Differential regulation and synthetic lethality of exclusive \textit{RB1} and \textit{CDKN2A} mutations in lung cancer

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Abstract. Genetic alterations in lung cancer are distinctly represented in non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). Mutation of the \textit{RB1} and \textit{CDKN2A} genes, which are tightly associated with cell cycle regulation, is exclusive to SCLC and NSCLC cell lines, respectively. Through the systematic analysis of transcriptome and proteome datasets for 318 cancer cell lines, we characterized differential gene expression and protein regulation in \textit{RB1}-mutant SCLC and \textit{CDKN2A}-mutant NSCLC. Many of the genes and proteins associated with \textit{RB1}-mutant SCLC cell lines belong to functional categories of gene expression and transcription, whereas those associated with \textit{CDKN2A}-mutant NSCLC cell lines were enriched in gene sets of the extracellular matrix and focal adhesion. These results indicate that the loss of \textit{RB1} and \textit{CDKN2A} function induces distinctively different signaling cascades in SCLC and NSCLC cells. In addition, knockdown of the \textit{RB1} gene in \textit{CDKN2A}-mutant cell lines (and vice versa) synergistically inhibits cancer cell proliferation. The present study on the exclusive role of \textit{RB1} and \textit{CDKN2A} mutations in lung cancer subtypes demonstrates a synthetic lethal strategy for cancer regulation.

Introduction

Understanding heterogeneous genetic alterations in tumors is recognized as a key factor in advancing cancer therapy (1-3). Lung cancers are classified into two subtypes, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC), which harbor exclusive specific mutations: \textit{RB1} in SCLC and \textit{CDKN2A} (p16\textsuperscript{INK4a}) in NSCLC (4). Both the \textit{RB1} and \textit{CDKN2A} genes are tightly associated with cell cycle regulation, and \textit{CDKN2A} regulates \textit{RB1} phosphorylation through cyclin E and D1 (5,6). The crucial role of \textit{RB1} as a regulator in cell cycle progression has been intensively investigated (7-9). Accumulated data have demonstrated mutually exclusive mutation patterns for genes encoding proteins that function in the same biological pathway. For instance, mutations of the \textit{KRAS} or \textit{BRAF} gene, which are downstream of the EGFR signaling pathway, have not been found in \textit{EGFR}-mutated NSCLC (10,11), and co-mutations of the \textit{TP53} and \textit{PIK3CA} pair (12) or the \textit{RB1} and \textit{CDKN2A} pair (13) rarely occur in the same tumors. However, the biological meaning of such mutually exclusive mutation patterns is not fully understood, even though this exclusiveness does serve as an attractive target for the development of novel therapeutics (14).

An understanding of differential regulation along with distinct mutations in \textit{RB1} and \textit{CDKN2A} is required to identify molecular characteristics of the progression of SCLC and NSCLC subtypes. Large-scale cell line-based high-throughput transcriptome and proteome datasets facilitate the understanding of molecular characterization of cancers through genome-wide functional analyses. The National Cancer Institute (NCI) released well-annotated sets of both DNA microarray data to detect the gene expression and reverse-phase protein array (RPPA) data to detect the total protein and phosphorylation on 60 well-characterized cancer cell lines (15). Diverse omics datasets on an expanded panel of >300 cancer cell lines were also generated by GlaxoSmithKline (GSK) (16). Together with these datasets, the extensive mutation profile on individual cell lines is available from the COSMIC (Catalogue of Somatic Mutations in Cancer) database (17). Through mutation-oriented association studies on cell line-based omics data, we have reported new targets and mechanisms for cancer regulation (3,18,19).

In the present study, the regulation of gene and protein levels driven by \textit{RB1} or \textit{CDKN2A} mutations in lung cancer was analyzed using transcriptome and proteome datasets obtained from 318 diverse cancer cell lines. We attempted to identify the differentially regulated gene/protein signatures and functional pathways specific to \textit{RB1} and \textit{CDKN2A} mutations. Furthermore, we experimentally investigated whether double or complementary knockdown of \textit{RB1} or \textit{CDKN2A} gene expression has a specific effect on the reciprocal mutant subtype in lung cancer cell lines. We expect that this study will provide a useful resource for the regulation of lung cancer.
progression using synergistic mechanisms of exclusive RB1 or CDKN2A mutations.

Materials and methods

Data acquisition. The large-scale transcriptome dataset on 318 cancer cell lines was obtained from the Cancer Biomedical Informatics Grid (caBIG) website (https://cabig.nci.nih.gov/caArray_GSKdata) (16). This dataset, also known as the GlaxoSmithKline (GSK) dataset, has 950 arrays performed in triplicate for each cell line with the Affymetrix U133 Plus 2.0 Array chip. It was normalized to MAS5 and then transformed to a log2 scale.

The reverse-phase protein array (RPPA) dataset to detect protein expression and phosphorylation was generated in the Functional Proteomics Core of the M.D. Anderson Cancer Center using a total of 179 cancer cell lines, which were included in the transcriptome dataset. These cell lines were purchased from several vendors (American Type Culture Collection; Developmental Therapeutics Program, National Cancer Institute; German Resource Centre for Biological Material and European Collection of Animal Cell Cultures) and grown in standard culture media as recommended by the vendor. The genetic identity of cell lines was determined by cross comparing all cell lines in this set (16,20). The cells were maintained in RPMI-1640 supplemented with 5% fetal bovine serum at 37˚C in a humidified atmosphere at 5% CO2.

Proteins were harvested when the cells reached ~70% confluence. The cells were lysed in buffer containing 1% Triton X-100, 50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1 mM ethylene glycol tetraacetic acid, 100 mM NaF, 10 mM Na3VO4, 10% glycerol, 1 mM Na2VO4 and complete protease inhibitor cocktail (Roche Diagnostics). Protein supernatants were isolated using standard methods (21), and the protein concentration was determined using the bicinchoninic acid assay (22). Samples were diluted to a uniform protein concentration and denatured in 1% sodium dodecyl sulfate for 10 min at 95˚C. Samples were stored at -80˚C until use. RPPA analysis was performed as described previously (21,23,24). A logarithmic value reflecting the relative amount of each protein in each sample was generated for subsequent analyses. The RPPA analysis was performed using a total of 115 antibodies.

The annotation of somatic mutation on all cell lines was organized by the COSMIIC (Catalogue of Somatic Mutations in Cancer) database (http://cancer.sanger.ac.uk/cosmic) (17).

Enrichment analysis of somatic mutations. To describe the selectivity of mutation occurrence, we calculated enrichment scores using an odds ratio between the observed odds and expected odds. The observed odds score is the ratio for the number of mutated cell lines in a specific cancer type via the number of cell lines in a specific cancer type. The expected odds score is the ratio for the number of mutated cell lines vs. the total number of cell lines. In addition, the probability of an odds ratio was calculated by the Fisher exact test using the R open-source computing language, version 2.15. The Fisher exact test uses a hypergeometric distribution to determine the significance of the agreement between individual question pairs (25).

Mutation-specific gene and protein expression analysis. For the selection of RB1 and CDKN2A mutation-specific gene and protein expression markers together with excluding the subtype-dependent expressions, lung cancer cell lines were classified into two groups: NSCLC and SCLC. Then, we divided the cell lines of each subtype into two groups based on the mutational status of RB1 and CDKN2A. CDKN2A-mutant and wild-type cell lines were mainly considered in the NSCLC type. RB1-mutant and wild-type cell lines were considered in the SCLC type. As a result, in the transcriptome dataset, we classified 9, 16, 22 and 24 cell line samples into the following four groups, respectively: RB1wt SCLC; RB1mt SCLC; CDKN2Awnt NSCLC; and CDKN2Amnt NSCLC. In the RPPA dataset, we classified 4, 7, 4 and 16 cell line samples into four groups, respectively: RB1wt SCLC; RB1mt SCLC; CDKN2Awnt NSCLC; and CDKN2Amnt NSCLC. The gene expression was detected using a log2 fold change value for the average difference of mutant and wild-type cell lines. The significance was confirmed by a t-test.

The patterns of gene expression were analyzed through a hierarchical clustering method. The clustering and its visualization on a heatmap were performed using the software QCanvas (26). QCanvas can be downloaded freely from the website http://compbio.sookmyung.ac.kr/~qcanvas.

Gene set enrichment analysis. Pathway analysis was performed using the GSEA (Gene Set Enrichment Analysis) method (27). Gene sets, integrated from Reactome, PID, KEGG, and Biocarta database, were obtained from the online pathway database, MSigDB v3.1 (http://www.broadinstitute.org/gsea/msigdb). The significantly (p<0.01) enriched gene sets among the results of the GSEA were reorganized based on major functional categories in each database.

Cell culture. NCI-60 lung cancer cell lines (NCI-H460, A549, NCI-H322M, NCI-H226, EKVX, and NCI-H23) were obtained from National Cancer Institute (NCI DTP), USA. NCI-H1993, NCI-H1935, NCI-H82 and NCI-H524 were obtained from American Type Culture Collection (ATCC). All cells were grown in RPMI-1640 medium (HyClone, USA) with 10% FBS (HyClone) and 1% penicillin/streptomycin (Gibco, USA), and maintained at 37˚C in a humidified atmosphere at 5% CO2.

siRNA transfection and cell viability assay. To detect cell viability after siRNA transfection, the cells were seeded in a 96-well plate at a density of 5,000 cells per well. After adhering for 24 h, target siRNAs were added in transfection medium (Gibco) for 6 h at 37˚C in a CO2 incubator. siRB1 (L-003296-02), siCDKN2A (L-011007-00) and non-targeting siRNA (D-001810-10) were purchased from Drmacon Inc. (Lafayette, CO, USA). After being cultured for 72 h at 37˚C, 5% CO2, cell viability was detected using a CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA).

Results and Discussion

RB1 and CDKN2A mutations in SCLC and NSCLC cell lines. Genetic alterations affecting the same biological pathway are generally not found in the same cancer cell. Accordingly, exclusive mutation patterns of RB1 and CDKN2A genes
have been observed in the lung cancer subtypes SCLC and NSCLC (4,13). Based on the analysis of mutation frequencies across 318 cell lines, we found the general exclusiveness of \(RB1\) and \(CDKN2A\) mutations in diverse cancer lineages (Fig. 1A). \(RB1\) mutations were significantly enriched in urinary tract and lung cancer cell lines yet rarely found in liver, renal, pancreatic and skin cancers, in which \(CDKN2A\) mutations were frequent.

Furthermore, among 71 lung cancer cell lines, 25 SCLC-derived cells were significantly enriched with \(RB1\) mutations, whereas 46 NSCLCs predominantly contained \(STK11\), \(KRAS\) and \(CDKN2A\) mutations (Fig. 1B). Taken together, the mutations of \(RB1\) and \(CDKN2A\) genes, which belong to a common functional pathway, were clearly exclusive from each other among frequently mutated genes in diverse cancer cell lines (Fig. 1C).

**Differential gene expression profiles between \(RB1\)mt SCLCs and \(CDKN2A\)mt NSCLCs.** To find lineage-independent, mutation-specific gene expression patterns, we classified 9, 16, 22 and 24 cell line samples into four groups, \(RB1\)wt SCLC, \(RB1\)mt SCLC, \(CDKN2A\)wt NSCLC and \(CDKN2A\)mt NSCLC, and analyzed the group-specific gene expression patterns using DNA microarray data. There was no general correlation of gene expression between the SCLC and NSCLC cell lines (Fig. 2A), and significantly enriched gene sets were also different between the lung subtypes. However, \(RB1\)mt SCLC...
and CDKN2A\textsuperscript{mt} NSCLC cells showed a negative correlation in gene expression (Fig. 2B), whereas RB1\textsuperscript{wt} SCLC and CDKN2A\textsuperscript{wt} NSCLC exhibited a positive correlation (Fig. 2C). This observation indicated that RB1 and CDKN2A mutations caused lineage-specific distinctive changes in gene expression.

Our analysis showed that the lineage difference was generally more important than RB1 and CDKN2A mutational status in the differential gene expression pattern (Fig. 3A). Thus, we attempted to identify RB1\textsuperscript{mt} and CDKN2A\textsuperscript{mt}-specific gene signatures by separately analyzing SCLC and NSCLC cells (Fig. 3B). As a result, we were able to identify distinct mutation-specific gene signatures for which expression was significantly regulated (>2-fold change and p<0.05) in each subtype (Tables I and II). Of note, the significantly over-enriched (p<0.01) gene sets (functional categories of selected gene signatures) generally did not overlap between the two mutation groups (Fig. 3B). The upregulated gene sets with RB1 mutation in SCLC cell lines mainly belonged to functional categories of transcription. The hit list included known target genes of E2F, which are released and activated upon RB1 inactivation (28). The upregulated genes upon CDKN2A mutation in NSCLC cell lines were largely enriched in the gene sets of extracellular matrix and metabolism. Genes related to the extracellular matrix are known to be important factors for enhancing tumorigenicity and promoting metastasis (29).

Specific change in total proteins and phosphoproteins in RB1 and CDKN2A mutations. We characterized the differential regulation of RB1 and CDKN2A mutations at the protein level using RPPA data of 77 pan- and 38 phospho-antibodies for 89 proteins across 179 cancer cell lines. Consistent with the patterns of gene expression data, the overall protein expression and phosphorylation status were inversely correlated between RB1\textsuperscript{mt} SCLC and CDKN2A\textsuperscript{mt} NSCLC cell lines (Fig. 4). Thus, the mutational effect of RB1 and CDKN2A genes were separately analyzed in SCLC and NSCLC cell lines (Fig. 5). The results showed that \(\beta\)-catenin was commonly overexpressed in both RB1 and CDKN2A mutants. \(\beta\)-catenin overexpression has been extensively reported in lung cancer (30), and the overexpression of \(\beta\)-catenin might be maintained by the mutational effect of both RB1 and CDKN2A genes. The RB1 mutation specifically regulated PTEN, STAT, mTOR, p53 expression and MAPK phosphorylation in SCLC cells. However, the CDKN2A mutation altered the expression of JNK2 and cKIT and the phosphorylation status of AKT, STAT3 and AMPKa.

MAPK (T202), which is significantly (p<0.05) phosphorylated in RB1-mutated SCLC cancer cell lines, has an important role in transcriptional regulation of targeting transcription factors such as c-Jun, c-Fos, and c-Myc (31). This observation is consistent with the DNA microarray data (Fig. 3B) for RB1\textsuperscript{mt} SCLC cells, which are enriched in the functional categories of transcription. AKT is specifically phosphorylated (S473, T308) in CDKN2A\textsuperscript{mt} NSCLC and related to focal adhesion (32), which is the enriched gene set of CDKN2A\textsuperscript{mt} NSCLC from
Table I. The RB1 mt-specific gene signatures in SCLC.

| ProbeID | Symbol | log₂ fold change | p-value | ProbeID | Symbol | log₂ fold change | p-value |
|---------|--------|------------------|---------|---------|--------|------------------|---------|
| 231736_x_at | MGST1 | 2.083 | 0.001 | 202834_at | AGT | -3.059 | 0 |
| 218847_at | IGF2BP2 | 2.058 | 0.011 | 1566674_at | MACC1 | -2.16 | 0.035 |
| 202620_s_at | PLOD2 | 2.01 | 0.002 | 205501_at | PDE10A | -2.159 | 0.004 |
| 213139_at | SNAI2 | 2.002 | 0.016 | 204044_at | QPRT | -2.149 | 0.004 |
| 206332_s_at | IFI16 | 1.879 | 0.03 | 239503_at | Unknown | -2.041 | 0 |
| 235763_at | SLC4A4 | 1.829 | 0.009 | 208891_at | DUSP6 | -2.019 | 0.006 |
| 204646_at | DPYD | 1.828 | 0.005 | 1560652_at | Unknown | -1.943 | 0.019 |
| 202016_at | MEST | 1.817 | 0.003 | 203881_s_at | DMD | -1.937 | 0.006 |
| 226225_at | MCC | 1.717 | 0.045 | 208892_s_at | DUSP6 | -1.921 | 0.005 |
| 217028_at | CXCR4 | 1.675 | 0.023 | 206218_at | MAGEB2 | -1.732 | 0.013 |
| 214597_at | SSTR2 | 1.655 | 0.038 | 203132_at | RB1 | -1.709 | 0.006 |
| 210839_s_at | ENPP2 | 1.557 | 0.04 | 205305_at | FGL1 | -1.67 | 0.006 |
| 203038_at | PTPRK | 1.531 | 0.001 | 201328_at | ETS2 | -1.663 | 0.005 |
| 225553_s_at | OXR1 | 1.528 | 0.003 | 205110_s_at | FGFI3 | -1.651 | 0.036 |
| 1558217_at | SLFN13 | 1.515 | 0.045 | 209365_s_at | ECM1 | -1.61 | 0.014 |
| 1565162_s_at | MGST1 | 1.493 | 0.016 | 210102_at | WVASA | -1.597 | 0.005 |
| 204620_s_at | VCAN | 1.47 | 0.011 | 209468_at | LRP5 | -1.583 | 0.001 |
| 221731_s_at | VCAN | 1.44 | 0.018 | 1558882_at | LOC401233 | -1.574 | 0.032 |
| 218197_s_at | OXR1 | 1.409 | 0.006 | 219750_at | TMEM144 | -1.572 | 0.034 |
| 205229_s_at | COCH | 1.338 | 0.006 | 223748_at | SLC4A4 | -1.552 | 0.002 |
| 203184_at | FBN2 | 1.338 | 0.026 | 205601_s_at | HOXB5 | -1.511 | 0.023 |
| 205027_s_at | MAP3K8 | 1.311 | 0.004 | 209803_s_at | PHLD2 | -1.495 | 0.038 |
| 204030_s_at | SCHIP1 | 1.308 | 0.038 | 212268_at | SERPINB1 | -1.467 | 0.001 |
| 241400_at | Unknown | 1.296 | 0.006 | 1569191_at | ZNF826 | -1.448 | 0.022 |
| 1555788_a_at | TRIB3 | 1.274 | 0.034 | 212188_at | KCTD12 | -1.43 | 0.002 |
| 211675_s_at | MDFIC | 1.272 | 0.012 | 241672_at | C13orf36 | -1.414 | 0.033 |
| 229465_s_at | PTPRS | 1.255 | 0.008 | 219305_s_at | FBXO2 | -1.337 | 0.015 |
| 225093_at | UTRN | 1.255 | 0.042 | 1554472_a_at | PHF20L1 | -1.317 | 0 |
| 205122_at | TMEFF1 | 1.251 | 0.01 | 203028_s_at | CYBA | -1.308 | 0.047 |
| 219489_s_at | NXN | 1.238 | 0.035 | 228726_at | Unknown | -1.303 | 0.01 |
| 225056_s_at | SIA1L2 | 1.237 | 0.011 | 204158_s_at | TCC1 | -1.302 | 0.006 |
| 208949_s_at | LGALS3 | 1.235 | 0.021 | 211538_s_at | HSPA2 | -1.279 | 0.035 |
| 201063_at | RCN1 | 1.229 | 0.033 | 220082_at | PPP1R14D | -1.259 | 0.008 |
| 235244_at | CCDC58 | 1.184 | 0.032 | 203005_at | LTBR | -1.257 | 0.011 |
| 210978_s_at | TAGLN2 | 1.184 | 0.005 | 229964_at | C9orf152 | -1.23 | 0.036 |
| 233903_s_at | SGEF | 1.182 | 0.003 | 203961_at | NEBL | -1.212 | 0.032 |
| 205123_s_at | TMEFF1 | 1.177 | 0.019 | 224577_at | ERGIC1 | -1.206 | 0.002 |
| 200897_s_at | PALLD | 1.164 | 0.018 | 238021_s_at | CRNDE | -1.189 | 0.022 |
| 200916_at | TAGLN2 | 1.161 | 0.015 | 223041_at | CD99L2 | -1.182 | 0.001 |
| 215127_s_at | RBMS1 | 1.143 | 0.03 | 205586_x_at | VGF | -1.182 | 0.008 |
| 202887_s_at | DDI74 | 1.141 | 0.005 | 239278_at | Unknown | -1.163 | 0.013 |
| 212636_at | QKI | 1.137 | 0.014 | 213689_x_at | FAM69A | -1.157 | 0.005 |
| 214877_at | CDKAL1 | 1.134 | 0.03 | 232099_at | PCDHB16 | -1.153 | 0.028 |
| 227197_at | SGEF | 1.129 | 0.005 | 219256_s_at | SH3TC1 | -1.153 | 0.005 |
| 224918_x_at | MGST1 | 1.12 | 0.02 | 227943_at | Unknown | -1.141 | 0.004 |
| 227522_at | CMBL | 1.08 | 0.007 | 210538_s_at | BIRC3 | -1.138 | 0.024 |
Table I. Continued.

| ProbeID  | Symbol       | log₂ fold change | p-value | ProbeID     | Symbol       | log₂ fold change | p-value |
|----------|--------------|------------------|---------|-------------|--------------|------------------|---------|
| 206385_s_at | ANK3         | 1.073            | 0.042   | 1568838_at  | LOC100132169  | -1.117           | 0.032   |
| 226464_at  | C3orf58      | 1.072            | 0.01    | 229872_s_at | LOC100132999  | -1.099           | 0.021   |
| 1568720_at | ZNF506       | 1.054            | 0.04    | 1555579_s_at| PTPRM        | -1.09            | 0.043   |
| 201656_at  | ITGA6        | 1.04             | 0.028   | 224997_s_at | H19         | -1.082           | 0.032   |
| 212190_at  | SERPINE2     | 1.034            | 0.037   | 229190_s_at | KANK1        | -1.081           | 0.01    |
| 204995_at  | CDK5R1       | 1.022            | 0.017   | 219371_s_at | KLF2         | -1.076           | 0.013   |
| 210512_s_at| VEGFA        | 1.02             | 0.037   | 37408_at    | MRC2         | -1.074           | 0.01    |
| 226419_s_at| FLJ44342     | 1.015            | 0.001   | 224391_s_at | SIAE         | -1.059           | 0.01    |
| 210735_s_at| CA12         | 1.011            | 0.032   | 201329_s_at | ETS2         | -1.053           | 0.022   |
| 65588_at   | LOC388796    | 1.002            | 0.009   | 205016_at   | TGFA         | -1.049           | 0.007   |
| 213857_s_at| CD47         | 1.001            | 0.002   | 227384_s_at | LOC727820    | -1.043           | 0.002   |
| 208622_s_at| EZR          | 1.001            | 0.001   | 228010_at   | PPP2R2C      | -1.033           | 0.031   |
|           |              |                  |         | 209500_s_at | TNFSF12/     | -1.031           | 0.019   |
|           |              |                  |         |             | TNFSF13      |                  |         |
|           |              |                  |         | 224576_at   | ERGIC1       | -1.031           | 0.007   |
|           |              |                  |         | 236719_at   | Unknown      | -1.025           | 0.004   |
|           |              |                  |         | 227001_at   | NIPAL2       | -1.021           | 0.006   |
|           |              |                  |         | 230722_at   | BNC2         | -1.019           | 0.047   |
|           |              |                  |         | 204682_at   | LTBP2        | -1.007           | 0.024   |

Figure 4. Comparison of protein expression and phosphorylation in SCLC and NSCLC with the mutational status of RB1 and CDKN2A. Protein expression change of a total of 77 pan-antibodies was compared between (A) SCLC and NSCLC, (B) RB1-mutated SCLC and CDKN2A-mutated NSCLC, and (C) SCLC and NSCLC with RB1 and CDKN2A wild-type cell lines, respectively. Protein phosphorylation change of a total of 38 phospho-antibodies was compared between (D) SCLC and NSCLC, (E) RB1-mutated SCLC and CDKN2A-mutated NSCLC, and (F) SCLC and NSCLC with RB1 and CDKN2A wild-type cell lines, respectively. The phosphorylation change for each protein phospho-antibody was calculated by the log₂ fold change via the median across 179 cell lines. The r value represents the Pearson correlation coefficient.
DNA microarray analysis. Furthermore, PTEN, which was overexpressed in RB1<sup>mt</sup> SCLC cells (Fig. 5A), is a well-known negative regulator of AKT activation (33), suggesting that AKT-mediated signaling might be exclusively activated by CDKN2A<sup>mt</sup> in NSCLC, not by RB1<sup>mt</sup> in SCLC. Both proteome and transcriptome data analyses demonstrated that exclusive RB1 and CDKN2A mutations in different subtypes of lung cancer included a differential change of gene expression and protein regulation, even though RB1 and CDKN2A are in the same cell cycle-related pathway.

**Synthetic lethality of reciprocal regulation of RB1 and CDKN2A expression.** Through the systematic analysis of transcriptome and proteome data, we found unique mRNA and protein regulation patterns induced by the mutation of either the RB1 gene or the CDKN2A gene (Fig. 6A). Furthermore, we investigated the synergistic negative effect on cancer growth by simultaneous functional loss (or knockdown) of these two genes. We performed a viability assay with diverse lung cancer cell lines with the combined knockdown of RB1 and CDKN2A genes using siRNA-mediated gene depletion. As a result, the knockdown of one of these genes decreased the viability of cells harboring a mutation of the other gene (Fig. 6B). The viability of CDKN2A<sup>mt</sup>-mutant cell lines was significantly decreased by knockdown of RB1; similarly, RB1<sup>mt</sup>-mutant cell lines were inhibited by CDKN2A depletion. Consistently, the simultaneous depletion of RB1 and CDKN2A genes significantly decreased the viability of lung cell lines harboring wild-types of these genes (Fig. 6C). However, the single knockdown of either the RB1 gene or the CDKN2A gene did not effectively reduce viability in these wild-type cell lines. In conclusion, the functional inhibition of the RB1 or CDKN2A gene in CDKN2A<sup>mt</sup> or RB1<sup>mt</sup> cancer cells, respectively, might be a promising therapeutic approach in SCLC or NSCLC lung cancers. The present

| ProbeID   | Symbol | log<sub>2</sub> fold change | p-value |
|-----------|--------|-----------------------------|---------|
| 236694_at | CYorf15A | 2.585                      | 0.001   |
| 211980_at | COL4A1  | 1.978                      | 0.006   |
| 213725_x_at | XYL11 | 1.654                      | 0.023   |
| 204971_at | CSTA    | 1.615                      | 0.022   |
| 209970_x_at | CASP1 | 1.566                      | 0.001   |
| 225688_s_at | PHLD2B | 1.412                      | 0.017   |
| 202638_s_at | ICAM1 | 1.388                      | 0.011   |
| 222453_at | CYBRD1  | 1.377                      | 0.016   |
| 156202_at | AKR1C1  | 1.344                      | 0.048   |
| 208782_at | FSTL1   | 1.312                      | 0.014   |
| 211340_s_at | MCAM | 1.299                      | 0.002   |
| 210004_at | OLR1    | 1.299                      | 0.01    |
| 202008_s_at | NID1 | 1.286                      | 0.004   |
| 20350_s_at | MATN2   | 1.197                      | 0.012   |
| 239999_at | Unknown | 1.126                      | 0.033   |
| 205407_at | RECK    | 1.117                      | 0.014   |
| 203304_at | BAMB1   | 1.113                      | 0.012   |
| 228698_at | SOX7    | 1.104                      | 0.014   |
| 227051_at | Unknown | 1.088                      | 0.036   |
| 201939_at | PLK2    | 1.082                      | 0.017   |
| 209087_x_at | MCAM | 1.081                      | 0.007   |
| 206165_s_at | CLCA2 | 1.067                      | 0.025   |
| 227178_at | CUGBP2  | 1.067                      | 0.012   |
| 227253_at | CP      | 1.044                      | 0.015   |
| 212262_at | QKI     | 1.043                      | 0.002   |
| 202998_s_at | LOXL2 | 1.039                      | 0.006   |
| 214022_s_at | IFITM1 | 1.021                      | 0.034   |
| 211366_x_at | CASP1 | 1.019                      | 0.001   |
| 222446_s_at | BACE2 | 1.014                      | 0.009   |

**Table II. The CDKN2A<sup>mt</sup>-specific gene signatures in NSCLC.**
study on differential proteome and transcriptome profiles between two mutant groups provides mechanistic insights into the synthetic lethality of RB1 and CDKN2A mutations.

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