Crebbp Loss Drives Small Cell Lung Cancer and Increases Sensitivity to HDAC Inhibition

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ABSTRACT

CREBBP, encoding an acetyltransferase, is among the most frequently mutated genes in small cell lung cancer (SCLC), a deadly neuroendocrine tumor type. We report acceleration of SCLC upon Crebbp inactivation in an autochthonous mouse model. Extending these observations beyond the lung, broad Crebbp deletion in mouse neuroendocrine cells cooperated with Rb1/Trp53 loss to promote neuroendocrine thyroid and pituitary carcinomas. Gene expression analyses showed that Crebbp loss results in reduced expression of tight junction and cell adhesion genes, including Cdh1, across neuroendocrine tumor types, whereas suppression of Cdh1 promoted transformation in SCLC. CDH1 and other adhesion genes exhibited reduced histone acetylation with Crebbp inactivation. Treatment with the histone deacetylase (HDAC) inhibitor Pracrinostat increased histone acetylation and restored CDH1 expression. In addition, a subset of Rb1/Trp53/Crebbp-deficient SCLC exhibited exceptional responses to Pracrinostat in vivo. Thus, CREBBP acts as a potent tumor suppressor in SCLC, and inactivation of CREBBP enhances responses to a targeted therapy.

SIGNIFICANCE: Our findings demonstrate that CREBBP loss in SCLC reduces histone acetylation and transcription of cellular adhesion genes, while driving tumorigenesis. These effects can be partially restored by HDAC inhibition, which exhibited enhanced effectiveness in Crebbp-deleted tumors. These data provide a rationale for selectively treating CREBBP-mutant SCLC with HDAC inhibitors.

INTRODUCTION

Recent identification of the genomic alterations in small cell lung cancer (SCLC), a deadly type of lung cancer, may provide new opportunities for therapeutic intervention (1-3). Critical challenges remain, however, as few of these SCLC alterations are readily actionable, and a majority of them have not been validated for their roles in disease initiation and progression (4). Along with Rb1 and TP53 inactivation, mutations in the CREBBP and EP300 acetyltransferases are among the most frequent in SCLC, appearing in 15% to 17% and 5% to 13% of tumors in patients with SCLC, respectively (1, 2, 5, 6). In SCLC, deletions and truncating mutations in CREBBP and EP300 genes along with missense mutations in the histone acetyltransferase (HAT) domain are frequent, and these occur in a mutually exclusive manner. For CREBBP, HAT domain mutations observed in SCLC samples have been shown to abrogate CREBBP-mediated histone acetylation (6). CREBBP and EP300 acetylation of lysine residues on histone tails neutralizes their positive charge and can increase chromatin accessibility. Acetylation of a specific histone residue, histone H3 lysine 27 (H3K27), by CREBBP/EP300 can promote transcriptional enhancer function (7), and deletion of Crebbp/Ep300 in mouse fibroblasts eliminates the vast majority of H3K27 acetylation (8). CREBBP/EP300 also acetylates nonhistone proteins, such as p53 and BCL6 (9, 10). CREBBP is mutated in lymphomas, urothelial carcinoma, and other human tumor types (11-13). Studies employing mouse models have demonstrated that Crebbp functions as a tumor suppressor in leukemia and lymphoma (14-17). However, in vivo evidence that CREBBP functions as a tumor suppressor in solid tumors is lacking. In lymphoma, it has been postulated that loss of CREBBP-mediated acetylation and activation of p53 drives tumorigenesis (13, 17). p53-dependent mechanisms of tumor suppression mediated by CREBBP are likely not relevant to tumors such as SCLC that almost invariably harbor TP53 mutations (1). Thus, elucidating roles for p53-independent tumor-suppressive activities of CREBBP in SCLC is important. In this study, we demonstrate p53-independent Crebbp tumor-suppressor function not only in SCLC but across multiple neuroendocrine tumor types. We report CREBBP control of adhesion-related transcript expression, including Cdh1, encoding E-Cadherin, as contributing to tumor suppression, and we identify a potential therapeutic approach for treating CREBBP-deficient SCLC.

RESULTS

Crebbp Mutation Promotes Tumorigenesis of Prenostlastic Neuroendocrine Cells

To study the potential role of Crebbp in SCLC tumor suppression, we mutated Crebbp in a cell-based model of early-stage...
SCLC that we previously described (18). Rb1/Trp53/Rbl2-deficient “preSC” cells, derived from a mouse SCLC model at an early stage in tumorigenesis, become fully transformed with ectopic expression of SCLC oncogenes such as MYCL (18). Here, we expressed Cas9 and two single-guide RNAs (sgRNA) targeting DNA sequences encoding the HAT domain of the murine Crebbp gene and validated loss of CREBBP protein (Supplementary Fig. S1A and S1B). We found that Crebbp-mutant preSC cells formed more and individually larger colonies in soft agar compared with control preSC cells (Fig. 1A). When preSC cells were injected into the flanks of immune-compromised mice, tumors emerged at 30 to 50 days in the sites injected with the Crebbp-mutant preSC cells but not in those injected with control cells (Fig. 1B). Further aging of the mice injected with control preSC cells showed that these cells also formed tumors, with delayed kinetics (Fig. 1C). Hematoxylin–eosin (H&E) staining showing typical SCLC morphology and immunostaining showed the expression of UCHL1 and CGRP, markers of both SCLC and normal pulmonary neuroendocrine cells (Fig. 1D, Supplementary Fig. S1C). These data support a role for CREBBP in SCLC tumor suppression.

**Crebbp Inactivation Accelerates SCLC in an Autochthonous Mouse Model**

To further investigate the contribution of Crebbp inactivation to SCLC development in vivo, we employed an autochthonous model. We performed a genetic cross to incorporate a floxed Crebbp allele (14) into an Rb1/Trp53-deleted model of SCLC that develops lung tumors with histopathologic and molecular features of human SCLC (19, 20). Vi

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Figure 1. Inactivation of Crebbp accelerates SCLC in mouse models. A, Representative images of control and Crebbp-targeted preSC cells in soft agar 3 weeks after seeding of 1 × 10⁴ cells. Two independent single-guide RNAs were employed (sgCrebbp-1, sgCrebbp-2). Bottom, quantification of colonies > 0.1 mm in diameter (n = 4). Scale bar, 0.5 mm. *P < 0.001. Student t-test. B, Images of preSC-derived allografts, 40 days after s.c. injection of cells. Scale bars, 1 cm. C, Kaplan–Meier overall survival curves of mice injected with control-preSC cells (Control, n = 6) and mice injected with Crebbp-knockout preSC cells (n = 5 each). Statistical significance was calculated using the log-rank (Mantel–Cox) test. D, Images of UCHL1-stained sections of Crebbp-mutant tumor and normal lung. Arrow points to neuroepithelial body in the airway. Scale bars, 100 μm. Representative section of Crebbp-deficient tumors stained with H&E. Scale bar, 20 μm. E, Kaplan–Meier tumor-free survival curves of Rb1/Trp53- and Crebbp-mutant (red, n = 28) mice from autochthonous model infected with Ad-CGRP-Cre (day 0). Statistical significance was calculated using the log-rank (Mantel–Cox) test. F, Representative immunoblotting results of CREBBP protein levels in 5 lung tumor tissues from each cohort (Rb1/Trp53 vs. Rb1/Trp53/Crebbp). β-actin was used as a loading control. G, Representative H&E-stained section of SCLC in each cohort (Rb1/Trp53 vs. Rb1/Trp53/Crebbp). H, Representative immunofluorescence for SCLC markers TTF-1 and CGRP in each cohort (Rb1/Trp53 vs. Rb1/Trp53/Crebbp). DAPI was used as a nuclear stain. Original magnification, ×40.
CREBBP Suppresses SCLC and Other Neuroendocrine Tumors

To further evaluate whether Crebbp acts as a tumor suppressor across multiple neuroendocrine tissue compartments, we employed a tamofoxifen (TAM)-inducible neuroendocrine-expressing Cre driver strain (Ascl1Cre-ERT2 knockin allele; ref. 22) and generated Ascl1Cre-ERT2/Rb1/Trp53 and Ascl1Cre-ERT2/Rb1/Trp53/Crebbp mice. Following TAM injection into the peritoneum to delete conditional alleles, we monitored mice for tumor-associated morbidity. In the control group, Ascl1Cre-ERT2/Rb1/Trp53 mice became moribund at a median of 128 days after TAM injection, with 100% incidence of massive pituitary tumors that distorted the brain (Fig. 2A; Supplementary Fig. S4A and S4B). Histologic review of these tumors, performed by a veterinary pathologist (S.P.S. Pillai), indicated pituitary carcinoma of the intermediate lobe, a neuroendocrine tumor type previously described in Rb1- and Rb1/Trp53-mutant mouse models (23–25). The tumors exhibited multifocal invasion of the overlying neural parenchyma. We also observed invasion of the underlying sphenoid bone with islands of neoplastic cells within bone marrow (Supplementary Fig. S4C). Ascl1Cre-ERT2/Rb1/Trp53 mice also exhibited thyroid neuroendocrine C-cell adenomas, characterized by discrete nodular foci composed of well-differentiated C cells that compressed adjacent follicles (Supplementary Fig. S4D and S4E). Thyroid C-cell tumors have also been previously shown to be frequent in mice with Rb1 and Trp53 mutation (26). Ascl1Cre-ERT2/Rb1/Trp53/Crebbp mice became moribund significantly more rapidly than Rb1/Trp53-mutant controls (Fig. 2A), as pituitary tumor burden led to euthanasia at a median of 99 days (P < 0.0001, log-rank test). The pituitary carcinomas in Rb1/Trp53/Crebbp-mutant mice were histologically similar to those from the Rb1/Trp53-mutant model (Supplementary Fig. S4F and S4G). In contrast to small thyroid C-cell adenomas in Rb1/Trp53 mutants, the Rb1/Trp53/Crebbp-mutant animals invariably exhibited large, bilateral, and invasive thyroid medullary C-cell carcinomas with multifocal necrosis, marked nuclear atypia, and high mitotic rates (Supplementary Fig. S4H and S4I). The tumor cells also showed vascular invasion (Supplementary Fig. S4J). We confirmed complete loss of CREBBP protein expression in the pituitary and thyroid tumors from Ascl1Cre-ERT2/Rb1/Trp53/Crebbp mice (Fig. 2B and C).

To determine how Crebbp inactivation accelerated tumor development in these tissues, we examined affected pituitary at 3 months after TAM and found significantly larger pituitary tumor size in the triple-mutant compared with the double-mutant mice (Fig. 2D and E). Immunohistochemistry for PCNA, a marker of proliferating cells, showed a higher rate of proliferation in the Ascl1Cre-ERT2/Rb1/Trp53/Crebbp compared with Ascl1Cre-ERT2/Rb1/Trp53 pituitary tumors (Fig. 2F and G), explaining the faster tumor growth upon Crebbp deletion. Although Ascl1Cre-ERT2/Rb1/Trp53 mice exhibited hyperplastic medullary thyroid lesions or small early adenomas at 3 months after TAM, the Ascl1Cre-ERT2/ Rb1/Trp53/Crebbp group exhibited large bilateral thyroid C-cell carcinomas (Fig. 2H). These data indicate that Crebbp cooperates with Rb1 and Trp53 to suppress tumorigenesis not only in SCLC, but across multiple neuroendocrine cell types.

Crebbp-Regulated Transcriptional Changes in Neuroendocrine Tumors

We hypothesized that Crebbp loss cooperates with Rb1/Trp53 deletion to promote SCLC, thyroid medullary C-cell, and pituitary carcinomas (all neuroendocrine tumors) through control of gene expression. We performed RNA sequencing (RNA-seq) analyses to compare Rb1/Trp53 versus Rb1/Trp53/Crebbp tumors, initially examining each of these three tumor types individually. To identify pathways and gene sets enriched in the Crebbp-mutant tumors, we performed gene set enrichment analyses (GSEA; ref. 27). We queried the 50 gene-set “Hallmark” gene signatures from MSigDB (28). For SCLC, thyroid and pituitary tumor types, gene sets related to E2F_TARGETS, G2–M_CHECKPOINT, and MITOTIC_SPINDLE were commonly enriched in the Rb1/Trp53/Crebbp compared with the Rb1/Trp53 group (Fig. 3A–D; Supplementary Fig. S5). This result is consistent with the increased proliferation observed in vivo with Crebbp inactivation in pituitary carcinomas (Fig. 2F and G). Next, to identify genes significantly deregulated upon Crebbp inactivation, we used edgeR (29) and found 847 differentially expressed genes between the double-mutant and triple-mutant SCLC, and 3,313 and 3,078 genes similarly altered in pituitary and thyroid tumors, respectively, with FDR < 0.05 (Fig. 3E; Supplementary Tables S1–S3). The large number of differentially expressed genes in any given tumor type complicated determination of which genes were likely to be functionally important. We hypothesized that key mediators of Crebbp tumor-suppressive activity are likely to be commonly deregulated across the three different Rb1/Trp53-deleted neuroendocrine tumor types that were each accelerated upon Crebbp genetic inactivation. We identified a
Figure 2. CREBBP inactivation accelerates pituitary and thyroid neuroendocrine tumors. A, Kaplan–Meier tumor-free survival curves of double-knockout mice (Rb1lox/lox;Trp53lox/lox;ASCL1CreERT2, green, n = 18) and triple-knockout mice (Rb1lox/lox;Trp53lox/lox;Crebbplox/lox;ASCL1CreERT2 red, n = 20). Log-rank (Mantel–Cox) test was used to determine the significance of tumor-free survival between the cohorts. B, Representative immunoblotting results of CREBBP protein levels in pituitary tumors from 5 mice in each cohort. Normal mouse pituitary was used as a control. RPC represents Rb1/Trp53/Crebbp; RP represents Rb1/Trp53. C, Representative immunoblotting showing CREBBP protein levels in thyroid tumors from 3 mice in each cohort. D and E, Mice with the indicated genotypes were euthanized 3 months after TAM injection. D, Representative H&E-stained sections showing normal pituitary gland and pituitary tumors; scale bars, 500 μm. E, Average area of pituitary and pituitary carcinomas in each cohort quantified with 5 mice in each cohort. Statistical significance was determined by two-tailed unpaired Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. F and G, Representative IHC of PCNA in normal pituitary and in pituitary tumors from each cohort. Scale bars, 20 μm. G, Quantification of positive PCNA staining in normal pituitary tissue and pituitary tumors from mice in each cohort, 3 months after TAM injection. Scale bars, 500 μm. Also see related Supplementary Fig. S4 for images of tumor histology at time of animal morbidity.
Figure 3. Gene expression analyses of CREBBP-perturbed neuroendocrine tumors. A–C, GSEA identifies biological processes and pathways enriched in Crebbp-deleted tumors across 3 murine neuroendocrine tumor types: (A) SCLC (n = 7 Rb1/Trp53/Crebbp vs. 7 Rb1/Trp53), (B) pituitary carcinomas (n = 8 Rb1/Trp53/Crebbp vs. 9 Rb1/Trp53), and (C) thyroid c-cell tumors (n = 5 Rb1/Trp53/Crebbp vs. 7 Rb1/Trp53). Red bars show 3 gene sets shared among the three murine neuroendocrine tumor types with FDR < 0.05. D, GSEA plots showing the top gene set enriched in the Crebbp-deficient neuroendocrine tumors, “E2F_ Targets.” E, Summary of commonly dysregulated genes upon Crebbp deletion in three neuroendocrine tumor types (SCLC, pituitary, and thyroid tumors) and preSC cells. F, Venn diagram showing 66 genes commonly deregulated in 3 Crebbp-deficient neuroendocrine tumor types compared with Crebbp wild-type controls as well as in preSC cells with lentiviral Crebbp shRNA expression (3 different shRNA sequences included in analysis). G, Heat map of the 66 commonly dysregulated genes with Crebbp suppression in SCLC primary tumors and preSC cells. Red denotes high expression and blue denotes low expression. RPC represents Rb1/Trp53/Crebbp, RP represents Rb1/Trp53. H, KEGG pathway enrichment analysis of 66 differentially expressed genes identified significantly enriched biological pathways such as tight junctions and cell adhesion molecules. Top enriched pathways shown.
set of 141 genes consistently regulated in a Crebbp-dependent manner across the three tumor types (Fig. 3E and F). To further define a core set of Crebbp-regulated transcripts relevant to SCLC, we knocked down Crebbp expression in murine preSC cells using lentiviral shRNA vectors and identified 1,355 differentially expressed genes (Supplementary Table S4), with 66 genes commonly downregulated across each of the 4 Crebbp-perturbed comparisons (mouse SCLC, pituitary tumors, thyroid tumors, and preSC cells; Fig. 3E and F). A heat map of this core set of 66 Crebbp-dependent genes in the mouse SCLC samples and in preSC cells is shown in Fig. 3G. Interestingly, there were 6-fold more core genes downregulated upon Crebbp deletion than upregulated, consistent with CREBBP acting, in general, as a positive regulator of gene expression. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of these 66 genes indicated significant changes in tight junction and cell adhesion genes, reflecting decreased expression of functionally related genes such as Cdh1, Cldn3, Cldn6, Cldn9, and Tjp3 (Fig. 3H). Data from cross-tumor analyses indicate that CREBBP expression in neuroendocrine tumor cells promotes a cellular adhesion-related transcriptional program.

Features of Epithelial–Mesenchymal Transition in Crebbp-Deleted SCLC

CREBBP-dependent expression of CDH1 was of particular interest, as CDH1 is a tumor-suppressor gene that controls epithelial-to-mesenchymal transitions (EMT; ref. 30). E-Cadherin is normally expressed in lung neuroendocrine cells, the cells of origin for a majority of SCLC (21), but transient suppression of E-Cadherin and concomitant upregulation of mesenchymal proteins occurs when neuroendocrine cells migrate toward developing neuroendocrine bodies (31). We wanted to determine whether Crebbp loss in SCLC was associated with additional features of EMT. Immunoblot analysis confirmed reduced E-CADHERIN level in Rb1/Tip53/Crebbp primary tumors relative to Rb1/Tip53 SCLC (Fig. 4A). Furthermore, in the Rb1/Tip53/Crebbp-null mouse SCLC tumors, we observed increased expression of proteins associated with EMT, including ZEB1, N-CADHERIN, VIMENTIN (VIM), and SLUG (Fig. 4A). Cell lines derived from Rb1/Tip53/Crebbp-deficient mouse tumors also exhibited decreased CDH1 and increased expression of EMT-associated proteins while maintaining ASCL1 expression, an indication that neuroendocrine features were still maintained (Fig. 4B). We noted that the magnitude of EMT marker changes upon Crebbp deletion was enhanced in the cell lines compared with primary tumors. Reduction in CDH1 and increased expression of VIMENTIN and ZEB1, two mesenchymal markers, were confirmed using immunofluorescence in SCLC tumors from the Rb1/Tip53/Crebbp model (Fig. 4C; Supplementary Fig. S6). We noticed nonuniform CDH1 distribution in the Rb1/Tip53/Crebbp model, and the E-Cadherin–low cells maintained CGRP expression (Supplementary Fig. S6). Mining data from human SCLC in which CREEBBP mutation status was annotated, 11 of 81 tumors with associated RNA-seq data exhibited CREEBBP mutation (1). GSEA revealed a trend (FDR = 0.19) toward enrichment in the Hallmark “EMT” gene set in the CREEBBP-mutant samples (Supplementary Fig. S7). Knockdown of CREEBBP in preSC cells using two different shRNAs led to reduced expression of ZEB1 and increased expression of ASCL1, features of an epithelial cell morphology (Fig. 4D). Similarly, knockdown of CREEBBP in the human SCLC cell line DMS53 also led to reduced CDH1 and increased ZEB1 expression (Fig. 4E). Importantly, knockdown of CREEBBP in DMS53 was associated with increased growth, whereas overexpression of CDH1 had the opposing effect (Supplementary Fig. S8). Collectively, these findings indicate that CREBBP inactivation can induce a partial EMT program with reduced expression of CDH1.

CREEBBP Restoration Results in Increased CDH1 Expression and Impaired Proliferation

To better understand the functional effects of CREBBP inactivation in SCLC, we performed a rescue experiment using lentiviral expression of CREEBBP in NCI-H1882, a human SCLC cell line that lacks expression of CREBBP owing to a large deletion (Supplementary Fig. S9A). Restoration of CREBBP expression in NCI-H1882 resulted in reduced cell proliferation, diminished growth capacity in soft agar, reduced colonies upon low-density plating, and increased CDH1 expression (Fig. 5A–C). CREBBP reexpression also led to decreased proliferation and reduced CDH1 and ZEB1 expression in the latter (Fig. 5A). We wanted to determine whether this axis of expression downstream of CREBBP, also phenocopied the acquisition of epithelial cell morphology seen in the CREBBP-restored cells (Fig. 5H). To determine whether this axis of CDH1 expression downstream of CREBBP is functionally important, we reexpressed CREBBP and simultaneously suppressed CDH1 using lentiviral shRNAs. CDH1 knockdown partially blocked the decrease...
in LU505 proliferation mediated by CREBBP restoration (Fig. 5I). Consistent with the notion that changes in the EMT pathway can enable tumor-invasive abilities, overexpression of either CREBBP or CDH1 decreased the migratory capacity of LU505 cells in a transwell migration assay (Supplementary Fig. S12A and S12B). Taken together, our findings suggest that CREBBP suppresses tumorigenesis and restrains EMT in part through positive regulation of CDH1.

**Crebbp Loss Leads to Epigenetic Suppression of CDH1 and Other Cell Adhesion Genes**

CREBBP acetylates multiple histone residues, including histone H3K27, a key site for transcriptional enhancer activation (7). We hypothesized that the reduced histone acetylation following Crebbp deletion might contribute to the reduced expression of CDH1. Crebbp knockdown in preSC cells resulted in a global decrease in acetylated H3K27 (H3K27Ac; Fig. 6A, also see Fig. 4D). A similar result was seen with CREBBP deletion in DMS53 cells (Fig. 6B). We performed chromatin immunoprecipitation sequencing (ChIP-seq) analyses to examine H3K27Ac levels surrounding the transcriptional start sites (TSS) across the genome of preSC cells and found Crebbp knockdown did not correlate significantly with a reduction in H3K27Ac in these regions (Fig. 6C). However, limiting our analysis to the 57 genes consistently downregulated upon Crebbp knockdown in preSC cells and in the neuroendocrine tumors previously described (Fig. 3), we found that Crebbp knockdown in preSC cells exhibited reduced H3K27Ac associated with these core CREBBP-regulated genes (Fig. 6C). Further examining the pattern of H3K27Ac at the Cdh1 gene, Crebbp knockdown in preSC cells resulted in reduced H3K27Ac levels in potential enhancer regions extending from intron 1 through intron 250.
low-density plating (Fig. 6F and G). These results indicate soft agar and increased their colony-forming capacity upon deletion pro-lentiviral CRISPR/Cas9 vectors and verified that this led to decreased CDH1 expression (Fig. 6E). We show that CREBBP inactivation leads to reduced acetylation of H3K27 and transcriptional repression of CDH1. If reductions in acetylation upon CREBBP deletion contribute to reduced gene expression, then the effects could potentially intron 2 (Fig. 6D). Other core Crebbp-regulated adhesion genes such as Cldn6, Cldn3, Cldn9, Tjp3, and Sdc4 also exhibited reduced H3K27 acetylation in response to Crebbp knockdown (Supplementary Fig. S13). These results suggest a role for CREBBP-mediated H3K27 acetylation in regulating the expression of CDH1 and other CREBBP-regulated cellular adhesion genes.

**CDH1 Suppression Increases Transformation**

To evaluate the importance of Crebbp downregulation for cellular transformation, we deleted Crebbp in preSC cells using lentiviral CRISPR/Cas9 vectors and verified that this led to decreased CDH1 expression (Fig. 6E). Crebbp deletion promoted anchorage-independent growth of targeted preSCs in soft agar and increased their colony-forming capacity upon low-density plating (Fig. 6F and G). These results indicate that the inactivation of Cdh1 promotes cell transformation, a necessary step toward full-blown tumor development. On the other hand, reexpression of CDH1 inhibited proliferation and eliminated tumor-initiating ability of CREBBP-deficient LU505 cells (Fig. 5F and G). Thus, Cdh1 itself exhibits properties of a tumor-suppressor gene in SCLC.

**Histone Deacetylase Inhibition Suppresses Effects of Crebbp Deletion**

The balance of histone acetylation and deacetylation plays critical roles in the regulation of gene expression. Effects of HATs such as CREBBP are opposed by histone deacetylases (HDAC). We show that CREBBP inactivation leads to reduced acetylation of H3K27 and transcriptional repression of CDH1. If reductions in acetylation upon CREBBP deletion contribute to reduced gene expression, then the effects could potentially...
CREBBP Suppresses SCLC and Other Neuroendocrine Tumors

To determine whether Pracinostat exhibits therapeutic efficacy in SCLC, we employed the autochthonous Rb1/Trp53/Crebbp models. We screened mice by MRI for lung tumor burden and entered mice into treatment groups upon detection of adequately sized tumors (see Methods). Mice underwent subsequent MRI at 2 and 3 weeks of treatment followed by animal euthanasia and tumor analyses (Fig. 7C–F). Focusing on the 3-week time point, in saline-treated Rb1/Trp53 and Rb1/Trp53/Crebbp models, all mice exhibited progressive disease (PD; >30% increase in tumor volume; Fig. 7D–F). In the Rb1/Trp53 model treated with Pracinostat, only 1 of 8 animals exhibited PD, whereas 6 of 8 exhibited stable disease and 1 of 8 a partial response (PR; >30% decrease in tumor volume). Thus, Pracinostat exhibits efficacy as monotherapy in the Rb1/Trp53-deleted autochthonous model. We also tested Pracinostat in the Rb1/Trp53/Crebbp-deleted model. Of 12 Pracinostat-treated Rb1/Trp53/Crebbp mice, 4 exhibited partial or complete responses, which included 2 complete regressions and 2 strong regressions (84% and 62% reductions in tumor volume). Stable disease was seen in an additional 5 mice (Fig. 7D and E). We could not study molecular properties of the exceptional responders, owing to a lack of tumor material available; however, of the tumors we could analyze, Pracinostat treatment in...
both Rb1/Trp53 and Rb1/Trp53/Crebbp models resulted in reduced proliferation, as shown by reduced phospho Ser-10 histone H3–positive cells, and increased apoptosis, as shown by cleaved caspase-3 immunostaining (Fig. 7G and H). In the Pracinostat-treated Rb1/Trp53/Crebbp SCLC tumors, we also observed increased expression of CDH1 (Fig. 7I). Thus, although Pracinostat exhibits efficacy as monotherapy regardless of Crebbp status, we found that Crebbp deletion in SCLC results in the potential to elicit striking responses, including complete regressions.

**DISCUSSION**

**CREBBP Is a Tumor-Suppressor Gene in SCLC**

CREBBP is one of the most frequently mutated genes in human SCLC, yet the functional contribution of CREBBP...
inactivation to SCLC has been largely uncharacterized. This study definitively demonstrates that CREBBP functions as a tumor suppressor not only in SCLC but also across other neuroendocrine tumor types. CREBBP tumor-suppressor activity has been best described in lymphoma and leukemia, where Crebbp inactivation in mouse models led to tumor acceleration (13, 15–17). This was in part mediated by impaired acetylation and reduced activity of another tumor suppressor, p53 (13, 17). In contrast, CREBBP perturbation in neuroendocrine cell/tumor consistently leads to p53-independent changes in the expression of genes related to tight junctions and cell adhesion, including CDH1, and we show that CDH1 itself exhibits properties of a tumor suppressor in SCLC. Our observation that CREBBP controls adhesion molecule expression in driving SCLC is a novel finding that has not been described in CREBBP-mutant leukemia/lymphoma. These findings illustrate the importance of cellular context for CREBBP function as a tumor suppressor.

**CREBBP Regulation of CDH1 and Cellular Adhesion Genes in SCLC**

Neuroendocrine tumors have common features, and we leveraged the generation of three different Rb/Trp53-deleted neuroendocrine tumor types that were each accelerated by Crebbp loss to hone in on consistently regulated CREBBP targets. We further refined our identification of core CREBBP-regulated transcripts by suppressing CREBBP expression in preSC cells. Among the 66 genes commonly deregulated with Crebbp suppression across each of these 4 comparisons, we found that tight junction and adhesion genes were strongly enriched (Fig. 3H). We were particularly interested in the reduced expression of Cadh1 upon Crebbp loss, as we initially hypothesized that CREBBP positively controls tumor-suppressor pathways relevant to SCLC, and CDH1 is a tumor-suppressor gene, mutated in gastric, breast, bladder, and other cancers (34–36). Although CDH1 itself is not a target of inactivating mutations in SCLC, loss of CDH1 expression has been implicated in SCLC chemoresistance (37) and in advanced murine SCLC (38). Moreover, downregulation of CDH1, a key regulator of EMT, and upregulation of EMT-associated genes occurs in normal neuroendocrine cell development as ASCL1-positive lung neuroendocrine cells migrate to form neuroendocrine cell bodies (31). Crebbp suppression in SCLC may contribute to epigenetic reactivation of a pathway active in developing neuroendocrine cells, which are likely major cells of origin for SCLC (21). CREBBP suppression resulted in increased expression of EMT markers such as Vimentin and ZEB1 (Fig. 4) with the converse effects observed upon CREBBP reintroduction to CREBBP-null SCLC cells (Fig. 5). It has increasingly become appreciated that functionally important EMT-like programs in cancer are often only partially activated, without overt mesenchymal transformation (39, 40). Importantly, ASCL1 and other neuroendocrine markers were found to be coexpressed with EMT markers in Crebbp-deleted SCLC. These data suggest that CREBBP loss drives partial acquisition of mesenchymal programs, while still maintaining neuroendocrine features. This is consistent with the “classic” SCLC histology observed in the Rb1/Trp53/Crebbp Ad-CGRP Cre mouse model that was similar to Rb1/Trp53 controls.

Our finding that CREBBP inactivation leads to a reduction in CDH1 expression and partial acquisition of mesenchymal features has important clinical relevance. In clinical samples, positive expression of E-Cadherin was significantly associated with a better outcome in samples of patients with SCLC, whereas expression of mesenchymal markers, such as Vimentin and SNAIL1, was associated with a worse outcome (41). Factors upstream of E-Cadherin expression in SCLC have been poorly understood, and we link a major SCLC driver gene as a key regulator of E-Cadherin expression. The full breadth of phenotypes caused by CREBBP loss in SCLC remains to be determined. In a set of 51 SCLC cell lines, expression of E-Cadherin was a top marker of sensitivity to cisplatin (37). It will be critical for future studies to determine whether reduced E-Cadherin upon CREBBP loss contributes to chemoresistance in SCLC. Notably, we found a high rate of liver metastasis in both the Rb1/Trp53/Crebbp and Rb1/Trp53 models without clear differences between these genotypes (Supplementary Fig. S2). Nonetheless, detailed examination of the consequences of Crebbp loss on each step in the metastatic cascade is warranted.

HDAC inhibitors have been employed in clinical trials without success in SCLC, but there have been no attempts to select subsets of patients with SCLC for clinical trial entry. Our study suggests a new avenue to target CREBBP-mutant SCLC, using inhibitors of HDACs as a strategy to restore H3K27Ac and CDH1 expression following CREBBP deletion. Pracinostat, an HDAC inhibitor with excellent pharmacokinetic characteristics, has yielded promising data in AML that has led to its current testing in a phase III clinical trial for this tumor type. Our observations of efficacy in the Rb1/Trp53 autochthonous model support investigation of Pracinostat broadly in SCLC. Moreover, our observations of examples of complete regressions in the context of Crebbp-inactivated SCLC further suggest that targeting this approach to subsets of patients with SCLC most likely to respond would increase potential for clinical success. We focused our study on CREBBP, but mutations in either CREBBP or the related acetyltransferase EP300, both prevalent in human SCLC (1), could be used to stratify patients to treatment in SCLC. Future studies using the Crebbp-deficient mouse model should also test Pracinostat efficacy together with cisplatin–etoposide chemotherapy, the mainstay of first-line SCLC therapy in the clinic. The preclinical mouse model we developed will also be invaluable to test other ideas to specifically target the substantial subset of SCLC that harbors CREBBP inactivation. For example, observed synthetic lethality between CREBBP and EP300 in some contexts could lead to a specific sensitivity of CREBBP-deleted SCLC toward pharmacologic inhibition of EP300 (42, 43).

In summary, we have demonstrated that CREBBP plays a critical role in SCLC tumor suppression. The mouse model of SCLC that we have generated will help delineate mechanisms underlying CREBBP tumor suppression in SCLC and will be an ideal tool to test novel approaches to treating CREBBP-mutant SCLC.

**METHODS**

**Mice**

We thank Drs. Tyler Jacks, Anton Berns, and Jane Johnson for Rb1lox/lox, Trp53lox/lox, and Avid1-CreERT2 strains, respectively. We thank Dr. Jan van Deursen (Mayo Clinic) for providing the Crebbp...
Crebbp

3,000 mm³ when 1.0 Crebbp, CDH1 or Athymic were injected into the flanks of immune-compromised mice (Hsd: × Foxn1nu) purchased from Envigo. The injected mice were monitored by MRI scan (ICON small animal MRI system, Bruker Biospin) to identify mice with lung tumor burden. Respiratory gating was employed, and a total of 15 slices of 1 mm thickness was acquired to cover the entire lung volume. Mice were anesthetized with isoflurane during imaging. Tumor volume per animal was quantified using 3D ImageJ Suite with manual quantification of consecutive axial image slices. Once tumor size met defined criteria (present on 3 consecutive 1-mm slices), mice were treated with saline (control) or Pracinostat for 3 weeks. Pracinostat was provided by MEI Pharma. Note that 10 mg/mL Pracinostat was prepared in 0.5% methylcellulose and 0.1% Tween 80 in sterile water. Pracinostat was given using oral gavage 100 mg/kg for 3 weeks (daily, 5 days per week). Saline was also given for 3 weeks (daily, 5 days per week). MRI was performed to follow tumor volume after treatment start, and weights were monitored daily during the course of treatment.

Histology and Immunohistochemistry

Mouse lungs, lung tumors, pituitary and thyroid tumors were fixed in NBF for 24 hours and then transferred to 70% ethanol before paraffin embedding. Tissue sections (4 µm thick) were stained with H&E or used for immunohistochemistry performed using standard procedures. Anti-PCNA (1:1,000; Rabbit polyclonal, ab18197; Abcam) and anti–phospho histone H3 (1:500; 06-570; EMD Millipore) were used as markers of proliferating cells. Anti–cleaved caspase 3 (1:100; #9661; Cell Signaling Technology) was used as a marker of apoptosis. Immunofluorescence using paraffin sections was performed on preSC allografts with anti-UCHL1 (Sigma; HPA005993) primary antibody and Alexa Fluor 488-conjugated secondary antibody (Invitrogen). For immunofluorescence of mouse SCLC tumors, fresh lung tumor tissues were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, transferred to 30% sucrose at 4°C overnight, and then embedded in Tissue-Tek OCT solution and stored at –80°C. Serial sections (8 µm thick) were cut using a Leica CM1950 cryostat at –20°C. For immunofluorescence staining of cultured adherent cells, cells were first seeded onto 8-well chamber slides (35-6688; Corning). After cells adhered, slides were washed with PBS and fixed in 4% PFA for 10 minutes at room temperature. Immunofluorescence staining was performed with standard procedures. The following primary antibodies were used: anti-CGRP (1:1,000, Rabbit polyclonal; Sigma), anti-CGRP (1:100, Guinea Pig; Peninsula Laboratories), anti–TTF-1 (1:100, Rabbit polyclonal; Cell Signaling Technology), anti–E-CADHERIN (1:200; Rabbit polyclonal; Cell Signaling Technology), anti–CREBBP (1:100; Rabbit polyclonal; Cell Signaling Technology), anti–VIMENTIN (1:200; Rabbit polyclonal; Cell Signaling Technology), and anti–ZEB1 (1:50, Rabbit polyclonal, NB1-P05987; Novus). Dylight 554 Phallolidin was used to stain F-actin (1:200; Cell Signaling Technology). Tissues/cells were dual-labeled using secondary antibodies: Alexa Fluor 488 Goat Anti-Rabbit IgG (A11034; Thermofisher) or Alexa Fluor 594 Goat Anti-Guinea Pig (A11076; Thermofisher). Fluorescence images were obtained using a Zeiss LSM 700 confocal microscope. For preserving fluorescence and nuclear counter staining, antifade reagent with DAPI (Vector Laboratories) was used. H&E and immunostained images were acquired using Nikon Eclipse microscope.

Protein Extraction and Western Blot Analyses

The T-PER Tissue protein extraction reagent (Thermofisher) was used to extract protein from mouse tumor tissues and cultured human and murine cell lines following the manufacturer’s procedure. The immunoblot experiments were performed with standard procedures. The following antibodies were used in this study: anti–CREBBP (7389; Cell Signaling Technology), anti–E-CADHERIN (3195; Cell Signaling Technology), anti–N-CADHERIN (13116; Cell Signaling Technology), anti–ZEB1 (3396; Cell Signaling Technology), anti–SLUG (9585; Cell Signaling Technology), anti–VIMENTIN (5741; Cell Signaling Technology), anti–ASCL1 (556604; BD Biosciences), anti–NEUROD1 (ab60704; Abcam), and anti–β-ACTIN (A3854; Sigma).

Histone Extraction

Total histone proteins were extracted by acid extraction as described previously (45). Briefly, cells were suspended in hypotonic buffer and lysed at 4°C for 30 minutes. Lysates were centrifuged at 10,000 g for 5 minutes, and the supernatant was discarded. The pellets were resuspended in 400 µL of 0.4 N H2SO4 and incubated on a rotator overnight at 4°C. Samples were centrifuged at 10,000 g for 10 minutes at 4°C, and the supernatant was transferred to a new tube. To precipitate histones, 132 µL trichloroacetic acid was added solution mixed by inverting. The mixtures were incubated on ice for 1 hour. Total histones were pelleted by centrifugation at 10,000 g for 10 minutes at 4°C, and washed twice with ice-cold acetone. Histone pellets were dissolved in H2O. Primary antibodies against H3K27ac

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(8173, Cell Signaling Technology) and total Histone H3 (3638, Cell Signaling Technology) were used in the immunoblot experiments.

**Plasmids and Reagents**

The plasmids used in this study are summarized in Supplementary Table S5. The lentivirus vectors pLKO.1-TRC (#10879, Addgene, a gift from David Root) and pLKO.1-neo (#13425, Addgene, a gift from Shelia Stewart, Washington University) were used to knock down mouse Crebbp and human CDH1 genes in SCLC cells, and the shRNA sequences are provided in Supplementary Table S5. The pLenti-puro lentiviral vector (#39481, Addgene, a gift from Jennifer Stow, University of Queensland) into the pLenti-puro vector.

**Cell Lines, Cell Culture, and Generation of Knockout and CRISPR-Mediated Knockout Cells**

Human SCLC cell lines (DMS53 and NCI-H1882) and 293FT cells, obtained from the ATCC, were cultured in normal DMEM media supplemented with 10% FBS and pen/strep. Murine SCLC cell lines and the human PDX LU505 SCLC model were cultured in DMEM supplemented with 15% FBS, 1 mmol/L sodium pyruvate, 100 μg/mL penicillin, and streptomycin. The LU505 cells were extracted from an SCLC PDO model generated and utilized as previously described (46). preSC cells were cultured in RPMI-1640 media supplemented with 10% FBS and pen/strep. No Mycoplasma testing was performed. The HDAC inhibitor Pracinostat (SAF2000, Sigma-Aldrich) and Puromycin (A6500, Molecular Probes) were used to select stably transduced cells.

**Chromatin Immunoprecipitation and ChIP-Seq Library Preparation and Sequencing**

ChIP experiments in preSC cells were performed using the ChIP-IT High Sensitivity Kit (3040, Active Motif) following the manufacturer’s manual with some modifications. Briefly, 20 million cells were fixed using the cell-fixative solution provided by the kit followed by nuclear preparation and sonication (Covaris E220; Covaris) to obtain short-fragment chromatin (~200 bp). H3K27ac antibody (5 μg; 39885, Active Motif) was used to lysate of sheared chromatin and incubated at 4°C overnight. Enriched chromatin was collected by Protein G agarose beads and then processed by reverse cross-link and DNA purification. ChIP-seq libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (E7530L; New England Biolabs Inc.) following the manufacturer’s protocol. Illumina HiSeq 2500 system. Reads were mapped to the mm9 genome using TopHat (47), and the fragment per kilobase per million (FPKM) values were generated using Cuffdiff (48). Differentially expressed genes regulated by CREBBP were identified using the edgeR package (29). FPKM values of each sequenced sample were used for GSEA (http://www.broadinstitute.org/gsea/; ref. 27). For all the mouse tumor tissues and matched preSC cell lines, gene set permutation type was used. The Hallmark gene sets in the Molecular Signatures Database collection were selected for each of the analyses (28). Genomic data have been deposited to an appropriate repository with Gene Expression Omnibus accession number GSE117552.

**Nucleic Acid Extraction, RT-qPCR, and RNA-seq Experiments**

Total genomic DNA was isolated from cells, and PCR was performed to amplify fragments containing target areas using specific primer sets described in Supplementary Table S5. The same primers were also used to sequence the PCR fragments. The sequencing results were visualized using Snapgene software. Total RNA from tumors or cultured cells was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. DNA was synthesized using the Protocscript II First Strand cDNA Synthesis Kit (New England Biolabs) or Script reverse transcription supermix for rt-qPCR (Bio-Rad). Quantitative PCR was performed with All-in-One qPCR mix (GenecoTopea). Quantitative expression data were acquired and analyzed with a 7900 Real-time PCR System (Applied Biosystems) using power SYBR Green PCR master mix (Thermo Fisher Scientific) and StepOnePlus System (Applied Biosystems). Primers were designed to detect targeted genes, and the sequences of each primer are provided in Supplementary Table S5. RNA-seq libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (E7530L; New England Biolabs Inc.) following the manufacturer’s protocol. Oligo dT–purified mRNA from 500 ng of total RNA was used as starting material. Libraries were submitted to the genomics core facility at Fred Hutchinson Cancer Research Center. 50-μg single-end sequencing was performed on an Illumina HiSeq 2500 system. Reads were mapped to the mm9 genome using TopHat (47), and the fragment per kilobase per million (FPKM) values were generated using cuffdiff (48). Differentially expressed genes regulated by CREBBP were identified using the edgeR package (29). FPKM values of each sequenced sample were used for GSEA (http://www.broadinstitute.org/gsea/; ref. 27). For all the mouse tumor tissues and matched preSC cell lines, gene set permutation type was used. The Hallmark gene sets in the Molecular Signatures Database collection were selected for each of the analyses (28). Genomic data have been deposited to an appropriate repository with Gene Expression Omnibus accession number GSE117552.

**Cell Proliferation, Colony Formation, and Anchorage-Independent Growth Assays**

Cell viability experiments were performed using the CellTiter-Glo Luminescent Cell Viability Assay following the protocol provided by the manufacturer with some modifications (Promega). Briefly, 100 μL...
of cell suspension was seeded in white-walled 96-well plates. The CellTiter-Glo reagent was added directly to each well at a 1:1 ratio. The plate was then mixed on a plate shaker for 5 minutes and incubated at room temperature for another 5 minutes before being recorded on a plate reader (BioTek). All of the experiments were performed in biological triplicates. For the colony formation assay experiments in Fig. 5C, control and CREBBP-overexpressed NCI-H1882 cells were seeded at 10,000 cells/well in technical triplicates in 6-well plates. Three weeks later, cells were washed twice with PBS, fixed in 4% PFA at room temperature for 10 minutes, and stained with crystal violet (0.05%). Experiments were repeated twice, and 3 representative wells of each condition are shown. For the colony-forming assays in Fig. 6G, control and Cdh1-knockout preSC cells were seeded at 6,000 cells/well in technical triplicates in 6-well plates. Two weeks later, cells were fixed and stained with crystal violet (0.05%). Four fields representing the entire well were counted. Experiments were performed in biological triplicates and fields from representative wells shown. For anchorage-independent growth assays in Fig. 1A, cells were plated in at least triplicate in 6-well plates in 0.5 mL of growth medium containing 0.3% agar and seeded on top of a 0.5 mL base layer of medium containing 0.5% agar. The medium was regularly changed every 3 days for 3 weeks. Colonies were stained with 0.05% crystal violet after fixation in 4% PFA. For experiments in Figs. 5B and 6F, cells were seeded in 0.3% low-melting-point SeaPlaqueTM agarose (Lonza; Catalog no: 50101) on top of 0.6% low-melting-point SeaPlaqueTM agarose layer. Low-melting-point agarose was premixed with DMEM 2X (Fisher Scientific; SLM202B) complemented with 20% FBS, sodium pyruvate (2 mmol/L), 200 U/mL penicillin, and 200 μg/mL streptomycin. Cells were allowed to grow at 37°C with 5% CO2 for 2 to 4 weeks. For each well, colonies from at least 5 random fields were counted (a total of at least 15 fields/condition). Representative microscopic images are displayed.

**Transwell Migration Assay**

Cell migration assay was performed using 8-μm cell culture insert (08-771-21; Falcon). Briefly, 7.5 × 10^4 cells in serum-free DMEM media were seeded per well into the inserts, and 10% FBS DMEM media were used as chemokine added into the plate wells. The inserts were washed with PBS after 17-hour incubation, and cells migrated to the basal side of the membrane were fixed using 4% PFA and then stained with 0.05% crystal violet. For each well, at least 5 random fields were photographed and counted. The experiments were performed using biological triplicates.

**Statistical Analyses**

Statistical analyses and graphical presentation were performed with GraphPad Prism 7.0. The results are presented as the mean ± SD or the mean ± SEM and evaluated using an unpaired Student t test (two-tailed; P < 0.05 was considered to be significant). Kaplan–Meier curves of lung tumor-free survival were generated using Prism 7.0 (log-rank test; P < 0.05 was considered to be significant).
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