations in GNAS that occur in SCLC patients can significantly enhance SCLC progression in a mouse model, defining Gxs as an oncogene in SCLC.

**Identification of a High-Confidence Proximal PKA Signaling Network in SCLC Cells**

We next performed a systems-level proteomic and transcriptomic analysis of PKA in SCLC cells and tumors to identify the proximal PKA pathway and direct kinase substrates, map how the phosphoproteome changes in response to PKA activation, and determine how PKA activation alters the biology of SCLC. First, we sought to identify the PKA interactome in SCLC cells. We expressed LAP-tagged (localization and affinity purification tag) forms of PKA-Cz, PKA-Cj, and PKA-R1A in NCI-H446 SCLC cells (Torres et al., 2009) and performed mass spectrometry on the eluted fractions after purification (Figures S5A and S5A). This analysis identified 128 candidate interactors (Table S2). We found complex interactions between catalytic and regulatory PKA subunits, suggesting that loss or inhibition of a single catalytic enzyme may not fully reveal the function of PKA in SCLC cells (Figure S5B). We compiled a high-confidence protein-protein interaction network containing proteins that were identified with at least two baits (Figure 5B). This proximal network...
contained core components of the PKA pathway such as A-kinase anchoring proteins (AKAPs), PKA catalytic and regulatory subunits, and the cAMP-specific phosphodiesterase 7A (PDE7A), as well as GPR161, a PKA- and cilia-associated protein GPCR (Bachmann et al., 2016; Logue and Scott, 2010; Mukhopadhyay et al., 2013) (Figure 5C). Notably, we found that PKA interacts with SOX2, which is an oncogenic driver in SCLC (Rudin et al., 2012). Furthermore, PKA interacts with PDE4DIP (phosphodiesterase 4D interacting protein), which is recurrently mutated in SCLC (George et al., 2015); inactivating mutations in PDE4DIP may affect PKA signaling directly as an AKAP (Uys et al., 2011) or indirectly by changing the function of PDE4, which is a regulator of cAMP levels (Houslay and Adams, 2003).

Together, this analysis further places PKA downstream of GPCR/Gαs signaling networks in SCLC cells and links PKA signaling to additional genetic alterations in primary human SCLC tumors.

Global Proteomic Analysis of Direct PKA Substrates and Downstream Phosphoproteome

When we knocked down the PKA target CREB in murine SCLC cells, we did not observe any inhibition of SCLC cell growth in culture or in mice (Figures S6A and S6D). These results suggested that PKA promotes SCLC growth largely through other targets. To identify potential direct PKA substrates in SCLC cells, we used the PKA-CαM120A AS form of PKA. This gatekeeper...
mutation results in a kinase that cannot efficiently use ATP but instead allows for the incorporation of bulky ATP analogs such as Bn-ATP\(^S\), which are inefficiently used by wild-type kinases and can serve to identify direct kinase substrates following the covalent capture of thiophosphorylated peptides (Hertz et al., 2010) (Figure 6A). We selected two PDX models (NJH29 and LX102), one allograft model (derived from a TKO mouse, 5BI), and one human cell line (NCI-H82), which are representative of the diversity of molecular phenotypes in human SCLC (Rudin et al., 2019) (Figure S6E). As expected, recombinant active PKA (rPKA) was better able to phosphorylate substrates with ATP while recombinant AS-PKA (rAS-PKA) (PKA-C\(^{aM120A}\)) was better at using Bn-ATP\(^S\) (Figures 6B, 6C, and S6F–S6G). Large-scale labeling reactions were performed, and mass spectrometry identified ~200 substrates that were labeled by rAS-PKA but not by wild-type rPKA (Table S3). Because there was little overlap in the identity of the substrates between the different tumors used, and because known substrates such as CREB and VASP were not found in this analysis, this number of substrates likely underestimates the total number of PKA substrates in SCLC cells. Among the identified substrates, at least 31 have been described before (Baba et al., 2011; Imamura et al., 2014; Isobe et al., 2017; Moore et al., 2009; Nagai et al., 2016) (Table S3). The majority of substrates had the canonical PKA motif RXXS/T (Figure 6D). Gene ontology (GO) term analysis showed enrichment in multiple signaling pathways involved in cell cycle, gene expression, and metabolic processes (Figure 6E). Together, these data indicate that PKA phosphorylates hundreds of substrates in SCLC and identify a broad role for PKA in cell-cycle and cell-growth mechanisms that can promote SCLC expansion.

To gain further insight into the downstream consequences of PKA activation in SCLC cells, we next performed a global phosphoproteomic analysis in NCI-H526 cells (highly dependent on PKA activity, Figures 2G–2I) and NCI-H69 cells (which have relatively high PKA activity, Figure S6H). Upon PKA activation with a long-acting analog of cAMP (8-Br-cAMP) (Figure 6F), we identified thousands of phosphorylation sites, both gains and losses, which changed in response to 8-Br-cAMP (Table S4). The functional significance of the changes in phosphorylation that we observed upon PKA activation remains unknown for nearly all the proteins analyzed. However, GO term analysis of the phosphoproteome affected by cAMP in both cell lines showed enrichment for a variety of pathways implicated in cell proliferation and cancer (Table S4; Figures 6G and S6I), similar to the pathways enriched in the AS-PKA experiments. Fifty-four PKA substrates identified using the AS-PKA approach were also identified using the global phosphoproteomic approach (Table S5). Knockdown
of some of these high-confidence targets, including ribosome subunits (RPL34, RPS3A, RPS6), decreases the growth of human SCLC cell lines in the DepMap dataset (Figure 6H). This multifaceted approach identified a large number of substrates for PKA, and the results of this analysis indicate that PKA activation promotes SCLC growth by regulating multiple signaling networks.

**PKA Activity Promotes a Cancer Stem Cell State in SCLC**

To investigate the cellular consequences of PKA activation in SCLC cells, we expressed a Dox-inducible version of PKA-Cα with reduced capability for inhibition by regulatory subunits (active PKA [aPKA]) (Orellana and McKnight, 1992) or a GFP control in human NCI-H82 and NJH29 SCLC cells, which have relatively low PKA activity when compared with other SCLC cell lines (Figure S6H). Expression of aPKA led to a moderate increase in PKA signaling and a significant increase in the growth of xenografts (Figures 7A and S7A–S7D). We performed RNA sequencing (RNA-seq) of these xenografts (Table S6). PANTHER pathway analysis of the genes downregulated in both models (p < 0.05) revealed changes in various signaling pathways, including cell adhesion and cell death (Figure S7E). Analysis of the upregulated genes in aPKA-expressing tumors revealed pathways associated with neuronal and neuroendocrine features (Figure S7F). In particular, we noticed a significant upregulation of a number of markers of SCLC stem cells (Jahchan et al., 2016), including NCAM1, DLL3, MYCL, and CD24 (Table S6; Figures 7B, S7G, and S7H). This connection between PKA activation and cancer stem cell phenotypes was supported by the interaction between PKA and SOX2 (Figure 5B), which is a known regulator of stem cells (Novak et al., 2019), as well as the identification of the histone demethylase PHF2 as a direct PKA substrate (Table S3), PHF2 having been implicated in the expansion of breast cancer stem cells (Pattabiraman et al., 2016).

These observations led us to test the possibility that a key output of PKA is the promotion of cancer stem cell phenotypes in SCLC. We tested this idea in two human tumor model systems. First, as we observed an increase in tumor growth and upregulation of cancer stem cell markers such as CD24 (Jahchan et al., 2016) or DLL3 (Saunders et al., 2015) in NJH29 xenografts upon PKA activation (Figure 7A), we performed an extreme limiting dilution analysis by transplanting dilutions of NJH29-GFP and NJH29-aPKA cells into NSG mice. Activation of PKA was sufficient to enhance the frequency of SCLC stem cells in this context (Figure 7C). Conversely, knockdown of PKA-Cα in NCI-H526 cells, which are dependent on PKA activity (Figures 2G–2I), decreased the expression of cancer stem cell markers...
Growth, differentiation, and decreased the frequency of tumor-initiating cells (Figure 7F). The expression of cancer stem cell markers was also significantly reduced in the resulting tumors (Figure 7G). Thus, alterations in PKA activity in SCLC cells result in changes in signaling networks that control gene expression programs governing the cancer stem cell state.

**DISCUSSION**

SCLC is one of the most fatal forms of cancer. In contrast to many other cancer types, DNA- and RNA-seq of SCLC tumors have identified few actionable therapeutic targets that may apply to a large number of patients. Using a proteomics approach, we...
identified 20 candidate kinases that are more active in SCLC cells than in normal lungs or NSCLC cells. Among these kinases, we confirmed that PKA is active in a large fraction of SCLC cases and that PKA activity is required for the optimal growth of SCLC tumors.

A striking association exists between PKA activity and endocrine and neuroendocrine tumors. First, activating mutations in PRKACA were identified in Cushing’s syndrome, which includes abnormal growth of endocrine cells in the adrenal gland (Beuschlein et al., 2014; Cao et al., 2014; Goh et al., 2014; Kirschner, 2014; Sato et al., 2014). Second, inactivating mutations in a regulatory subunit of PKA (PRKAR1A gene) in Carney complex are associated with the development of tumors from endocrine tissues such as the pituitary gland (Horvath et al., 2010; Kirschner et al., 2000). Third, somatic activating mutations in GNAS in McCune-Albright syndrome result in multiple endocrine neoplasia (Salpea and Stratakis, 2014; Weinstein et al., 1991). Finally, increased expression of PKA-Cα from a DNAJB1-
**PRKACA** fusion leads to the development of fibrolamellar hepatocellular carcinoma, a rare form of liver cancer with neuroendocrine features (Honeyman et al., 2014; Xu et al., 2015). In mice, knockout of *Prkar1a* initiates neuroendocrine cancer in the pancreas (Salousostros et al., 2017). These data indicate that PKA may act as an oncogene in the initiation of infrequent neuroendocrine cancers. A study with a dominant-negative form of PKA suggested that PKA might also be important in SCLC cells (Xia et al., 2018). Similarly, loss of *Prkar1a* was found to promote the growth of SCLC cells in culture (Li et al., 2019). Our work extends these observations and conclusively demonstrates in multiple contexts that PKA kinase activity is critical for the development and the maintenance of SCLC, the most common and lethal form of neuroendocrine cancer.

How is PKA activated in SCLC cells? Recurrent alterations in genes such as GNAS or *PDE4DIP* may lead to PKA activation in 5%–10% of SCLC cases, and it is possible that other recurrent genetic events contribute to activation of PKA signaling, including mutations in GPCRs upstream of Gαs and PKA (Rudin et al., 2012). A genomic region encompassing Gnas is amplified in mouse SCLC tumors (McFadden et al., 2014), and we speculate that Gnas in this amplicon is the driver of this chromosomal alteration. A number of GPCRs are expressed and active in SCLC cells (Teicher, 2014). We previously showed that SCLC cells may activate these GPCRs via autocrine mechanisms and that the simultaneous inhibition of several of these GPCRs with imipramine, a tricyclic antidepressant, could inhibit the growth of SCLC, which correlated with decreased PKA activity (Jahchan et al., 2013). We note that imipramine may also directly activate PP2A, which may have contributed to its anticancer activity (Kastrinsky et al., 2015). Based on our RNA-seq data that activation of PKA can result in the increased expression of genes coding for GPCRs (e.g., DRD2, CHRM4, or GRM2), these autocrine/paracrine loops may be maintained by constant positive feedback, thereby sustaining high PKA activity in SCLC cells even in the absence of genetic alterations in this pathway.

Why PKA is oncogenic in neuroendocrine tumors but tumor-suppressive in some other contexts is not completely elucidated, but our work suggests a possible model. PKA activation correlates with more epithelial, more neuroendocrine state in SCLC cells, a state that we and others previously found was associated with increased tumorigenicity (Augustyn et al., 2014; Borromeo et al., 2016; Jahchan et al., 2016). Similarly, PKA activation is sufficient to reprogram hepatocytes toward a more neuroendocrine state in fibrolamellar hepatocellular carcinoma (Kastenhuber et al., 2017) and for the acquisition of neuroendocrine-like features in prostate cancer (Deebel et al., 2007). Interestingly, in breast cancer cells, PKA activation promotes a mesenchymal-to-epithelial transition associated with decreased tumorigenicity (Pattabiraman et al., 2016). Therefore, PKA may generally promote a more epithelial state in cancer cells, but this state may correspond to less aggressive cancer cells in cancers where loss of epithelial features accompanies tumor progression, and more aggressive cancer cells in neuroendocrine cancer where tumor-propagating cells are more epithelial.

PKA is ubiquitously expressed and essential for many cellular processes, including embryonic development (Skalhegg et al., 2002). However, our data showing that a 50% decrease in PKA activity is sufficient to significantly inhibit tumor growth in mice suggest that there may be a therapeutic window for PKA inhibitors, especially in combination with other agents. Whether specific and potent PKA inhibitors may one day be available to treat cancer patients is unknown, but an alternative pathway to inhibit PKA in SCLC and other neuroendocrine cancers may be through activation of PP2A, which dephosphorylates PKA substrates and has other tumor-suppressive effects (Mazhari et al., 2019). While PKA inhibitors and PP2A activators need to be further developed and tested in preclinical models, our efforts highlight the possibility of targeting this signaling axis to thwart SCLC progression. Importantly, our work underscores the possibility of targeting non-mutated, ubiquitous signaling pathways to treat specific cancer types such as SCLC, and raises the possibility that genetic alterations in these pathways might help identify patients most responsive to these therapeutic strategies.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at [https://doi.org/10.1016/j.ccell.2020.05.003](https://doi.org/10.1016/j.ccell.2020.05.003).

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
M.O. acknowledges Partnership for New York City for a BioAccelerate Award, funding large-scale synthesis of SNAP-1154. Mt. Sinai has filed patents on behalf of M.O. on SNAP-1154 and SNAP-DT-061. CWRU has filed a patent on behalf of M.O. and G.N. describing combinations of 1154 and 061 with kinase inhibitors. G.N. has an ownership interest in RAPPThera Therapeutics LLC. D.K.N. is a co-founder, shareholder, and scientific adviser for Artris Therapeutics and Frontier Medicines. J. Sage receives research funding from Stemcentrx/Abbvie, Pfizer, and Revolution Medicines and owns stock in Forty Seven Inc.

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REFERENCES


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**Experimental Models: Cell Lines**

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| Mouse: KP11 | (Park et al., 2011) | N/A |
| Mouse: Kras<sup>G12D</sup>,Trp53<sup>-/-</sup> lung adenocarcinoma cell lines | Leanne Sayles and Alejandro Sweet-Cordero | N/A |
| Human: A549 | ATCC | CCL-185 |
| Human: NCI-H69 | ATCC | HTB-119 |
| Human: NCI-H82 | ATCC | HTB-175 |
| Human: NCI-H187 | ATCC | CRL-5804 |
| Human: NCI-H446 | ATCC | HTB-171 |

**Experimental Models: Organisms/Strains**

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| Mouse: TKO;Prkaca<sup>M120A</sup> | This paper | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

### Lead Contact
Information regarding the request of materials and reagents should be directed to and will be fulfilled by the lead contact and corresponding author, Julien Sage (julsage@stanford.edu).

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Plasmids generated in this study are available upon request. Please email the lead contact and corresponding author, Julien Sage (julsage@stanford.edu) for requests.

Data and Code Availability
The proteomics datasets generated in this study are available at ProteomeXChange: PXD018209 and PXD017704, http://www.proteomexchange.org/.

The RNA sequencing datasets generated in this study are available at GEO: GSE126353, https://www.ncbi.nlm.nih.gov/geo/.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Mouse Models
All experiments regarding the use of mice were performed per protocols set in place by the National Institute of Health at Stanford’s Research animal facility or the University of Michigan. All treatments were performed un-blinded, but all measurements and quantification were performed in a blinded fashion. The generation and initiating of SCLC tumors in TKO mice with conditional alleles of Rb1, Trp53, and Rb1 mutant, DKO), and KP11 (Rb1/Trp53 mutant, TKO) mouse SCLC cells were generated from single tumors (Park et al., 2011). Allografts tested in the multiplexed inhibitor beads inhibitors assays were derived from single tumors. NJH29 cells were previously generated in our laboratory from a PDX model (Jahchan et al., 2013), and A549, NCI-H69, NCI-H82, NCI-H187, and NCI-H446 were purchased from ATCC. Cells were grown in RPMI-1640 media supplemented with 10% bovine growth serum (BGS, Plasmids generated in this study are available upon request. Please email the lead contact and corresponding author, Julien Sage (julsage@stanford.edu) for requests.

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KP1 (Rb1/Trp53 mutant, DKO), and KP11 (Rb1/Rb2/Trp53 mutant, TKO) mouse SCLC cells were generated from single tumors (Park et al., 2011). Allografts tested in the multiplexed inhibitor beads inhibitors assays were derived from single tumors. NJH29 cells were previously generated in our laboratory from a PDX model (Jahchan et al., 2013), and A549, NCI-H69, NCI-H82, NCI-H187, and NCI-H446 were purchased from ATCC. Cells were grown in RPMI-1640 media supplemented with 10% bovine growth serum (BGS, Plasmids generated in this study are available upon request. Please email the lead contact and corresponding author, Julien Sage (julsage@stanford.edu) for requests.

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tein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and 10 μM Cells or tumors were lysed and sonicated in RIPA lysis buffer (Pierce) with protease and phosphatase inhibitor tablets (Roche). Pro-

**Single Cell Suspension**

To generate single cell suspensions and to isolate primary tumor cells for in vitro assays, TKO control and PrkacaM120A mutant SCLC tumors were collected from mice at ~6 months following tumor initiation and tumor tissue was digested with Collagenase/Dispase (Roche) as described by (Jahchan et al., 2016). Cells were plated in 6 well plates and SCLC cells growing in suspension were selected for so that after 6 passages cultures were depleted of contaminating stromal cells. Tumor cells were genotyped using standard methods. All SCLC cells isolated from both control and PKA-CαM120A-mutant tumors had undergone recombination in the conditional alleles. Cells were then used in cell viability and kinase assays.

**Cell Viability Assays**

Either 8×10⁢³ human or 10⁴ mouse SCLC suspension cells were plated in flat bottom 96 well plates in 200 μL of media containing 2% BGS and plates were incubated for 2-6 days before AlamarBlue (Thermo Fisher Scientific) reagent was added and plates were read after 2 hours according to manufactures specifications. Plates were not re-fed.

**RNA Purification, RT-qPCR, and RNA Sequencing**

Frozen tumor samples were ground with a mortar and pestle and total RNA was extracted using the RNeasy Mini Kit (Qiagen). For RT-qPCR, 1 μg of total RNA was used to make cDNA using the NEB ProtoScript cDNA synthesis kit, and cDNA was diluted 1:20 before use. Gene specific RT-qPCR primers for mouse Gnas were described in Chen et al., 2005 (Table S7). The remaining gene specific RT-qPCR primers were obtained from the Mass General Primer Bank and are described in (Table S7).

For RNA-seq analysis, 2 μg of total RNA was used to prepare cDNA libraries with the TruSeq Stranded Total RNA LT Kit (Illumina). Libraries were sequenced at the Stanford Functional Genomics Facility using a NextSeq sequencing system. Reads were mapped to human reference genome hg19 with STAR2.5.1b using default settings. Genes that have at least ten reads in three out of six samples in one condition were used for further analysis. Differentially expressed genes were obtained using DEseq2. Panther pathway analysis was performed using the Enrichr platform (Chen et al., 2013). The accession number for the RNA-Seq results is GEO: GSE126353.

**Immunoblot Analysis, Immunoassays, and Immunostaining**

Cells or tumors were lysed and sonicated in RIPA lysis buffer (Pierce) with protease and phosphatase inhibitor tablets (Roche). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and 10 μg of protein was analyzed by immunoblot. For immunobassays, 1 μg of protein was analyzed using the Simple Western quantitative immunoassay and the Compass software, according to the manufacturer’s protocol. Immunoblotts were quantified in Image J and target proteins were normalized to loading control for each sample.

Tumor samples were fixed in 4% formalin and embedded in paraffin before staining with hematoxylin and eosin or immunostaining (immunohistochemistry), as previously described. (Lim et al., 2017) Images were quantified using ImageJ. Tissue microarrays were stained and scored by a board-certified pathologist, C.S. Kong.

TUNEL staining was performed as previously described using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer’s protocol (Sangodkar et al., 2017). DAPI was used to counterstain the DNA.

**In Vitro Kinase and Phosphatase Assays**

For testing the kinase activity of endogenous PKA-Cα in TKO and TKO;PrkacaM120A SCLC tumor cells, cells were lysed and sonicated in cell lysis buffer (CST # 9803) with protease and phosphatase inhibitor tablets (Roche). 1 mg of extract was incubated with 10 μL of PKA-Cα antibody (CST# 4782) or normal rabbit IgG (CST #2729) overnight with gentle rotation. The following day 50 μL pre-washed protein A-agarose beads (Sigma #P3476) were added and incubated at 4⁰ C for 60 min with gentle shaking. Beads were washed then incubated with 20 μg of NJH29 PDX native tumor cell extract (isolated with CST #9803), ATP (CST #9804), and 1X kinase buffer (CST # 9804, 9802) at 37⁰ C for 60 min. Samples were then incubated with 6X Laemmli buffer and analyzed by immunoblot using the phosho-PKA substrate antibody (CST# 9624). Signal intensity was measured using Image J software and normalized to PKA-Cα expression. For testing the kinase activity of recombinant enzymes 1 μg of recombinant rPKA or rAS-PKA (M120A mutant) was used in the kinase assays.
Total cellular PKA kinase and PP2A phosphatase activity was measured using commercially available kits (PKA Colorimetric Activity Kit, Thermo Fisher Scientific; PP2A Immunoprecipitation Phosphatase Assay Kit, Millipore) according to the manufacturers’ instructions.

**Purification of Recombinant Enzymes**

The PDK1–PKA co-expression system was used to purify active recombinant rPKA and rAS-PKA as previously described (Schauble et al., 2007). The PKA-Cz-M120A expression vector was generated by synthesizing a fragment containing the M120A mutation that was flanked between NdeI and HindIII digest sites. The fragment was then digested and gel purified and ligated into PetI15B. Vectors for His- PKA-Cz and GST-PDK1 or His- PKA-Cz-M120A and GST-PDK1 were then co-transformed into BL21 bacteria and placed on 1 mM IPTG to induce expression. His-tagged proteins were then purified using Ni-NTA beads (Thermo Fisher Scientific) and immunoblots confirmed that GST-PDK1 did not co-purify with PKA.

**Kinome Profiling Sample Processing**

Allografts and PDX samples were ground into a fine powder using a mortar and pestle and continuously kept frozen with liquid nitrogen. Powder was then gathered into microcentrifuge tubes, on ice, in 1 mL of lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA) with Roche PhosSTOP and Complete Protease Inhibitor tablets added as manufacturer instructs (Sigma-Aldrich). After vortexing lightly, lysates were incubated on ice for 20 min, and re-vortexed lightly every 10 min. Lysates were centrifuged twice at 13,000 RPM for 10 min at 4°C in a tabletop microcentrifuge, and supernatant was removed. Protein concentration was measured by Bradford reagent (CWA #100514-184), and samples were snap-frozen in liquid nitrogen.

**Multiplexed Inhibitor Beads (MIBs) Assays**

Kinase chromatography, mass spectrometry and analytical processing were performed as described previously (Roskoski, 2016; Sos et al., 2014) and coupled to sepharose using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide chemistry. Cell lysates were diluted in binding buffer with 1 mol/L NaCl, and affinity purification was performed with gravity chromatography after pre-clearing. The bound kinases were stringently washed and then eluted with SDS followed by extraction/precipitation, tryptic digest and desalting. Liquid chromatography-tandem mass spectrometry (LC/MS-MS) was performed on a Velos Orbitrap (Thermo Fisher Scientific) with in-line high-performance liquid chromatography (HPLC) using an EASY-spray column (Thermo Fisher Scientific). Label-free quantification was performed with Skyline (Schilling et al., 2012), and statistical analysis with MSstats (Choi et al., 2014).

**Covalent Capture**

Labeling experiments for covalent capture enrichment were performed on 2 mg of protein lysate per sample (n=2/line) as previously described (Levin et al., 2016). Briefly, samples were incubated in lysis buffer supplemented with 250 μM Bn-ATPγS, 250 μM ATP, 5 mM GTP, 10 mM MgCl2, and 20 μg of purified PKA as indicated. Labeling reactions were performed at 37°C for 60 min before quenching with 50 mM EDTA. Thirty-microliter aliquots of each reaction were alkylated with 2 μL of 100 mM p-nitro mesylate (PNBM) for 30 min at room temperature. Thiophosphorylation was detected by immunoblot with the antithiophosphate ester antibody (110C).

Covalent capture of thiophosphorylated proteins was performed as described previously (Hertz et al., 2010). Briefly, lysates were denatured by adding 60% (wt/vol) solid urea, 10 mM final TCEP, and incubating at 55°C for 30 min. Samples were diluted to 2 M urea with 50 mM ammonium bicarbonate, brought to a pH of 8, and digested overnight at 37°C with trypsin (Promega) at a 1:20 ratio. Peptides were acidified with trifluoroacetic acid, desalted on a SepPak C18 column (Waters), and speed-vacuumed to dryness. Peptides were resuspended in 50 mM HEPES and 50% (vol/vol) acetonitrile and adjusted to pH 7. The peptide solution was incubated overnight rocking with 100 μL of iodoacetetyl Sepharose resin in the dark (Thermo Fisher Scientific). Beads were washed by gravity flow with water, 5 M NaCl, 50% (vol/vol) acetonitrile, 5% (vol/vol) formic acid, and 10 mM DTT followed by elution with 1 mg/mL oxone (Sigma). Peptides were desalted with ZipTips (Millipore) and speed-vacuumed until dry.

**LC-MS/MS Analysis and Data Processing**

All desalted peptides were resuspended into 10 μL of 0.1% formic acid. Peptides were loaded on to a nanoACQUITY (Waters) UPLC instrument for reversed-phase chromatography with a C18 column (BEH130, 1.7-μm bead size, 100 μm x 100 mm) in front of an LTQ Orbitrap Velos. The LC was operated at a 600-nL/min flow rate and peptides were separated over an 80-min gradient from 2 to 50% buffer B (buffer A: water and 0.1% formic acid; buffer B: acetonitrile and 0.1% formic acid). Survey scans were recorded over a 350–1,800 m/z range and MS/MS fragmentation was performed using HCD on the top eight peaks. A second injection (i.e., technical replicate) of each sample was performed using ETD fragmentation on the top six peaks. Peak lists were generated with an in-house software called PAVA and searched against the SwissProt Homo sapiens database (downloaded June 27, 2013; 20,264 entries) using Protein Prospector (v5.10.10). Data were searched with a 20-ppm tolerance for parent and fragment ions (HCD or 20 ppm/0.6 Da ETD), allowing for standard variable modifications and S/T/Y phosphorylation. Filtering of background peptides and phosphopeptides was accomplished using an in-house R script described previously (Lipp et al., 2015). The AS-PKA logo motif was generated using the Berkeley Berkeley’s WebLogo generator (Crooks et al., 2004).
Global Phosphoproteomics

Cells were plated in media containing 2% BGS (described above) and the following day cells treated with vehicle or 8-Br-cAMP for 60 min. Cells were then collected, lysed, and subjected to tryptic digest. Samples were desalted and lyophilized, and phospho-enrichment was performed with immobilized metal affinity chromatography following established protocols (Swaney and Villen, 2016). Phosphopeptides were then additionally desalted and data acquired using a Thermo Q Exactive (Thermo Fisher Scientific). Mass spectrometry was performed at the Thermo Fisher Scientific Proteomics Facility for Disease Target Discovery at UCSF and the J. David Gladstone Institutes. Prospector was used for peptide assignment (http://prospector.ucsf.edu/prospector/mshome.htm) and Skyline was used for all quantification (https://skyline.ms/project/home/software/Skyline begins.html). Statistical comparison was done using MS Stats (Choi et al., 2014). All gene ontology networks were generated using ClueGO version 2.5.3 through CytoScrape 3.7. Biological Process terms were identified for each group of proteins (phosphorylated, de-phosphorylated, both phosphorylated and de-phosphorylated and direct hits). We used GO Tree Interval min:2 max:5, 8 minimal genes per GO term, kappa score of 0.45 and GO Term fusion. Only p values below 0.05 are displayed.

GO term enrichment analysis was performed on top hits using Metascape with the default settings in the “Express Analysis” function in August 2019 (Zhou et al., 2019).

DepMap Analysis

To determine which PKA substrates play key roles in regulating SCLC cell proliferation or survival, we examined dependency scores associated with each gene using the data available from Cancer Dependency Map portal (https://depmap.org/portal/download) (McFarland et al., 2018). Gene dependency scores were derived from DEMETER2 algorithm applied to combined RNAi screen data (Marcotte et al., 2016). A negative gene dependency score corresponds to greater gene essentiality, such that median gene dependency score of pan-essential genes is normalized to -1 and that of negative control genes is set to 0. We analyzed the distribution of dependency scores from SCLC cell lines (n = 25) for downstream substrate (n = 48 – some of the genes in the common list of candidate targets were not screened by RNAi in DepMap). Genes were organized in an ascending order by median gene dependency scores.

Tandem Affinity Purification and Mass Spectrometry

The lentiviral backbone vector (pWPXLd/LAP-N/puro/DEST) vector was created by inserting EGFP-TEV cleavage site-S tag-Prescision cleavage site/DEST/Puromycin resistance cassette of pG-LAP6/puro vector into pWPXLd vector, a gift from Prof. Didier Trono (Addgene plasmid #12258). pDONR223-PRKACA-WT was a gift from Jesse Boehm & William Hahn & David Root (Addgene plasmid #82311). pDONR221-PRKACB (HsCD00296755) and pENTR221-PRKAR1A (HsCD00043858) were obtained from Harvard PlasmID. These vectors were transduced into NCI-H446 human SCLC cells using standard techniques. Tandem affinity purification and analysis were performed as previously described (Kanie et al., 2017; Li et al., 2017). The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD017704 and 10.6019/PXD017704.

Large Scale Synthesis of JNS-1-40

N-benzyl-2-chloro-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acetamide (JNS-1-40) (Figure S3E, left panel): To a round bottom flask charged with a stir bar and 4 Å molecular sieves was added 1,4-Benzodioxan-6-amine (2.267 g, 15 mmol, 1.5 equiv.). The flask was then put under vacuum and back-filled with nitrogen, and dry DCM (20 mL) was added. To the resulting brown solution was added benzaldehyde (1.02 mL, 10 mmol, 1 equiv.). After 20 min of stirring solid sodium triacetoxyborohydride (3.179 g, 15 mmol, 1.3 equiv.) was gradually adding to the stirring mixture. The reaction was allowed to warm to room temperature overnight. The resulting product was transferred to a stir bar-charged flask and dissolved in 10 mL of dry DCM. Triethylamine (1.9 mL, 13.65 mmol, 1.5 equiv.) was added to the stirring solution. After putting the reaction on ice, chloroacetyl chloride (0.95 mL, 11.96 mmol, 1.3 equiv.) was gradually adding to the stirring mixture. The reaction was allowed to warm to room temperature overnight. The reaction mixture was diluted to a total volume of 50 mL DCM and partitioned with 125 mL H2O. The aqueous layer was back-extracted and the combined organic layers were washed 1x with 50mL DCM and dried over MgSO4 and concentrated in vacuo. The resulting dark brown crude was purified by column chromatography (15% EtOAc:hexane) to afforded Si-1 (2.28 g, 94%) as a yellow oil.

The resulting product was transferred to a stir bar-charged flask and dissolved in 10 mL of dry DCM. Triethylamine (1.9 mL, 13.65 mmol, 1.5 equiv.) was added to the stirring solution. After putting the reaction on ice, chloroacetyl chloride (0.95 mL, 11.96 mmol, 1.3 equiv.) was gradually adding to the stirring solution. The reaction was allowed to warm to room temperature overnight. The reaction mixture was diluted to a total volume of 50 mL DCM and partitioned with 50 mL saturated NaHCO3. The aqueous layer was back extracted with and additional 50mL DCM and the combined organic layers were washed 1x with 50mL DCM and dried over MgSO4. The crude product was concentrated in vacuo and purified by column chromatography (30% EtOAc:hexane) to afford JNS-1-40 (2.57 g, 89%) as a yellow wax. Yield over the two-step reaction was 2.57 g, 84%. The product was analyzed by Nuclear magnetic resonance (NMR) (Figure S3E, right panels).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was assayed with GraphPad Prism software. Data are represented as mean ± SEM. Unpaired t-tests were performed with all individual data points from all the biological replicates. If F-test (for variance) reported a significantly different distribution between the test groups (F-test P < 0.05), the nonparametric Mann-Whitney p value is reported.
Supplemental Information

Unbiased Proteomic Profiling Uncovers a Targetable GNAS/PKA/PP2A Axis in Small Cell Lung Cancer Stem Cells

Garry L. Coles, Sandra Cristea, James T. Webber, Rebecca S. Levin, Steven M. Moss, Andy He, Jaya Sangodkar, Yeonjoo C. Hwang, Julia Arand, Alexandros P. Drainas, Nancie A. Mooney, Janos Demeter, Jessica N. Spradlin, Brandon Mauch, Vicky Le, Yan Ting Shue, Julie H. Ko, Myung Chang Lee, Christina Kong, Daniel K. Nomura, Michael Ohlmeyer, Danielle L. Swaney, Nevan J. Krogan, Peter K. Jackson, Goutham Narla, John D. Gordon, Kevan M. Shokat, and Julien Sage
Supplementary Fig. 1, related to Fig. 1: MIBs kinome profiling identifies PKA and other active kinases in SCLC

(A) Unsupervised hierarchical clustering of patient-derived xenografts (PDXs) using the 60 kinases with greatest variance across samples separates SCLC and NSCLC samples. Protein values were median-centered for each protein, with the lowest values in blue and the highest in red. (B) Unsupervised hierarchical clustering of GEMM (genetically-engineered mouse model)-derived allografts using the 60 kinases with greatest variance across samples separates SCLC from NSCLC and mouse normal lungs (mNL). Protein values were median-centered for each protein, with the lowest values in blue and the highest in red. (C) RNA-seq expression levels of PKA catalytic and regulatory subunit genes in 48 human SCLC cell lines from the Cancer Cell Line Encyclopedia (CCLE) database. There are no data (nd) for PRKACG in this dataset. (D) PKA activity scored by immunohistochemistry of phosphorylated CREB1 (pCREB) on human SCLC tumors. Negative staining was based on no signal (n=53) or <5% nuclear staining (n=13). Positive staining was based on 5-50% nuclear staining (n=10) or >50% nuclear staining (n=4). Note that serine 133 in CREB1 may also be phosphorylated by other kinases. Scale bar, 100 µm.
Supplementary Fig. 2, related to Fig. 2: PKA is required for the growth of SCLC

(A) Representation of the inducible Prkaca<sup>M120A</sup> allele. (B) Images of TKO and TKO;Prkaca<sup>M120A</sup> SCLC cells in culture. Scale bar, 300 µm. (C) PCR analysis of the Rb, p53, p130, and Prkaca alleles in TKO ("WT") and TKO;Prkaca<sup>M120A</sup> ("Mutant") SCLC cells. (D) AlamarBlue survival assay of TKO and TKO;Prkaca<sup>M120A</sup> SCLC cells (n=4). M120A-1 day 2 p=0.002**, M120A-1 day 4 p<0.001**, M120A-2 day 2 p<0.001***, M120A-2 day 4 p<0.001***. (E) Immunoblot for PKA-Cα and cleaved PARP (Cl-PARP) on control (shGFP) and PKA knock-down (shPrkaca, 2 independent shRNAs) murine KP11 SCLC cells. Tubulin is a loading control. (F) AlamarBlue survival assay of control and Prkaca knock-down KP11 cells (n=4). shPrkaca-1 p<0.0001***, shPrkaca-2 p<0.0001***. (G) Immunoblot for PKA-Cα and the Cl-PARP on shGFP and shPrkaca murine KP1 SCLC cells. Tubulin is a loading control. (H) AlamarBlue survival assay for control and Prkaca knock-down KP1 cells (n=4). shPrkaca-1 day 0 p=0.03*, day 6 p=0.0001***, shPrkaca-2 day 6 p=0.0001***. (I) Growth of shGFP (control) and shPrkaca KP1 allografts in NSG mice. n=9-10 tumors/group, p=0.001**. (J) Immunoblot for PKA-Cα, Cl-PARP, cleaved caspase 3 (CC3, apoptosis marker), the mitosis marker phospho-histone H3 (pHH3), and the PKA substrate CREB1 (CREB) and phosphorylated CREB1 (pCREB) in control and Prkaca knock-down KP1 allografts. HSP90 is a loading control. (K) Immunoassay for PKA-Cα and Cl-PARP on control and PRKACA knock-down NCI-H526 xenografts (see Figure 2J). HSP90 is a loading control. All error bars represent SEM.
Supplementary Figure 3, related to Fig. 3: PP2A activation inhibits SCLC

(A) Quantification of PP2A activity using IP-phosphatase assays on NJH29 and NCI-H82 cells treated with 10 µM of the PP2A inhibitor (PP2Ai) Cantharidin (n=3). NJH29 p<0.0001***, NCI-H82 p<0.0001***. (B) Expression of the genes coding for PP2A α subunits in human SCLC tumors. (C) DepMap gene dependency score (RNAi) for PPP2R1A in human SCLC cells. (D) Immunoblot for the indicated proteins from NJH29 cells treated with various concentrations of the SMAP JNS-1-40 for 6 hours. Cleaved PARP is a marker of cell death. HSP90 is a loading control. (E) Large scale synthesis of JNS-1-40, chemical reactions (left) and NMR analysis of the synthesized product (right two panels). ¹H NMR (400 MHz, CDCl₃) δ 7.32 – 7.18 (m, 5H), 6.79 (d, J = 8.5 Hz, 1H), 6.56 (d, J = 2.5 Hz, 1H), 6.45 (dd, J = 8.5, 2.5 Hz, 1H), 4.84 (s, 2H), 4.29 – 4.21 (m, 4H), 3.90 (s, 2H).; ¹³C NMR (101 MHz, CDCl₃) δ 166.47, 143.98, 143.89, 136.72, 134.01, 128.97, 128.50, 127.67, 121.31, 118.05, 117.05, 64.26, 53.82, 42.18; HRMS (+ESI) calcd. for [C₁₇H₁₇ClNO₃]: m/z 318.0891, found 318.0898. (F) Immunoblot for the indicated proteins from NJH29 cells treated with 10 µM SMAP-1154 showing decreased c-MYC levels and phosphorylation, and decreased mTOR phosphorylation (as a marker of mTOR activity). HSP90 is a loading control. (G) Growth curve of NJH29 SCLC xenografts grown in NSG mice treated with JNS-1-40 (n=8-10, p=0.025*). (H) Phosphorylated PKA substrates (pPKA) measured relative to PKA-Cα levels by immunoassay in NJH29 xenografts treated with JNS-1-40 from (F) (n=4, p=0.02*). (I) Body weight of mice with NCI-H69 xenografts (Figure 3D-F) and
Supplementary Figure 4, related to Fig. 4: Gαs is activated in human SCLC an important for the growth of mouse SCLC

(A) RNA-seq expression levels for Gα subunits genes in 81 human SCLC tumors. (B) cBioPortal analysis of SCLC (MSKCC, n=319 tumors) for GNAS showing amplification (n=5) and a known oncogenic mutation (R201C) (n=1). (C) Schematic representation of the Gnas<sup>loxP</sup> allele. (D) RT-qPCR for Gnas in TKO (Rb<sup>flox/flox</sup>;p53<sup>flox/flox</sup>;p130<sup>flox/flox</sup>) and TKO;Gnas mutant tumors. n=10 per group, p<0.0001***. (E) Schematic representation of the alleles to express Gαs<sup>R201C</sup> after expression of Cre and treatment with doxycycline (Dox) to activate rtTA on the Tet-responsive element (TRE). (F) Representative images of tumor sections counterstained with hematoxylin and eosin (H&E) in TKO and TKO;Gnas<sup>R201C</sup> mice 3.5 months after cancer initiation. In TKO;Gnas<sup>R201C</sup> mice, a variety of non-SCLC (NSCLC) subtypes can be identified in addition to SCLC. Scale bar, 100 µm. (G) Representative images of lung sections immunostained for SYP and counterstained with hematoxylin from TKO and TKO;Gnas<sup>R201C</sup> mice 3.5 months after Ad-Cre and Dox treatment. Scale bar, 5 mm. (H-I) Quantification of tumor numbers (H, p=0.0141*) and tumor area (I, p=0.0062**) for SYP-positive lesions from (G) (n=10-11 mice/group). (J-M) Quantification of TUNEL-positive and Ki67-positive cells in tumor sections with loss of Gnas (J-K) or Gnas<sup>R201C</sup> (L-M). NS, not significant. J, n=5 mice/group, p=0.9031, K, n=4 mice/group, p=0.4296, L, n=3 mice/group, p=0.7000, M, n=5 mice/group, p=0.4235. Data are averages from n=3-5 fields representing independent tumors. Error bars represent SEM.
Supplementary Figure 5, related to Fig. 5: The PKA interactome in SCLC cells

(A) Silver stain of protein expression for LAP-tagged PKA subunits, as indicated. The expected sizes for tagged PKA-Cα, PKA-Cβ, and PKA-R1A are 49 kDa, 47 kDa, and 50 kDa, respectively. The TEV protease is 27 kDa. (B) Bubble plot showing the components of the PKA holoenzyme (prey) associated with each of the bait proteins.
Supplementary Figure 6, related to Fig. 6: PKA controls a large and diverse set of downstream processes in SCLC cells

(A) Expression of CREB1 and cleaved PARP (Cl-PARP, cell death marker) in control (shGFP) and Creb1 knock-down (shCreb1) murine KP1 SCLC cells measured by immunoassay. HSP90 is a loading control. (B) AlamarBlue cell survival assay for control and Creb1 knock-down KP1 cells (n=3). NS=not significant, error bars represent SEM. (C) Growth of KP1 allografts in NSG mice with control vector or Creb1 knock-down (n=8-10 tumors). (D) Expression levels of CREB1, cleaved PARP (Cl-PARP), and the cell proliferation marker PCNA in control and Creb1 knock-down KP1 tumors measured by immunoassay. (E) Immunoblots for ASCL1, NEUROD1, NFIB, DLL3, HES1, VIM, and PKA-Cα (2 exposures) from tumor extracts of NJH29 and LX102 PDXs, a TKO allograft, and NCI-H82 xenografts. HSP90 is a loading control. (F) Ponceau-stained membrane for recombinant PKA-Cα (rPKA) or recombinant analog sensitive PKA-Cα (rAS-PKA). (G) Immunoblot for PKA-Cα on varying amounts of rPKA or rAS-PKA, as indicated. (H) Immunoblot for PKA-Cα and phospho-PKA (pPKA) substrates in the human non-small cell lung cancer (NSCLC) cell line A549, and human NJH29, NCI-H69, NCI-H82, and NCI-H187 SCLC cell lines. HSP90 is a loading control. (I) Nodes of GO terms of biological processes altered by activation of PKA in NCI-H69 SCLC cells after analysis of the global phosphoproteomic data (cases with both increased and decreased phosphorylation or just decreased phosphorylation).
Figure S7, related to Fig. 7: PKA Activity promotes the expansion of SCLC cancer stem cells

(A-B) Expression of PKA-Cα in NJH29 (A) and NCI-H82 (B) cells expressing GFP or an active form of PKA-Cα (aPKA) measured by immunoassay (Dox=doxycycline). HSP90 is a loading control. (C) Expression of the ratio of pCREB1-Ser133 (a site phosphorylated by PKA) relative to total CREB1 in NCI-H82 cells measured by immunoassay, showing increased phosphorylation upon PKA activation. n=3, p=0.031*. Error bars represent SEM. (D) Growth of NCI-H82 xenografts expressing GFP or aPKA. n=12, Day 14 p=0.04*, Day 22 p=0.02*. Error bars represent SEM. (E-F) Panther analysis of pathways significantly altered in aPKA-expressing NCI-H82 and NJH29 tumors. (G) Immunoblot for the indicated proteins in NJH29 SCLC tumors expressing GFP or aPKA. HSP90 is a loading control. (H) Quantification of protein levels in NJH2-GFP and NJH2-aPKA tumors from (G) relative to HSP90. n=5, Cleaved PARP (Cl-PARP) p=0.002**, phospho-histone H3 (pHH3) p=0.45, not significant (NS), NCAM1 p=0.007**, DLL3 p=0.003**, INS1M1 p=0.002**, PKA-Cα p=0.002**.