Cells Lacking the RB1 Tumor Suppressor Gene Are Hyperdependent on Aurora B Kinase for Survival

Matthew G. Oser1,2,3, Raquel Fonseca1, Abhishek A. Chakraborty1,3, Rachel Brough4,5, Alexander Spektor6,7,8, Rebecca B. Jennings9, Abdallah Flaifel9, Jesse S. Novak9, Aditi Gulati4,5, Elizabeth Buss1,6, Scott T. Younger10, Samuel K. McBrayer1, Glenn S. Cowley10, Dennis M. Bonal11, Quang-De Nguyen11, Laura Brulle-Soumare12, Paula Taylor13, Stefano Cairo12, Colm J. Ryan14, Elizabeth J. Pease15, Kim Maratea16, Jon Travers13, David E. Root10, Sabina Signoretti1,9, David Pellman6,7, Susan Ashton13, Christopher J. Lord4,5, Simon T. Barry17, and William G. Kaelin Jr1,3,6,10

ABSTRACT
Small cell lung cancer (SCLC) accounts for 15% of lung cancers and is almost always linked to inactivating RB1 and TP53 mutations. SCLC frequently responds, albeit briefly, to chemotherapy. The canonical function of the RB1 gene product RB1 is to repress the E2F transcription factor family. RB1 also plays both E2F-dependent and E2F-independent mitotic roles. We performed a synthetic lethal CRISPR/Cas9 screen in an RB1−/− SCLC cell line that conditionally expresses RB1 to identify dependencies that are caused by RB1 loss and discovered that RB1−/− SCLC cell lines are hyperdependent on multiple proteins linked to chromosomal segregation, including Aurora B kinase. Moreover, we show that an Aurora B kinase inhibitor is efficacious in multiple preclinical SCLC models at concentrations that are well tolerated in mice. These results suggest that RB1 loss is a predictive biomarker for sensitivity to Aurora B kinase inhibitors in SCLC and perhaps other RB1−/− cancers.

SIGNIFICANCE: SCLC is rarely associated with actionable protooncogene mutations. We did a CRISPR/Cas9-based screen that showed RB1−/− SCLC are hyperdependent on AURKB, likely because both genes control mitotic fidelity, and confirmed that Aurora B kinase inhibitors are efficacious against RB1−/− SCLC tumors in mice at nontoxic doses.

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).
INTRODUCTION

Small cell lung cancer (SCLC) is a high-grade neuroendocrine cancer that accounts for 15% of lung cancers, with 33,000 new SCLC cases in the United States each year (1). Although 60% to 70% of patients with extensive-stage (i.e., metastatic) SCLC initially respond to conventional chemotherapy (cisplatin and etoposide), these responses are typically short-lived, and median overall survival is only 9 to 11 months (1). There are no approved targeted therapies for SCLC.

Identifying therapeutic targets in SCLC has been challenging, partly because driver mutations in SCLC are primarily loss of function (LOF), typically involving the tumor suppressor genes RB1 and TP53 (2–4). The importance of these two tumor suppressors in SCLC is underscored by genetically engineered mouse studies, where inactivation of Rb1 and Trp53 in the lung causes SCLC (5, 6). Although TP53 is highly mutated in many types of human cancers, RB1 is only frequently mutated in SCLC among adult cancers (2–4, 7).

The canonical function of RB1 is to repress E2F-dependent transcription (8). RB1 operates in a pathway that includes its upstream regulators p16, Cyclin D1, and CDK4, which control RB1 phosphorylation and consequently its ability to repress E2F (9). Many types of cancers have RB1 pathway mutations without a strong bias toward any individual pathway component. In contrast, almost all SCLCs harbor RB1 mutations, whereas CDKN2A (p16), CCND1 (Cyclin D1), and CDK4 mutations are conspicuously rare. This suggests a specific, perhaps E2F-independent, role for RB1 loss in SCLC pathogenesis that is not shared by its upstream regulators or that loss of these upstream regulators is antithetical to SCLC pathogenesis. With regard to the former possibility, a number of E2F-independent functions have been ascribed to RB1, including a role in maintaining mitotic fidelity (10–13).

Synthetic lethality provides a paradigm for targeting cancers that have sustained LOF mutations in tumor suppressor genes. In applying this paradigm, one looks for specific vulnerabilities that are created upon loss of the gene of interest. The classic example of a successful synthetic lethal approach in cancer is the use of PARP inhibitors to target BRCA-deficient tumors (14). BRCA-deficient tumors have defects in homologous recombination and become hyperdependent on...
PARP-dependent collateral DNA-repair mechanisms (15). PARP inhibitors are now approved for treatment of recurrent ovarian cancers and metastatic breast cancers harboring LOF BRCA mutations (16–18). It is unknown whether RB1 loss confers dependencies (i.e., synthetic lethality) in SCLC. Herein, we used an isogenic cell system and CRISPR/Cas9 to identify synthetic lethal targets that result from RB1 loss in SCLC.

RESULTS

RB1 Loss Is Synthetic Lethal with Multiple Genes That Regulate Chromosomal Segregation in SCLC

To identify synthetic lethal interactors with RB1 in SCLC, we first infected two RB1−/− SCLC cell lines (NCI-H82 and NCI-H69) with a lentivirus that expresses RB1 in the presence of doxycycline (DOX-On RB1) or with the corresponding empty vector (DOX-On EV). In all our experiments, virally infected cells were maintained as polyclonal pools under drug selection suitable for the corresponding virus.

As expected (19, 20), RB1, once reintroduced into NCI-H69 cells, was unphosphorylated and suppressed cell proliferation (Fig. 1A–C). In contrast, exogenous RB1 was partially phosphorylated in NCI-H82 cells and did not grossly suppress cell proliferation (Fig. 1A, B, and D). We then lentivirally infected the DOX-On RB1 and DOX-On EV NCI-H82 cells to express Cas9 and confirmed their ability to edit a GFP reporter plasmid within 13 days of receiving a short-guide (sg) GFP (Supplementary Fig. S1A and S1B).

Given that RB1 reexpression had no gross effect on cell proliferation in NCI-H82 cells, we used the DOX-On RB1 NCI-H82 cells to perform a RB1 synthetic lethal screen using CRISPR/Cas9 in the presence of doxycycline (DOX-On RB1) and DOX-On EV cells were grown in the presence of DOX for 28 days and then infected (day 0); multiplicity of infection (MOI) of 0.3) with a lentiviral sgRNA library targeting 1,350 epigenetic, cell-cycle, and cancer-relevant genes (6 sgRNAs per gene; Fig. 1E; Supplementary Table S1). The library also contained 1,000 nontargeting control sgRNAs. Thirty-six days later, the cells were split (50:50) into media that did or did not contain DOX, and passed until day 30. Cell aliquots were removed for genomic DNA (gDNA) isolation at days 13 and 30. RB1 expression was maintained in the cells continuously exposed to DOX (Fig. 1F).

We used deep sequencing of the gDNA samples and the RIGER-E log fold change (LFC) second best scoring algorithm to score the relative depletion or enrichment of the individual sgRNAs in the day 30 samples (±DOX) compared with the corresponding shared day 13 sample. Multiple sgRNAs targeting genes known to interact with RB1 (CDKN2A, E2F8, EID1, and RB1 itself) were enriched over time in the DOX-On RB1 cells (+DOX), but not in the DOX-On EV cells (+DOX; Supplementary Fig. S1C and S1D and Supplementary Tables S2 and S3; see Methods), implying that the RB1 induced by DOX was at least partially active and that our screening methodology was technically sound. The recovery of these well-annotated RB1 interactors (21, 22) suggests that many of the other genes that scored in this assay are likewise required for SCLC suppression by RB1.

Using this strategy, we also identified 104 genes that were synthetic lethal with RB1, based on sgRNA depletion in the RB1-deficient cells (DOX-On RB1; no DOX) compared with the RB1-proficient cells (DOX-On RB1; +DOX), using a P value cutoff of <0.05 (Fig. 1G; Supplementary Tables S2 and S4). Many of the top-scoring genes (P < 0.01; hereafter called “hits”) had 3 or 4 of their 6 sgRNAs in the library score in the top 500 (~top 5%) of differentially depleted sgRNAs (Fig. 1G). These hits were unlikely to be “noise” as very few nontargeting control sgRNAs were similarly depleted, and there were very few hits in the analogous DOX-On EV cell screen (Supplementary Fig. S1E and S1F). We obtained a very similar list of synthetic lethal hits using STARS (23), which is an orthogonal screen analysis algorithm, suggesting that our hit list was robust and independent of the algorithm chosen for analysis (Supplementary Table S4). Interestingly, many of the hits encode regulators of chromosomal segregation that functionally interact, including components of condensin complexes (SMC2, NCAPG, and SMC4) and their upstream regulators (AURKB, PLK1, and INCENP; refs. 24–28; Fig. 1H).

Aurora B Kinase Is Synthetic Lethal with RB1 in NCI-H82 Cells

We focused on AURKB because it was the highest-scoring “druggable” hit. To test whether AURKB was a true positive synthetic lethal hit, we first performed competition experiments with NCI-H82 cells that were infected to express both RB1 and GFP or to express tdTomato alone and then mixed 1:1 (Supplementary Fig. S2A and S2B). Treating this mixture with a lentivirus expressing one of two effective AURKB sgRNAs caused a dramatic outgrowth of the GFP-positive (RB1-proficient) cells compared with the nontargeting sgRNA control (Fig. 2A and B; Supplementary Fig. S2C–S2E). These effects were on-target because growth inhibition of the parental NCI-H82 cells by the AURKB sgRNAs was completely reversed by a DOX-inducible sgRNA-resistant AURKB variant (Fig. 2C and D).

In another variation of this experiment, the Cas9-positive DOX-On RB1 and DOX-On EV NCI-H82 cells were grown in the presence of DOX and infected with a lentivirus expressing an AURKB sgRNA or control sgRNA. Notably, there was progressive enrichment of cells that had failed to edit AURKB among the EV (RB1-deficient) cells compared with the RB1-proficient cells (Fig. 2E).

In a complementary set of experiments, the GFP (RB1-proficient) and tdTomato (RB1-deficient) cells were again mixed at approximately 1:1 and then treated with the Aurora B kinase–specific inhibitor AZD2811 (Supplementary Fig. S3A and S3B). Treatment with AZD2811 also caused a dramatic enrichment of GFP-positive (RB1-proficient) cells compared with the cells treated with DMSO (Fig. 3A and B). Furthermore, AZD2811 did not cause progressive enrichment of GFP-positive cells when neither the GFP nor tdTomato cells expressed RB1 (Supplementary Fig. S3C), and other antimitotic drugs including paclitaxel, vincristine, the PLK1 inhibitor BI 6727, and the Mps1 inhibitor CFI-402257 did not phenocopy AZD2811 (Fig. 3C; Supplementary Fig. S3D, S3E, S3I–S3L). The targeted sgRNA library we used for our screen did not contain sgRNAs targeting the Aurora B kinase paralog Aurora A kinase. The Aurora A kinase–specific inhibitor MK-S108 (ref. 29; Supplementary Fig. S3B and S3M) selectively depleted RB1−/− cells in both short-term assays and long-term mixing assays (Supplementary Fig. S3N and S3O), although in competition experiments, its effects were less pronounced than the Aurora B kinase inhibitor AZD2811 (compare Fig. 3B with Supplementary Fig. S3N).
Figure 1. RB1 is synthetic lethal with multiple genes that regulate chromosomal segregation. A, Immunoblot analysis of NCI-H69 and NCI-H82 RB1 SCLC lines that were infected with a DOX-On RB1 or DOX-On EV and then grown in the presence or absence of DOX, as indicated, for 48 hours. NCI-H82 (left) and NCI-H82 (right) cells grown as in A. Cell extracts were then treated with λ phosphatase in the presence or absence of a phosphatase inhibitor as indicated. C and D, Cell proliferation of NCI-H69 (C) and NCI-H82 (D) cells grown as in A. The values for each cell line were normalized to a day 0 value of 1. Where indicated, DOX was added on day 0. ** P < 0.01. E, Schema for the synthetic lethal CRISPR screen. An identical screen was performed with NCI-H82 cells infected with the DOX-On EV as a control. n = 3 biological replicates. F, Immunoblot analysis of NCI-H82 cells subjected to the protocol depicted in E. Note RB1 reexpression at day 13 before randomization to DOX or NO DOX and persistent RB1 expression at day 30 for DOX-On RB1 cells maintained in G. Top 21 synthetic lethal genes as determined by RIGER-E-LFC second best analysis followed by sorting based on the number of sgRNAs that scored in the top 500 of all sgRNAs (0.100) in the library. H, Protein-protein interaction network analysis (http://string-db.org/) of statistically significant synthetic lethal hits linked to chromosomal segregation.
Genetic inactivation of AURKB is synthetic lethal with RB1 loss in NCI-H82 cells. A, FACS analysis of NCI-H82 cells infected to produce both RB1 and GFP (infected with RB1-IRES-GFP lentivirus) or to produce tdTomato (infected with EV-IRES-tdTomato lentivirus) after being mixed at a 1:1 ratio and infected with a lentivirus expressing Cas9 and the indicated sgRNA. B, Quantification of the GFP:tdTomato ratio at the indicated time points of cells treated as in A n = 5 biological replicates. *, P < 0.05 of sgAURKB #2 at day 21 compared with sgControl. C, Immunoblot analysis, and D, cellular proliferation assays [based on cell count (fold change relative to day 0)] of NCI-H82 cells that were infected with a DOX-inducible sgRNA-resistant Aurora B kinase cDNA and superinfected with the indicated sgRNAs. The cells were selected in the presence of DOX (see Methods) to maintain expression of the sgRNA-resistant Aurora B kinase and then grown in the presence or absence of DOX to either maintain or withdraw expression of exogenous Aurora B kinase for 5 days, n = 4 biological replicates. ***, P < 0.01; ****, P < 0.001. E, gray arrow shows exogenous Aurora B kinase, and black arrow shows endogenous Aurora B kinase. F, Immunoblot analysis of NCI-H82 cells expressing Cas9 that were superinfected with DOX-On RB1 or DOX-On EV (see Supplementary Fig. S1A), grown in the presence of DOX, and then infected with a lentivirus expressing the indicated sgRNA. Cell extracts were harvested at the times indicated after introducing the sgRNAs.

To ask if the effects of AZD2811 were on target, we exploited the fact that the biochemical and cellular IC50 values for AZD2811 were lower in −/− SCLC cells than in −/− NSCLC cells, which likely contributes to the increased sensitivity of the former to AZD2811 in cellular fitness assays (Supplementary Fig. S4E and S4F). Nonetheless, 125 nmol/L AZD2811 caused nearly complete inhibition of Aurora B kinase in NCI-H1975 and PC-9 cells without suppressing their proliferation (Fig. 4B and Supplementary Fig. S4F), suggesting that pharmacodynamic differences do not fully account for the striking differential sensitivity of these cell lines to AZD2811.

**RB1 Is Synthetic Lethal with Aurora B Kinase in Other SCLC, NSCLC, and Breast Cancer Cell Lines**

The ability of NCI-H82 cells to proliferate after restoration of RB1 function raised the question of whether RB1 and Aurora B kinase would be synthetic lethal in other cellular contexts. We therefore asked whether inhibition of AURKB is synthetic lethal with RB1 loss in other SCLC, non-small cell lung cancer (NSCLC), and breast cancer cell lines. Consistent with our findings in NCI-H82 cells, 3 different RB1−/− SCLC lines (NCI-H69, NCI-H82, and GLC16) were highly sensitive to AZD2811 (IC50 < 50 nmol/L) compared with 3 different RB1+/− NSCLC lines (NCI-H1650, NCI-1975, and PC-9; Fig. 4A and B). This differential sensitivity was not due to gross differences in the cell-cycle distribution among the 6 cell lines (Supplementary Fig. S4A and S4D). The pharmacodynamic IC50 values for AZD2811 were lower in RB1−/− SCLC cells than in RB1+/− NSCLC cells, which likely contributes to the increased sensitivity of the former to AZD2811 in cellular fitness assays (Supplementary Fig. S4E and S4F). Nonetheless, 125 nmol/L AZD2811 caused nearly complete inhibition of Aurora B kinase in NCI-H1975 and PC-9 cells without suppressing their proliferation (Fig. 4B and Supplementary Fig. S4F), suggesting that pharmacodynamic differences do not fully account for the striking differential sensitivity of these cell lines to AZD2811.

c-MYC amplification has been correlated with sensitivity of SCLC cell lines to Aurora B kinase inhibitors, and the NCI-H82 cells we used in our screen are c-MYC amplified (31–34). To ask whether c-MYC status was a confounder in our studies, we identified multiple RB1−/− cancer cell lines, including NCI-H1876 SCLC
**Figure 3.** Pharmacologic inhibition of Aurora B kinase is synthetic lethal with RB1 loss in NCI-H82 cells. A, FACS analysis of NCI-H82 cells infected to produce both RB1 and GFP or to produce tdTomato after being mixed at a 1:1 ratio and treated with 16 nmol/L AZD2811 or DMSO for the indicated number of days. B, Quantification of the GFP:tdTomato ratio on day 8 of NCI-H82 cells as in A, n = 5 biological replicates. ***, P < 0.01. C, Quantitation of the GFP:tdTomato ratio on day 8 of NCI-H82 cells as in A treated with either 16 nmol/L AZD2811, 8 nmol/L paclitaxel, 2 nmol/L vincristine, or DMSO. n = 3 biological replicates. *, P < 0.05. D, Alignment of human Aurora B kinase residues 156–168 with the corresponding region of Aurora A kinase. E161 in Aurora B kinase (blue) forms a hydrogen bond with AZD2811 and is not conserved in Aurora A kinase. A nearby residue R159 in Aurora B kinase (blue) is also not conserved in Aurora A (30). A nearby residue R159 in Aurora B kinase (blue) forms a hydrogen bond with AZD2811 and is not conserved in Aurora A kinase. Given the high selectivity of AZD2811 for Aurora B kinase relative to Aurora A kinase, we mutated both of these residues in Aurora B kinase to the residues found in Aurora A kinase and made an AZD2811 drug-resistant Aurora B kinase mutant (R159L; E161T). E and F, Immunoblot analysis of NCI-H82 infected with a lentivirus that constitutively expresses Aurora B kinase (WT), Aurora B kinase (R159L; E161T), or the empty vector. In F, the cells were treated with the indicated concentrations of AZD2811 for 24 hours. G, Growth inhibition (%) based on viable cell numbers relative to untreated (DMSO) controls. H, Quantification of the percentage of cells with polyploidy, as determined by FACS of propidium iodide–stained cells that were treated with AZD2811 for 72 hours, as in E, n = 3 biological replicates. *, P < 0.05; ***, P < 0.01.

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We then asked whether RB1 status caused the differences in sensitivity to AZD2811 between RB1−/+ SCLC lines and RB1+/+ NSCLC lines. Stable reintroduction of RB1 into NCI-H82 and GLC16 RB1−/− SCLC cells conferred partial resistance to AZD2811 (Fig. 4C and D; Supplementary Fig. S4G and S4H), whereas inactivating RB1 using CRISPR/Cas9 in NCI-H1975 and PC-9 NSCLC cells had the opposite effect (Fig. 4E and F; Supplementary Fig. S4B and S4C). Furthermore, these differences were not caused by increases in c-MYC expression as a result of RB1 loss (Supplementary Fig. SSC and SSD). As an orthogonal approach, we used CRISPR/Cas9 to generate NCI-H1975 cells deleted for RB1 alone, AURKB alone, or both (Fig. 4G). In keeping with our pharmacologic results, RB1 inactivation enhanced the antiproliferative effects of deleting AURKB (Fig. 4H).
Figure 4. RB1 is synthetic lethal with AURKB in multiple SCLC and NSCLC cell lines. A, Immunoblot analysis of the indicated 3 RB1−/− SCLC cell lines and 3 EGFR-mutant RB1+/+ NSCLC cell lines. B, Growth inhibition (%), based on viable cell numbers relative to untreated controls, of the indicated cell lines treated with AZD2811 for 72 hours. n = 3 biological replicates. C, Immunoblot analysis of NCI-H82 cells infected with a lentivirus that constitutively expresses RB1 or EV. D, Growth inhibition (%), based on viable cell numbers relative to untreated (DMSO) controls, of the cells in C, treated with AZD2811 for 48 hours. n = 3 biological replicates. *, P < 0.05. E, Immunoblot analysis of NCI-H1975 RB1+/+ cells infected with a lentivirus that expresses Cas9 and an sgRNA targeting RB1 (sgRB1) or a nontargeting sgRNA (sgControl). F, Growth inhibition (%), based on viable cell numbers relative to untreated (DMSO) controls, of the cells in E, treated with AZD2811 for 48 hours. n = 4 biological replicates. *, P < 0.05. G, Immunoblot analysis of NCI-H1975 RB1+/+ cells that were first infected with a lentivirus that expresses Cas9 and an sgRNA targeting AURKB (sgAURKB) or a nontargeting sgRNA (sgControl) and then superinfected with an sgRNA targeting AURKB (sgAURKB) or a nontargeting sgRNA (sgControl) as indicated. H, Growth inhibition (%), based on viable cell numbers relative to sgControl cells in G, after growth in culture for 48 hours. n = 2 biological replicates. *, P < 0.05.
RB1-deficient breast cancer cell lines were likewise more sensitive than RB1+/− breast cancer lines to genetic or pharmacologic inhibition of Aurora B kinase (Supplementary Fig. S6A–S6F). Furthermore, inactivating RB1 in RB1+/− breast cancer cell lines with an sgRNA or an shRNA increased their sensitivity to various Aurora kinase inhibitors (Supplementary Fig. S6G–S6J). Therefore, RB1 and AURKB display a synthetic lethal relationship in multiple cancer cell lines of different lineages.

**RB1 Loss Exacerbates Mitotic Abnormalities Caused by Aurora B Kinase Inhibition**

Aurora B kinase inhibition causes polyploidy and apoptosis in many cancer cell lines (35). Two of the three RB1+/− SCLC cell lines (NCI-H82 and GLC16) became polyploid in the presence of low concentrations of AZD2811, whereas the third SCLC cell line (NCI-H69) arrested with 4N DNA content (Supplementary Fig. S7A), and all underwent time-dependent apoptosis at low concentrations of AZD2811 (Supplementary Fig. S7C). In contrast, these concentrations of AZD2811 did not affect the cell-cycle distribution of the RB1+/− NSCLC lines, and a 4-fold higher concentration of AZD2811 caused only modest effects (Supplementary Fig. S7B).

Strikingly, RB1 reexpression in the DOX-On RB1 NCI-H82 cells suppressed the polyploidy caused by AZD2811 (Fig. 5A and B, Supplementary Fig. S7G) and decreased AZD2811-induced apoptosis (Fig. 5C and D). This was not because RB1 restored Aurora B kinase activity in the face of AZD2811 (Supplementary Fig. S7D and S7G), prevented cells from entering mitosis (Supplementary Fig. S7E and S7G), or increased expression of Aurora B or its paralog Aurora A (Supplementary Fig. S7F). Furthermore, the AZD2811-resistant Aurora B kinase (R159L; E161T) suppressed AZD2811-induced polyploidy to a comparable degree in both RB1-deficient (−DOX) and RB1-proficient (+DOX) cells (Supplementary Fig. S7H). Collectively, these results argue that the ability of RB1 to suppress the AZD2811-induced polyploidy caused by AZD2811 is not because RB1 alters the ability of AZD2811 to inhibit Aurora B kinase or due to gross alterations in the fraction of cells in M-phase.

RB1 loss in nontransformed retinal pigment epithelial (RPE-1) cells causes chromosomal missegregation and aneuploidy (12, 13). We next infected RPE-1 cells that (i) stably express GFP-H2B to facilitate imaging of chromosomes and (ii) express Cas9 in the presence of DOX (see Methods) with a lentivirus expressing an RB1 sgRNA or a control sgRNA (Fig. 5E). We then performed live-cell imaging of RB1 sgRNA-infected cells that were grown in the presence (RB1-deficient) or absence (RB1-proficient) of DOX, synchronized in late G2–M using the CDK1 inhibitor R0–3306 (36), and then released into AZD2811 or DMSO. AZD2811 caused mitotic abnormalities in both RB1-proficient and RB1-deficient cells, as most daughter cell nuclei in both conditions were abnormally shaped (Fig. 5F). However, significantly more RB1-deficient cells treated with AZD2811 failed to enter an identifiable anaphase and instead underwent mitotic slippage and induced p53 (Fig. 5G–I).

**RB1 Status Affects Multiple Genes Involved in the Mitotic Spindle and Chromosomal Segregation in SCLC**

Given that RB1 loss exacerbates the mitotic abnormalities caused by Aurora B kinase inhibition (Fig. 5), we next asked whether RB1 status affects, directly or indirectly, genes linked to mitosis by performing RNA sequencing (RNA-seq) with DOX-On RB1 NCI-H82 cells that were grown in the presence or absence of DOX for 96 hours (Supplementary Table S5). Despite the partial phosphorylation of exogenous RB1 (and absence of an overt cell-cycle block) after restoring RB1 expression in these cells, gene set enrichment analysis (GSEA) identified multiple RB1-regulated gene sets in the DOX-treated cells, including signature E2F target genes (Fig. 6A), consistent with our sgRNA enrichment data described above (Supplementary Fig. S1C). Interestingly, 7 of the 10 most upregulated gene sets in the RB1-deficient cells compared with RB1-proficient cells are linked to mitotic fidelity and chromosome segregation (Fig. 6B and C; Supplementary Table S5).

To explore the convergent effects of RB1 and Aurora B kinase on mitosis further, we again used RNA-seq followed by GSEA to assess transcriptional changes, whether direct or indirect, in DOX-On RB1 NCI-H82 cells that were grown in the presence or absence of DOX and then treated with AZD2811 or DMSO (Fig. 6D; Supplementary Table S5). AZD2811 induced statistically significant changes in 29 gene sets in RB1-deficient cells. Remarkably, only 3 of these gene sets remained statistically significant in RB1-proficient cells, and AZD2811 did not statistically alter any gene sets in RB1-proficient cells that it did not also affect in the RB1-deficient cells (Fig. 6D, Supplementary Table S5). The 26 gene sets that were selectively altered by AZD2811 in RB1-deficient cells included gene sets linked to the G2–M checkpoint and the mitotic spindle (Fig. 6E; Supplementary Table S5). These data show that RB1 and Aurora B kinase have partially redundant roles with respect to the control of mitosis in SCLC, possibly explaining their synthetic lethal relationship.

**Aurora B Kinase Inhibition Has Efficacy in Mouse Models of RB1-Deficient Neuroendocrine Cancers In Vivo**

Next, we treated several RB1+/− SCLC cell line xenograft models with the AZD2811 prodrug AZD1152 (37) or with AZD2811 encapsulated in a nanoparticle (AZD2811 NP; ref. 38). AZD2811 NP displays improved pharmacokinetics and efficacy in mouse models compared with AZD1152 (38). Both AZD1152 and AZD2811 NP were highly active against NCI-H82, NCI-H69, NCI-H417a, and NCI-1048 xenografts (Fig. 7A–D; Supplementary Fig. S8A and S8B) without causing overt toxicity (Supplementary Fig. S8C–S8H). Treatment of NCI-H417a xenografts with AZD2811 NP decreased phospho-histone H3 levels, consistent with inhibition of Aurora B kinase activity in vivo, and increased polyploidy and apoptosis (Supplementary Fig. S9A–S9D). Importantly, NCI-H82 xenografts expressing Aurora B R159L; E161T, but not WT Aurora B, were resistant to AZD2811 NP (Supplementary Fig. S9E–S9G), demonstrating that AZD2811 NP’s antitumor effects in vivo were due specifically to Aurora B kinase inhibition.

AZD2811 NP, using a variety of doses and treatment schedules, was also highly active in the RB1+/− SC61 SCLC patient-derived xenograft (PDX) model (Fig. 7E–G; Supplementary Figs. S8I and S10A–S10H; Supplementary Table S6) and, in 8 of 10 cases, caused sustained complete remissions after only two doses of AZD2811 NP (25 mg/kg on days 1 and 3; Fig. 7G). AZD2811 NP and AZD1152 also
slowed the growth of RB1−/− SCLC SC74 PDX tumors, but were ineffective in the RB1−/− SCLC SC6 PDX model (Fig. 7H and I; Supplementary Figs. S8 and S10A–H; Supplementary Table S6). Finally, AZD1152 was active against autophthous pituitary and thyroid neuroendocrine tumors arising in Rb1−/− mice (Fig. 7J–L; Supplementary Fig. S8K–S8M; ref. 39). Together, these data show that Aurora B kinase inhibition has efficacy against RB1−/− SCLC cell line xenografts, RB1−/− SCLC PDXs, and autophthous Rb1−/− neuroendocrine tumors.

**DISCUSSION**

Our screen unexpectedly revealed differential dependence of RB1-deficient and RB1-proficient cells on genes, such as AURKB and PLK1, that are essential during embryogenesis and usually considered essential in somatic cells (40, 41). This might have been due to CRISPR/Cas9’s ability to generate hypomorphic alleles, although the AURKB sgRNAs that scored in our primary screen targeted functionally important Aurora B subdomains and also profoundly lowered Aurora
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Figure 6. RB1 status affects multiple genes involved in the mitotic spindle and chromosomal segregation in SCLC. A and B, GSEA using the hallmark gene sets of E2F targets (A) and mitotic spindle genes (B) from an RNA-seq experiment performed in DOX-On RB1 NCI-H82 RB1+/− SCLC cells that were grown in the presence (RB1+) or absence (RB1−) of DOX for 96 hours. The accompanying heat maps show the top 10 enriched mRNAs (red) in the respective gene sets in RB1-deficient cells compared with the RB1-proficient cells. C, Top biological processes from Gene Ontology (GO) GSEA enriched in RB1-deficient cells (RB1−) compared with RB1-proficient cells (RB1+) from an RNA-seq experiment performed in DOX-On RB1 NCI-H82. RB1−/− SCLC cells that were grown in the presence (RB1+) or absence (RB1−) of DOX for 96 hours. The top 10 statistically significant GO gene sets are shown. Gene sets linked to chromosomal segregation are highlighted in red. For A–C, n = 2 biological replicates. D, Schema for the RNA-seq/GSEA experiment to identify gene sets regulated by AZD2811 in DOX-On NCI-H82 RB1−/− SCLC grown in the presence (RB1+, yellow) or absence (RB1−, blue) of DOX for 48 hours and then treated with AZD2811 (32 nM/L) for an additional 48 hours. Twenty-nine hallmark gene sets were altered by AZD2811 in RB1− cells, whereas only 3 hallmark gene sets were altered in RB1+ cells. E, GSEA showing behavior of two gene sets linked to mitosis (mitotic spindle and G2-M checkpoint) after treatment with AZD2811 in RB1− compared with RB1+ cells are shown. n = 2 biological replicates.

B kinase protein levels (Supplementary Fig. S1G). Another factor is that our screen measured relative dependence rather than absolute dependence. The AURKB sgRNAs were lost over time in the RB1-proficient NCI-H82 cells, but were lost more rapidly in the RB1-deficient cells. Thus, whereas RB1-proficient cells sustain a loss of fitness upon loss of Aurora B, the effect is far more pronounced in RB1-deficient cells. This translates into a quantitative difference in sensitivity to pharmacologic inhibition of Aurora B.

Our screen was conducted with a focused sgRNA library and can now be expanded to other libraries. In this regard, our library did not target TSC2 and SKP2, which have been reported to be synthetic lethal with RB1 (42, 43), and did not target the AURKB paralog AURKA, which is also synthetic lethal with RB1 (see Gong and colleagues in this issue, and Supplementary Fig. S3M–S3O). Another limitation of our screen was conducted with a focused sgRNA library (44). RB1 also reported to be synthetic lethal with RB1 (42, 43), and did not target TSC2 and SKP2, which have been reported to be synthetic lethal with RB1 (42, 43), and did not target the AURKB paralog AURKA, which is also synthetic lethal with RB1 (see Gong and colleagues in this issue, and Supplementary Fig. S3M–S3O). Another limitation of our study is that the partial phosphorylation of the exogenous RB1 in NCI-H82 cells might have masked certain differential dependencies.

RB1 represses the transcription of E2F-responsive mitotic genes such as MAD2, CENPE, and HEC1 (44). RB1 also directly promotes chromosomal condensation and cohesion.
Figure 7. Aurora B kinase inhibitors are efficacious in mouse models of RB1−/− neuroendocrine cancers in vivo. A, Caliper-determined tumor volumes of RB1−/− NCI-H82 xenografts grown subcutaneously in NCr nude mice treated with AZD1152 (25 mg/kg/day given by i.p. injection on days 4, 8–11, 15–18, and 22–25) or vehicle (30 mmol/L Tris pH 9). Treatment was initiated when tumors were visible by eye (~20 mm³, day 1); n = 20 tumors (AZD1152) and 20 tumors (vehicle). QWK, every week. B–D, Caliper-determined tumor volumes of RB1−/− (B) NCI-H69, (C) NCI-H417a, (D) NCI-H1048 xenografts grown subcutaneously in athymic nude mice treated with AZD2811 NP administered by tail-vein injection at the doses indicated or vehicle (0.9% saline for NCI-H69 and NCI-1048, unloaded NP for NCI-H417a). Treatment was initiated when tumors were ~100 mm³. For B, n = 10 tumors per arm. For C, n = 12 tumors (vehicle), 6 tumors (AZD2811 NP 25 mg/kg), and 6 tumors (50 mg/kg). For D, n = 10 tumors per arm. E–G, Caliper-determined tumor volumes of RB1−/− (E–G) SC61, (H) SC74, (I) SC6 PDx grown subcutaneously in athymic nude mice with treated with AZD2811 NP or AZD1152 administered by tail-vein injection at the doses indicated or vehicle (unloaded NP). Treatment was initiated when tumors were ~200 mm³. In G, treatment was a single cycle (2 doses for AZD2811 NP or 4-day osmotic mini-pump for AZD1152), and mice were monitored for 112 days. Eight of 10 mice treated with AZD2811 NP showed no regrowth of tumors during this time. For E, H, n = 20 tumors per arm. For F, n = at least 12 tumors per arm. For G, n = 10 tumors per arm. For A–4, upward arrows on the x-axis denote treatment days. For A–H, P < 0.05, **, P < 0.01; ***, P < 0.001, where indicated comparing AZD1152 or AZD2811 NP to vehicle. For I, P < 0.001 for both AZD1152 and AZD2811 NP for days 4, 8, and 11. J, Representative MRIs of pituitary tumors arising in RB1−/− mice that were treated with AZD1152 (25 mg/kg/day given by i.p. injection given 4 days a week for 8 weeks) or vehicle (30 mmol/L Tris pH 9) at the indicated number of days after initial treatment. Arrows show tumors. GEMM, genetically engineered mouse model. K, The percentage of increase in size of tumors (thyroid and pituitary) arising in RB1−/− mice after treatment for 8 weeks with AZD1152 or vehicle. n = 8 tumors (AZD1152) or 6 tumors (vehicle). *P < 0.01. L, Kaplan–Meier survival analysis of RB1−/− mice bearing pituitary or thyroid tumors treated with AZD1152 or vehicle for 8 weeks. n = 4 mice (AZD1152) and 5 mice (vehicle). *P < 0.05.
and thereby affects chromosomal segregation (10–13, 45, 46). For example, RB1 regulates condensin II localization to chromosomes by binding to CAP-D3 (11), a regulatory subunit of the condensin II complex. RB1 also binds to and promotes the activity of the H4K20 methyltransferases Suv4-20h1 and Suv4-20h2 (10), which are necessary for binding of cohesion to chromosomes (13). We documented transcriptional deregulation of multiple mitotic genes in RB1-deficient cells, which was exacerbated further by loss of Aurora B kinase activity. Whether these transcriptional changes are driven by E2F or are an indirect consequence of RB1’s biochemical functions during mitosis described above requires further study. Nonetheless, the fact that multiple mitotic genes scored as synthetic lethal with RB1 underscores the physiologic relevance of RB1’s control of mitotic fidelity.

The RB1 tumor suppressor pathway includes p16, Cyclin D1, and CDK4, which control RB1 phosphorylation and its ability to repress E2F (9). Many types of cancers have RB1 pathway mutations without a strong bias toward any individual pathway component. In stark contrast, SCLC’s stereotypically mutate RB1 (2–4). RB1’s mitotic functions are at least partially CDK-resistant, and we found that inactivating RB1 in p16−/− NSCLC cells (ref. 47; Supplementary Fig. S4K) made partially CDK-resistant, and we found that inactivating RB1 in p16−/− NSCLC cells (ref. 47; Supplementary Fig. S4K) made them hyperdependent on Aurora B. Perhaps RB1 loss causes greater E2F derepression than upstream RB1 pathway mutations, and E2F activity must exceed a certain threshold for SCLC pathogenesis and Aurora B kinase hyperdependence. Alternatively, RB1’s CDK-resistant mitotic functions might suppress SCLC and dependence on Aurora B.

We demonstrated Aurora B hyperdependence in multiple RB1-deficient cell types. It remains possible, however, that the genetic interaction between RB1 and AURKB is influenced by other SCLC driver mutations. For example, c-MYC and Aurora B have a synthetic lethal relationship (34), and c-MYC amplification in SCLC correlates with increased Aurora B dependence (31–33). Many SCLC tumors and cell lines, including NCI-H82 cells, are c-MYC amplified. However, we observed that RB1−/− cancer cells that did not overexpress c-MYC were still highly sensitive to Aurora B kinase inhibitors. Furthermore, AZD2811 NP was highly active in the SC61 SCLC PDX model, which is c-MYC unamplified (Supplementary Fig. S4K). AZD2811 NP was also active in the SC61 SCLC PDX model, which is c-MYC unamplified (Supplementary Fig. S4K). We demonstrated Aurora B hyperdependence in multiple RB1-deficient cell types. However, the genetic interaction between RB1 and AURKB is influenced by other SCLC driver mutations. For example, c-MYC and Aurora B have a synthetic lethal relationship (34), and c-MYC amplification in SCLC correlates with increased Aurora B dependence (31–33). Many SCLC tumors and cell lines, including NCI-H82 cells, are c-MYC amplified. However, we observed that RB1−/− cancer cells that did not overexpress c-MYC were still highly sensitive to Aurora B kinase inhibitors. Furthermore, AZD2811 NP was highly active in the SC61 SCLC PDX model, which is c-MYC unamplified, and had virtually no effect on the SC6 SCLC PDX model, which is c-MYC amplified (Supplementary Fig. S10A–S10H; Supplementary Table S6). Likewise, TP53 mutations, which are nearly universal in SCLC, increase Aurora kinase dependence in colorectal cancer cells (35). Clearly, additional work will be needed to understand how genetic context influences the dependence of RB1−/− SCLC cells on Aurora B, in part to explain the heterogeneous responses of RB1−/− SCLC tumors we observed preclinically and that are likely to be encountered clinically.

Aurora B kinase inhibitors are bone marrow–suppressive and display only modest activity in unselected patients with cancer at their maximal tolerated doses. Nonetheless, a recent all-comers phase I trial of the dual Aurora A/B kinase inhibitor alisertib (35) demonstrated clinical activity in a subset of heavily pretreated SCLCs (20% response rate) and breast cancers (48). Our findings suggest that the therapeutic index for Aurora B kinase inhibitors would be higher in patients with RB1−/− tumors. In addition to SCLC and pediatric retinoblastomas, RB1 mutations occur in a variety of cancers, including breast cancers, bladder cancers, prostate cancers, sarcomas, and are also emerging as a cause of acquired resistance to targeted agents such as androgen receptor antagonists, EGFR antagonists, and CDK4/6 inhibitors. Another way to improve their therapeutic index would be to optimize their biodistribution, such as is being tried with the AZD2811 NP (38).

METHODS

Cell Lines and Cell Culture

WERI-Rb-1 (obtained in June 2016), NCI-H1417 (June 2017), NCI-H1876 (November 2016), 293FT, and hTERT-RPE1 (January 2014) were all early-passage stocks on NCI-H69, NCI-H82, NCI-H1650, and NCI-H1975 cells by Genetica DNA Laboratories in September 2014 and was found to match the specifications listed in ATCC. NCI-H69, NCI-H82, GLC16, NCI-H1650, NCI-H1975, WERI-Rb-1, NCI-H1417, and PC-9 cells were maintained in RPMI-1640 media. 293FT, MDA-MB-436, and MDA-MB-468 cells were maintained in DMEM media. hTERT-RPE1 and NCI-H1876 cells were maintained in DMEM/F12 media. All media were supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin except for NCI-H1876 cells, where the media were supplemented with 5% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, and HITES [10 mmol/L hydrocortisone, insulin–transferrin–selenium (Sigma), and 10 mmol/L beta-estradiol]. Doubling times for cell lines in Fig. 4A are as follows: NCI-H69 (68 hours), NCI-H82 (26 hours), GLC16 (35 hours), NCI-H1650 (35 hours), NCI-H1975 (41 hours), and PC-9 (39 hours). MCF10A TP53−/− cells were purchased from Horizon Discovery and maintained in DMEM/F12 media supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 µg/mL insulin. All cell lines were maintained in 5% CO2 at 37°C. All cell lines when initially obtained were tested for Mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza; #LT07-418) and were negative. Early-passage cells of all parental cell lines listed above were frozen using Bambanker’s freezing media (Bulldog Bio). Cells were then maintained in culture for <4 months, at which point a new early-passage vial was thawed. Where indicated, the following chemicals (stored at −20°C) were also added to the media as indicated in the text: DOX (stock 1 mg/mL in H2O), AZD2811 (formerly AZD1152-HQPA; Selleck; cat no. #S1147, stock 1 mmol/L in DMSO), paclitaxel (Selleck; #S1150, stock 10 mmol/L in DMSO), vincristine (Selleck; #S1241, stock 10 mmol/L in DMSO), MK-5108 (Selleck; #S2770, stock 10 mmol/L in DMSO), BI 6727 (Selleck; #S2235, stock 5 mmol/L in DMSO), CFI-402257 (MedChem Express; #HY-101340, stock 5 mmol/L in DMSO), aphidicolin (Sigma; #A0781, stock 3 mmol/L in DMSO), nocodazole (Sigma; #M1404, stock 5 mmol/L in DMSO), and RO-3306 (Sigma; #SM5659, stock 10 mmol/L in DMSO).

Lentivirus and Retrovirus Production

Lentiviruses were made by Lipofectamine 2000–based cotransfection of 293FT cells with the respective lentiviral expression vectors and the packaging plasmids pPAX2 (Addgene; #12260) and pMD2.G (Addgene; #12259) in a ratio of 2:2:1. Virus-containing supernatant was collected at 48 and 72 hours after transfection,
pooled together (15 mL total per 10-cm tissue culture dish), passed through a 0.45-μm filter, aliquoted, and frozen at −80°C until use.

Retroviruses were made by Lipofectamine 2000-based cotransfection of 293FT with pBABE-H2BGFp plasmid (gift of Fred Dick; Addgene; plasmid #26790) along with the pUMVC and pSV-G packaging plasmids in a ratio of 4:3:1 according to the manufacturer’s instructions.

**Lentiviral and Retrovirus Infection**

Suspension cells were counted using a Vi-Cell XR Cell Counter (Beckman Coulter) and resuspended in 1 mL lentivirus with 5 μg/mL polybrene at the following concentrations in individual wells of a 12-well plate: 1 × 10^6 cells/mL for NCI-H82 cells, or 2 × 10^5 cells/mL for NCI-H69 and GLC16 cells. The plates were then centrifuged at 434 × g for 2 hours at 30°C. Twelve to 16 hours later, the virus was removed and cells were grown for 72 hours before being placed under drug selection.

Adherent cells (100,000 per well) were plated in 6-well plates in 2 mL of media. The following day, 500 μL of lentivirus was added together with 8 μg/mL polybrene, and the plates were centrifuged as above. The lentivirus was removed the next day, and the cells were grown for at least 24 hours before being placed under drug selection. Both suspension and adherent cells were selected by growth in puromycin (1 μg/mL), blasticidin (10 μg/mL), or G418 (800 μg/mL) and maintained in media containing puromycin (1 μg/mL), blasticidin (10 μg/mL), or G418 (400 μg/mL), respectively.

To make the RPE-1 cell line expressing H2B-GFP, RPE-1 cells were infected with retroviruses encoding H2B-GFP for 24 hours in the presence of 10 μg/mL polybrene, washed, and allowed to recover for 24 hours before selection by fluorescence cell sorting.

**Cell Proliferation Assays**

Cells were counted on day 0 using the Vi-Cell XR Cell Counter and plated in 12-well plates at 200,000 cells/mL in 1 mL of media per well for NCI-H69 cells or petri dishes at 32,500 cells/mL in 8 mL of media for NCI-H82 cells. Cell counts were then determined using the Vi-Cell XR Cell Counter every 3 days and normalized to day 0.

**Growth Inhibition Assays**

Cells were plated in RPMI media supplemented with 10% FBS in 6-well plates containing 2 mL of media per well at the following cell densities: NCI-H69 (200,000/mL), NCI-H82 (100,000/mL), GLC16 (200,000/mL), NCI-H1650 (100,000/mL), NCI-H1975 (100,000/mL), PC-9 (100,000/mL), MDA-MB-436 (100,000/mL), MDA-MB-468 (100,000/mL), WERI-Rb-1 (200,000/mL), NCI-H1417 (200,000/mL), and NCI-H1876 (500,000/mL). Viable cell counts at the indicated times were determined using a Vi-Cell XR Cell Counter. The percentage of growth inhibition was calculated as 100 × (1 – the ratio of the treatment sample value/control (DMSO or nontargeting sgRNA) sample value).

**MCF10A Cell Viability Assays**

Five hundred cells per well were seeded into 384-well plates. After 24 hours, cells were exposed to small-molecule inhibitors resuspended in DMSO using an Echo 550 liquid handler (Labcyte). Cells were incubated with the inhibitor for 5 days, after which cell viability was estimated using CellTiter-Glo (Promega). CellTiter-Glo luminescence data were normalized to the signal in DMSO-exposed cells to calculate surviving fractions (SF). GraphPad Prism software was used to convert SF into four parameter logistic regression dose/response curves.

**Lambda Phosphatase Assays**

Cell extracts were prepared in EBC lysis buffer (50 mM Tris Cl pH 7.5, 250 mM NaCl, 0.5% NP-40, 10% glycerol) without phosphatase inhibitors. Protein lystate (40 μg) was treated with λ-phosphatase and/or a phosphatase inhibitor for 1 hour at 30°C using a Lambda Protein Phosphatase kit (NEB #P0753) according to the manufacturer’s instructions. Extracts were then boiled in sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting as described in Supplementary Methods.

**GFP Reporter Assay for Cas9 Activity**

NCI-H82 DOX-On RB1 or EV cells previously infected with Lentivirus-Cas9-2A-Blast were superinfected with a lentivirus (pXPR_011; Addgene; #59702) expressing GFP that also encodes an sgRNA targeting GFP. Puromycin-resistant cells were analyzed by FACs for GFP. The GFP-negative population in the cells transduced with Lentivirus-Cas9-2A-Blast reflects the percentage of cells that successfully CRISPR-edited GFP.

**sgRNA Library Construction**

Gene-targeting sgRNAs and appropriate controls were designed using the rule set described at the GPP portal (http://portals.broadinstitute.org/gpp/public/; ref. 49). Oligonucleotides were flanked by PCR primer sites, and PCR was used to amplify DNA using NEBNext kits. The PCR products were purified using Qiagen PCR cleanup kits and cloned into pXPR_BRD003 using Golden Gate cloning reactions. Pooled libraries were amplified using electrocompetent Stbl4 cells. Viruses were generated as outlined at the GPP portal. The sgRNA library targeted epigenetics regulators, cell-cycle genes, and cancer-relevant genes. It consisted of 9,100 sgRNAs targeting 1,350 genes (6 sgRNAs targeting each gene) and 1,000 nontargeting sgRNAs.

**Isogenic RB1 Synthetic Lethal CRISPR Screen and Analysis**

NCI-H82 cells that had been infected with pTripZ-RB1 (or with the corresponding empty vector) were maintained in G418 and then superinfected with Lentivirus-Cas9-2A-Blast and placed under blasticidin selection. The successfully infected cells were grown in the presence of 0.5 μg/mL DOX, 10 μg/mL blasticidin, and 400 μg/mL G418 for 30 days using tetracycline-negative FBS (Gemini; #100-800). Prior to the initiation of the screen, a pilot experiment was performed to test the proliferation of DOX-On RB1 cells under the conditions that would be used during the screen. Specifically, DOX-On RB1 cells that were grown in the presence of DOX for 30 days were then split 50:50 and grown in the presence (RB1-ON) or absence (RB1-OFF) of DOX, and cell counts were determined every 3 days over the course of 14 days using a Vi-Cell XR Cell Counter. Doubling times calculated over the 14-day proliferation assay were 40.27 hours for the RB1-ON cells and 40.63 hours for RB1-OFF cells. We concluded the proliferation rates of the 2 arms of the screen would be similar and that differences in proliferation were unlikely to confound our results.

On day 0 (day of infection), ∼3 × 10^6 cells (∼3,000 cells.sgRNA) were resuspended in complete media with 10% FBS, 5 μg/mL puromycin at a concentration of 1 × 10^6 cells/mL in 50 mL conical tubes, and the lentiviral sgRNA expression library described above was added at an MOI of 0.3. The cells were then distributed onto low-adherence 6-well plates at a density of 2 × 10^4 cells per well, and the plates were centrifuged at 434 × g for 2 hours. The following morning (day 1), the virus was removed, and the cells were transferred to low-adherence 10-cm plates at a concentration of 0.4 × 10^6 cells/mL and maintained in DOX. On day 3, the cells were transferred to t175 cm non–tissue culture-treated flasks at a concentration of 0.2 × 10^6 cells/mL and maintained in DOX. On day 5, the cells were plated in fresh media in the presence of DOX and selected with 1 μg/mL puromycin for 72 hours. A parallel experiment was performed on day 5 to confirm the MOI. To do this, the cells infected with the sgRNA library and mock-infected cells were plated at 0.2 × 10^6 cells/mL in low-adherence 6-well plates in the presence or absence of puromycin. After 72 hours, cells were counted using the Vi-Cell XR Cell Counter, and the MOI...
was calculated using the following equation: (# of puromycin-resistant cells infected with the sgRNA library/# total cells infected with the sgRNA-infected cells) – (# of puromycin-resistant mock-infected cells/# total mock-infected cells).

After puromycin selection (day 8), puromycin-resistant cells were replated with fresh media and grown in the presence of DOX until day 13 after infection. On day 13, 2 × 10^7 cells were collected, washed in PBS, and frozen for gDNA isolation for the initial time point prior to DOX withdrawal. Cells were then split 1:1 to be grown in the presence (RB1-proficient) or absence (RB1-deficient) of DOX using RPMI supplemented with tetracycline-negative FBS and maintaining 3 × 10^7 cells per condition. Going forward, cells were passaged every 48 hours in the presence or absence of DOX, and a minimum of 3 × 10^7 cells per arm were maintained. At 30 days after infection, 2 × 10^7 cells were collected, washed in PBS, and frozen for gDNA isolation for the final time point. An identical screen was performed with NCI-H82 cells expressing Cas9 and an insertless version of the DOX-On lentivirus (pTriPZ-EV) to control for nonspecific effects of our screening strategy. The screen was performed in 3 biological replicates.

Following completion of the screen, gDNA was isolated using a Qiagen Genomic DNA midi prep kit (cat. # 51185) according to the manufacturer’s protocol. PCR of gDNA and pDNA (sgRNA plasmid pool used to generate virus) was performed as previously described (22). Raw Illumina reads were normalized between samples using Log2[(sgRNA reads/total reads for sample) × 1/e6] + 1. LFC calculations between isogenic pairs were generated for subsequent RIGER-E analysis. The initial time point data (day 13) was then subtracted from the end time point data to determine the relative fold depletion or enrichment of each individual sgRNA after DOX withdrawal. RIGER-E LFC second best algorithm was then used to compare the relative fold depletion or fold enrichment for each 2-class comparison.

For the synthetic lethal comparison in Fig. 1G and Supplementary Table S4, the LFC values for RB1-deficient cells (−DOX) at day 30 were compared with the LFC values for RB1-proficient cells (+DOX) at day 30, and the depletion of sgRNAs was analyzed using the RIGER-E LFC second best algorithm, which ranks the list in order of P value. For the analysis for enrichment of sgRNAs in RB1-proficient cells in Supplementary Fig. S1C and Supplementary Table S3, the LFC values for RB1-proficient cells (+DOX) at day 30 were compared with day 13, and the enrichment of sgRNAs was analyzed using the RIGER-E LFC second best algorithm. Although sgRNAs targeting genes that functionally interact with RB1 (Supplementary Fig. S1C) were enriched over time, the magnitude of the log2 fold enrichment for these sgRNAs was low [59 of 60 sgRNAs of the 10 genes had a log2 fold enrichment <1 over 17 days and only 1 sgRNA of 60 sgRNAs fell 3 standard deviations above the mean (which is 1.14) of the 1,000 nontargeting sgRNAs in the screen]. This result speaks more to the sensitivity of the CRISPR screening technology and demonstrates that our screening technology was able to detect subtle differences in enrichment over time. In particular, our methodology could detect enrichment of sgRNAs that negated RB1’s antiproliferative effects even in a cellular system where RB1 did not cause gross antiproliferative effects (see Figs. 1D, 2B, and 3B).

For both analyses, the list of hits was then further sorted for the number of sgRNAs (out of 6 sgRNAs in total for each gene) that scored in the top 5%. A P value cutoff of <0.05 was used to call hits (please see the Statistical Analysis section in Methods for how P values are calculated in RIGER-E). To determine whether the synthetic lethal hits we identified were independent of the algorithm used for screen analysis, we also used the STARS algorithm (23) to reanalyze the data (Supplementary Table S4). The STARS algorithm generated a hit list that was very similar to the hit list we obtained using the RIGER-E LFC second best algorithm. In fact, AURKB scored as the #1 hit using both the STARS algorithm to analyze median values. The data from all 3 biological replicates were used for all analyses.

The sgRNA library contained 6 sgRNAs targeting AURKB. There were 3 sgRNAs that scored in the top 500 (~top 5%) in the synthetic lethal analysis, which were then labeled as sg1, sg2 or sg3, sg2 and sg3 were used for all validation experiments, and an Aurora B kinase sgRNA-resistant cDNA was able to completely rescue the proliferative defects caused by sg2 and sg3, indicating that their antiproliferative effects were on-target. The other 3 sgRNAs (sg4, sg5, and sg6) dropped out in both the presence or absence of exogenous RB1 and therefore did not score as synthetic lethal. sg1 (CCAAACTTCCTAGCTGTGAGTTACCTGTG) targets AURKB nucleotides 247 to 266 in the catalytic domain and scored at 60 of 9,100 sgRNAs; sg2 (TCTAGAGTATGCCGAGCTCCAGATCATGG) targets nucleotides 459 to 478 in the catalytic domain and scored at 60 of 9,100 sgRNAs; and sg3 (TTGCAGATCATGTG) targets nucleotides 476 to 496 in the catalytic domain and scored at 6,144 of 9,100 sgRNAs.

Pharmacodynamic Studies of AZD2811, MK-5108, BI 6727, and CFI-402257 In Vitro

NCI-H82 cells were plated at 100,000 cells/mL on a petri dish and treated with AZD2811, MK-5108, BI 6727, or CFI-402257 at the indicated concentrations for 24 hours. Histones or soluble extracts were prepared, and immunoblot analysis was performed as described in Supplementary Methods.

FACS-Based Direct Competition Assay

NCI-H82 cells were infected with pLX304-RB1-IRES-GFP or pLX304-EV-GFP or pLX304-EV-tTomato lentiviruses, selected with 10 μg/mL of blasticidin, and then FACS sorted for GFP or tdTomato-positive cells. For the competition assay performed with pharmacologic inhibitors, cells were mixed at 1:1 ratios (pLX304-EV-tTomato:pLX304-RB1-GFP ratio) on day 0 (with exact proportions of cells after mixing determined by repeat FACS analysis), and plated on petri dishes at 100,000 cells/mL in 8 mL of media. Drugs were added to the cells at the concentrations indicated: AZD2811 (16 mmol/L), paclitaxel (8 mmol/L), vincristine (2 mmol/L), or DMSO. The AZD2811, paclitaxel, and vincristine drug concentrations were chosen based on the pharmacodynamic and EC50 assays in Supplementary Fig. S3A, S3B, and S3C. MK-5108, BI 6727, or CFI-402257 were used at the concentrations indicated in Supplementary Fig. S3. Two hundred thousand cells were then harvested every 2 to 3 days for FACS analysis, and the cells were replated in fresh drug in petri dishes again at 100,000 cells/mL.

For the competition assay using CRISPR genetic inhibition, pLX304-RB1-IRES-GFP and pLX304-EV-tTomato were mixed at 1:1 ratios on day 0 (with exact proportions of cells after mixing determined by repeat FACS analysis), infected with pLentiCRISPR lentiviruses expressing the indicated sgRNAs (sgAURKB #2, sgAURKB #3, or a nontargeting sgRNA), selected with puromycin at 1 μg/mL for 72 hours, and FACS analysis was performed and cells were replated as above.

All samples were analyzed by FACS using a BD-Fortessa. For analysis, at least 10,000 cells were analyzed per sample. Live cells were first gated, with doublets excluded, and the percentage of GFP-positive and tdTomato-positive cells was analyzed. The GFP:tdTomato ratio was calculated as a measure of RB1-proficient:RB1-deficient cells in the population. The GFP:tdTomato ratio for each time point was normalized to the GFP:tdTomato ratio at day 0 for each experiment. FlowJo was used for analysis.
FACS-Based Propidium Iodide Cell-Cycle Analysis

Cells were plated in RPMI media supplemented with 10% FBS at the following cell densities: NCI-H69 (200,000/mL), NCI-H82 (100,000/mL), GLC16 (200,000/mL), NCI-H1650 (50,000/mL), NCI-H1975 (50,000/mL), and PC-9 (50,000/mL). Forty-eight hours later, cells were washed once in ice-cold PBS and then fixed in ice-cold 80% ethanol (added dropwise) for at least 2 hours at −20°C. The cells were then centrifuged at 400 × g, washed once in PBS, centrifuged again at 400 × g, and then washed again in PBS containing 2% FBS. Finally, cells were centrifuged at 400 × g and stained with propidium iodide (PI; BD # 550825) for 15 minutes at room temperature prior to FACS analysis. For cell-cycle experiments in Supplementary Fig. S4A–S4D, ModFIT was used for quantitative cell-cycle analysis. For cell-cycle experiments following treatment with AZD2811 (Fig. S5, Supplementary Fig. S7), analysis was performed using FlowJo. The percentage of polyploidy was determined by gating on cells with >4N content.

FACS-Based Cleaved Cleaved PARP Apoptosis Analysis

NCI-H82 pTripZ-RB1 or pTripZ-EV cells were plated at 100,000 cells/mL and grown in the presence or absence of DOX for 48 hours. Cells were then treated with 64 nmol/L AZD2811 while being maintained in the presence or absence of DOX for another 48 hours. In Supplementary Fig. S7C, NCI-H69, NCI-H82, and GLC16 cells were plated at 100,000 cells/mL and grown in the presence of 64 nmol/L AZD2811 for the times indicated. Cells were then washed once in ice-cold PBS and then fixed in ice-cold 80% ethanol (added dropwise) for at least 2 hours at −20°C. The cells were then centrifuged at 400 × g, washed once in PBS, centrifuged again at 400 × g, and then washed again in PBS containing 2% FBS. Cells were then incubated with Alexa 647–conjugated cleaved PARP antibody (Cell Signaling, Asp214, D64E10, #6987) per the manufacturer’s instructions at a dilution of 1:50 for 1 hour at room temperature and then washed twice in PBS containing 2% FBS. FACS for Alexa 647 was then performed, and analysis was performed using FlowJo. The percentage increase in cleaved PARP was calculated by subtracting cleaved PARP-positive cells in the DMSO-treated samples from the AZD2811-treated samples.

FACS-Based Phospho-Histone H3 (Ser10) and Polyploidy Analysis

NCI-H82 pTripZ-RB1 (Dox-On) cells were superinfected with pLX304-CMV-EV, pLX304-CMV-AURKB WT, or pLX304-CMV-AURKB R159L; E161T and selected with blasticidin. Cells were plated at 200,000 cells/mL and grown in the presence or absence of DOX for 48 hours. The cells were then treated with the indicated concentrations of AZD2811 while being maintained in the presence or absence of DOX for another 24 hours. The cells were then washed once at room temperature in PBS and then fixed in 4% paraformaldehyde for 15 minutes at room temperature. The cells were then centrifuged at 400 × g at room temperature, washed once in PBS, centrifuged again at 400 × g, and then permeabilized with ice-cold methanol at −4°C for 30 minutes. The cells were then washed again in PBS and then incubated with Alexa 647–conjugated phospho-histone H3 (Ser10) antibody (Cell Signaling, #345B) at a dilution of 1:100 for 1 hour at room temperature, then washed once in PBS containing 0.5% BSA, centrifuged at 400 × g, and then stained with PI (BD # 550825) for 15 minutes at room temperature. FACS analysis was then performed to determine the percentage of positive phospho-histone H3 (Ser10) cells and cell-cycle distribution. The percentage of polyploidy was determined by gating of cells with >4N content, and the percentage of increase in polyploidy was calculated by subtracting the percentage of polyploidy in the DMSO-treated samples from the percentage of polyploidy in the AZD2811-treated samples.

Aurora B Kinase sgRNA Rescue Experiments

NCI-H82 cells were first infected with a DOX-On pTripZ lentiviral sgRNA-resistant Aurora B kinase cDNA expression vector, selected with G418, and then superinfected with pLentCRISPR V2 puromycin-based lentivirus encoding Cas9, a puromycin-resistance gene, and either 1 of 2 independent sgRNAs targeting AURKB (sgAURKB #2 or sgAURKB #3) or a nontargeting sgRNA (control sgRNA). The puromycin-resistant cells were selected with puromycin while being maintained in the presence of DOX at 1 μg/mL for 21 days. The cells were then grown in the presence or absence of DOX using RPMI with tetracycline-negative FBS (Gemini; #100-800). Immunoblot analysis and cell counts were performed 5 days after DOX withdrawal.

Aurora B Kinase AZD2811 Drug–Resistant Rescue Experiments

Given the high selectivity of AZD2811 for Aurora B kinase relative to Aurora A kinase, we mutated two residues in Aurora B kinase that are near the AZD2811-binding pocket (R159 and E161) to the residues found in Aurora A kinase and made an AZD2811 drug-resistant Aurora B kinase mutant (R159L, E161T). For the experiments performed in Fig. S3E–H, NCI-H82 cells were first infected with pLenti-EF1α-AURKB R159L, E161T, pLenti-EF1α-AURKB WT, or the empty pLenti-EF1α vector lentiviruses and selected with puromycin. These cells were then used for the in vivo xenograft experiments in Supplementary Fig. S9E–S9G. For the experiments performed in Supplementary Fig. S3F–S3H, NCI-H82 cells were first infected with pLX304-CMV-AURKB WT or pLX304-CMV-AURKB R159L; E161T and selected with blasticidin. Blasticidin-resistant cells were then superinfected with pLentCRISPR expressing the indicated sgRNAs (sgAURKB #3 targets an intron-exon junction in AURKB and therefore targets only endogenous and not exogenous AURKB) and selected with puromycin. Growth inhibition and cell-cycle experiments were performed 72 hours after treatment with AZD2811. The percentage of polyploidy was determined by gating on cells with >4N content. Pharmacodynamic experiments were performed 24 hours after treatment with AZD2811.

Time-Lapse Microscopy

iTERT-RPE-1 cells expressing DOX-inducible Cas9 (a kind gift of Dr. Iain Cheeseman; ref. 50) were superinfected with a retrovirus encoding H2B-GFP and sorted for GFP-positive cells by FACS. RPE-1 cells expressing DOX-inducible Cas9 and H2B-GFP were then superinfected with pLentiguide-Blast (a kind gift from Dr. Samuel McBrayer) containing an sgRNA targeting RB1 or a nontargeting sgRNA. Blasticidin-resistant cells were treated with 1 μg/mL DOX for 24 hours, and after an additional 72 hours, RB1 knockdown was confirmed by immunoblot analysis. Cells were then plated at a density of 30,000 cells per well in a 12-well MatTek plate containing glass coverslips and allowed to adhere overnight. The cells were then incubated in RO-3306 (9 μmol/L) for 18 hours to synchronize cells in late G2 phase of the cell cycle (36). The cells were released from RO-3306 by washing them 5 times in phenol red-free DMEM/F12 media for 1 minute per wash and then placed in phenol red-free DMEM/F12 media containing either AZD2811 (125 nmol/L) or DMSO. Live-cell imaging was performed on a Nikon TI-E inverted wide-field microscope equipped with a perfect focus and enclosed within a temperature- and CO2-controlled environment that maintained an atmosphere of 37°C and 5% humidified CO2. GFP fluorescence and differential interference contrast images were captured using Zyla sCMOS camera every 3 minutes with a 20X/0.45 Plan Fluor objective for at least 3 hours or until most cells had completed mitosis. Two independent wells per condition were imaged for each experiment, and two biological replicates were performed. Images were analyzed using H2B-GFP to visualize chromosomes for the timing of metaphase and anaphase onset, the presence of recognizable anaphase, and morphologic appearance of daughter nuclei.

RNA-seq and GSEA

For the RB1 reexpression RNA-seq experiment, NCI-H82 pTripZ RB1 cells were plated at 100,000 cells/mL in 8 mL of complete media and cell counts were performed 5 days after DOX withdrawal.
RB1−/− SCLCs Are Hyperdependent on Aurora B Kinase

in petri dishes and grown in the presence or absence of DOX (at 0.5 μg/mL) for 96 hours on petri dishes, changing the media and DOX at 48 hours. For the AZD2811 experiment, NCI-H82 TriIz RB1 cells were plated at 100,000 cells/mL. In the presence or absence of DOX (at 0.5 μg/mL) for 48 hours and then treated with AZD2811 (32 nmol/L) or DMSO for an additional 48 hours. After 96 hours, RNA was harvested using an RNeasy mini kit (Qiagen #74106), and RNA-seq was performed.

Libraries were prepared using Illumina TruSeq Stranded mRNA sample preparation kits from 500 ng of purified total RNA according to the manufacturer’s protocol. The finished dsDNA libraries were quantified by Qubit fluorometer, Agilent TapeStation 2200, and RT-qPCR using the Kapa Biosystems library quantification kit according to the manufacturer’s protocols. Uniquely indexed libraries were pooled in equimolar ratios and sequenced on an Illumina NextSeq500 with single-end 75 bp reads by the Dana-Farber Cancer Institute Molecular Biology Core Facilities.

Sequenced reads were aligned to the UCSC hg19 reference genome assembly, and gene counts were quantified using STAR (v2.5.1b). Differential expression testing was performed by DESeq2 (v1.10.1) as part of the VIPER analysis pipeline (https://bitbucket.org/cfca/viper/). Normalized read counts (RPKM) were calculated using cufflinks (v2.2.1).

For GSEA, software was downloaded from the GSEA website (http://www.broad.mit.edu/gsea/downloads.jsp). GSEA was performed using the “Gene-Ontology” or “Hallmark” gene sets for identification of enriched/depleted signatures. Gene sets with an FDR < 0.25 and a nominal P value of <0.05 were considered significant.

**Generation of SCLC PDX Models and Treatment Studies**

To generate the SC61 PDX model (derived from a previously untreated primary oat cell SCLC), SC74 PDX model (derived from a previously treated SCLC lymph node metastasis), and SC6 PDX model (derived from a previously untreated SCLC lymph node metastasis), tumors of the same passage were transplanted subcutaneously into 5 to 10 athymic nude donor mice (51). When these tumors reached 1,000 to 2,000 mm3, donor tumors were aseptically excised, and viable tumor was cut into fragments measuring approximately 20 mm3 and transplanted subcutaneously into experimental athymic nude mice. Growing tumors were allocated to treatment groups (vehicle [placebo NP], AZD1152, and/or AZD2811 NP groups) when tumors were in the range of 62.5 to 405 mm3 with 6 to 10 mice per group. The nanoparticles were diluted to required concentration in 0.9% physiologic saline. AZD1152 was diluted to required concentration in 30 mmol/L Tris buffer, pH 9. All agents were dosed at 25 mg/kg 4 days a week for 8 weeks by i.p. injection. Mice were monitored daily, tumor diameter was measured using calipers at the times indicated, and tumor volume was calculated: tumor volume (mm3) = (width)2 × length/2.

For the RB1 synthetic lethal CRISPR screen, RIGER-E second best Genetically Engineered Mouse Model Treatment Study

**Rb1−/− Genetically Engineered Mouse Model Treatment Study**

Rb1−/− mice (Jackson Laboratory stock number 002102) underwent monthly MRIs beginning at 9 months of age. Once tumors were detected, mice were randomized to treatment with AZD1152 or vehicle (30 mmol/L Tris pH 9) dosed at 25 mg/kg 4 days a week for 8 weeks by i.p. injection. Mice were randomized to treatment with AZD1152 or vehicle (placebo NP) for 96 hours, and then treated with AZD2811 NP groups, and treatment was administered at the times indicated as described in the PDX method section above. All mouse experiments using NCI-H82 xenografts and Rb1−/− mice that formed pituitary and thyroid tumors complied with NIH guidelines and were approved by the Dana-Farber Cancer Institute Animal Care and Use Committee (DFCI, protocol 03-105). All mouse experiments using NCI-H417a, NCI-H69, and NCI-H1048 xenografts and SC6, SC61, and SC74 SCLC PDX models were conducted in accordance with UK Home Office legislation, the Animal Scientific Procedures Act 1986, as well as the AstraZeneca Global Bioethics policy.

**Statistical Analysis**

For the RB1 synthetic lethal CRISPR screen, RIGER-E second best LFC algorithm was used to perform 2-class comparisons (e.g., RB1-ON vs. RB1-OFF) and determine a rank list of synthetic lethal genes ranked by P value where statistical significance was P < 0.05. The P value was assessed empirically against a null distribution created by scrambling the mapping of sgRNAs to genes. The actual score for each gene from the unscrambled real data was compared with the null score distribution; the fraction of scores from the scrambled null distribution that were as good as or better than the observed real score was used to determine the P value. Please see https://github.com/broadinstitute/riger for additional details on RIGER.

For the time-lapse imaging experiments, statistical significance was calculated using a two-sided t test. For the RNA-seq experiments, statistical significance with calculated using FDR corrected for multiple hypothesis testing where <0.25 is considered statistically significant.
For the in vivo efficacy experiments in Fig. 7B–I, tumor volume data were analyzed using the AstraZeneca regression tool. Tumor growth inhibition from the start of the treatment was assessed by comparison of the geometric mean change in tumor volume for the control and treated groups. Tumor regression was calculated as the percentage reduction in tumor volume from the pretreatment value: % Regression = (1 – RTV) × 100%, where RTV is the geometric mean relative tumor volume. Statistical significance was evaluated using a one-tailed Student t test.

Two-way ANOVA analysis was performed on dose-response curves in Supplementary Figs S6 and S9A–S9D. For all other experiments, statistical significance was calculated using an unpaired, two-tailed Student t test. P values were considered statistically significant if P < 0.05. For all figures: * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. Error bars represent SEM unless otherwise indicated.

Data and Materials Availability

Data generated from the RB1 synthetic lethal CRISPR screen are provided in Supplementary Tables S1 to S4. Data generated from the RNA-seq and GSEA analyses are provided in Supplementary Table S5. The RNA-seq data is deposited in the GEO repository (Accession Number GSE120562). Mutational/copy-number variation status of oncogenic drivers (RB1, TP53, and MYC) in SCLC cell lines and SCLC PDX models are provided in Supplementary Table S6 and Supplementary Fig. S10. All other data and materials can be requested from the corresponding author.

Disclosure of Potential Conflicts of Interest

M.G. Oser reports receiving commercial research support from AstraZeneca. A. Spektor has received honoraria from the speakers bureaus of Astellas Pharma, Bayer AG, and Janssen Pharmaceuticals. S.T. Barry has ownership interest (including stock, patents, etc.) in AstraZeneca.

W.G. Kaelin Jr is a board director at Lilly Pharmaceuticals, founder of Tango Therapeutics and Cedilla Therapeutics, Scientific Advisor at Nextech Invest, a future sponsored research agreement recipient from AstraZeneca, reports receiving a commercial research grant from AstraZeneca, has ownership interest (including stock, patents, etc.) in Lilly, Tango Therapeutics, Nextech Invest, and Cedilla Therapeutics, and is a consultant/advisory board member for Lilly Pharmaceuticals, Tango Therapeutics, Nextech Invest, and Cedilla Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M.G. Oser, E. Buss, S.K. McBrayer, W.G. Kaelin Jr

Development of methodology: M.G. Oser, R. Fonseca, A. Flaifel, J.S. Novak, E. Buss, G.S. Cowley


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.G. Oser, A. Flaifel, L. Brulle-Soumare, P. Taylor, J. Travers

Study supervision: M.G. Oser, P. Taylor, S. Ashton, W.G. Kaelin Jr

Other (conception and design of in vivo studies): S.T. Barry

Acknowledgments

W.G. Kaelin Jr is supported by the Howard Hughes Medical Institute (HHMI), the Breast Cancer Research Foundation, and an NCI/NIH R35 grant (no. R35CA210068). M.G. Oser is supported by an NCI/NIH K08 grant (no. K08CA222657) and the Lung Cancer Research Foundation. C.J. Lord is supported by Programme Funding from Breast Cancer Now and Cancer Research UK. C.J. Ryan is a Sir Henry Wellcome Fellow, funded by the Wellcome Trust. D. Pellman is an HHMI investigator and is supported by an NIH grant (no. CA213404-20). A. Spektor is supported by an NCI/NIH K08 grant (no. K08CA208008-01) and the Burroughs Wellcome Fund Career Award for Medical Scientists (CAMS). E. Buss was an HHMI Medical Research Fellow. Special thanks to Xentech for technical help with the mouse experiments, Vidyasagar Koduri, Wenhua Gao, and Gang Lu for generation of destination vectors used for recombination cloning, Zach Herbert and the Molecular Biology Core Facility at DFCI for RNA-seq analysis, Neil Umbreit for thoughtful discussions, and members of the Kaelin laboratory for critical reading of the manuscript.

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Received April 10, 2018; revised August 22, 2018; accepted October 5, 2018; published first October 29, 2018.

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RB1−/− SCLCs Are Hyperdependent on Aurora B Kinase


Cells Lacking the RB1 Tumor Suppressor Gene Are Hyperdependent on Aurora B Kinase for Survival


Cancer Discov Published OnlineFirst October 29, 2018.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-18-0389

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http://cancerdiscovery.aacrjournals.org/content/suppl/2018/10/26/2159-8290.CD-18-0389.DC1

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