Transposon Mutagenesis Screen Identifies Potential Lung Cancer Drivers and CUL3 as a Tumor Suppressor

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Abstract

Non–small cell lung cancers (NSCLC) harbor thousands of passenger events that hide genetic drivers. Even highly recurrent events in NSCLC, such as mutations in PTEN, EGFR, KRAS, and ALK, are detected, at most, in only 30% of patients. Thus, many unidentified low-penetrant events are causing a significant portion of lung cancers. To detect low-penetrance drivers of NSCLC, a forward genetic screen was performed in mice using the Sleeping Beauty (SB) DNA transposon as a random mutagen to generate lung tumors in a Pten-deficient background. SB mutations coupled with Pten deficiency were sufficient to produce lung tumors in 29% of mice. Pten deficiency alone, without SB mutations, resulted in lung tumors in 11% of mice, whereas the rate in control mice was approximately 3%. In addition, thyroid cancer and other carcinomas, as well as the presence of bronchiolar and alveolar epithelialization, in mice deficient for Pten were also identified. Analysis of common transposon insertion sites identified 76 candidate cancer driver genes. These genes are frequently dysregulated in human lung cancers and implicate several signaling pathways. Culin3 (Cul3), a member of a ubiquitin ligase complex that plays a role in the oxidative stress response pathway, was identified in the screen and evidence demonstrates that Cul3 functions as a tumor suppressor.

Implications: This study identifies many novel candidate genetic drivers of lung cancer and demonstrates that CUL3 acts as a tumor suppressor by regulating oxidative stress. Mol Cancer Res; 13(8); 1238–47. ©2015 AACR.

Introduction

Most lung cancers are caused by smoking, which explains the higher mutation load in lung cancer compared with most other major epithelial cancers (1). Even though individual lung cancers harbor a large number of mutations, comprehensive genetic analyses of these cancers have identified few recurrent drivers, such as activating mutations in EGFR and KRAS, inactivating mutations in TP53, and translocations affecting ALK. Except for TP53, the percentage of tumors with either one of these driver mutations is less than 25% (2, 3). Another recurrent event found in non–small cell lung cancers (NSCLC) and small cell lung cancers (SCLC) is activation of the PI3K/AKT pathway (3–5), which is frequently caused by loss of PTEN (6). Because of this confusing abundance of genetic mutations and other genetic anomalies, it is difficult to pinpoint specific drivers in any given patient and many lung cancer genetic drivers have yet to be identified (7).

Mouse models have been used to confirm that these altered proteins result in increased rates of lung cancer. For example, loss of Tp53 in the lungs results in adenocarcinomas after a long latency and, if Rb is simultaneously deleted in the lungs, tumor latency is decreased and the tumor phenotype switches to SCLC (8). If loss of one or both alleles of Pten are introgressed into this model, tumor latency is reduced even further (9). Decreasing Pten protein levels in wild-type mice by introducing a hypomorphic Pten allele also results in lung cancer in 28% of mice (10). Interestingly, lung-specific deletion of Pten in mice did not result in tumors, but when combined with lung-specific activation of Kras(11), tumor latency was significantly decreased (11).

The aim of our experiments was to discover new genetic drivers of lung cancer by performing forward genetic screens in mice using the SB DNA transposon as a mutagen in lung epithelial cells. We performed one genetic screen on a wild-type background and three additional screens using mice with predisposing mutations...
in Tp53, p14ARF, and Pten. Contrary to our expectations, the only screen that produced at least twice as many lung tumors in the experimental animals compared with controls was in mice deficient for Pten. We sequenced the tumors that arose in these mice and identified 78 common transposon insertion sites (CIS) and were able to identify 76 candidate cancer genes. Over 85% of these candidate genes have documented alterations in ten or more human lung cancers, and several have been strongly implicated as drivers of lung cancer. The three genes identified with the most transposon insertions were Serinc3, Magi1, and Nckap5. There is evidence that Serinc3 is an oncogene, while Magi1 has tumor-suppressive activities (12, 13). We performed functional tests on another of our candidate cancer genes, Cul3, and found that reducing levels of Cul3 and/or Pten resulted in cancer phenotypes in human lung cancer cell lines. Furthermore, analysis of gene expression patterns in cells deficient for Cul3, Pten, or both Cul3 and Pten suggests that this phenotype may be due to alterations in the NRF2 signaling pathway.

Materials and Methods

Mice

Pten floxed mice (PtenLoxP/; ref. 14) on the C57Bl6/J background were a generous gift of Pier Pandolfi (Memorial Sloan Kettering, New York, New York) and National Cancer Institute, Frederick, MD. Tp53flERT2/Cre mice [129S4-Tp53tm3Tyj/Nci] strain 01XM3 and p14ARF−/− mice [B6.129-Cdkn2atm1Cjs/Nci] strain 01XG7 were purchased from the National Cancer Institute Mouse Repository. Both Tp53flERT2/Cre and p14ARF−/− mice were backcrossed >10 generations to the C57Bl6/J background. Conditional Sleeping Beauty transposase mice (RosaSBaseLsL; ref. 15) and additional Sleeping Beauty transposase mice (Spc-Cre; ref. 16) on the ICR/FVB/n background were a generous gift of Brigid Hogan (Duke University, Durham, NC). T2/Onc15 mice were generated as described (rosa 68; Spc-Cre mice were backcrossed to C57Bl6/J wild-type mice >10 generations). T2/Onc1 mice were backcrossed to C57Bl6/J wild-type mice >10 generations. T2/Onc4 mice were backcrossed to C57Bl6/J wild-type mice >10 generations. T2/Onc4 mice were backcrossed to C57Bl6/J background as described (TG6070; ref. 18). Mice were genotyped using DNA from tail biopsies. PCR protocols and primer sequences are available in Supplementary Data. All mice protocols were approved by the University of Minnesota’s Institutional Animal Care and Use Committee.

Cells

All cell lines, except HBEC, were obtained from ATCC and the authenticity of these cell lines was verified by short tandem repeat analysis (Johns Hopkins, Baltimore, MD). Human bronchial epithelial cells immortalized with CDK4 and hTERT were provided by John Minna (UT Southwestern; Dallas, TX). Functional assays were conducted in stage II lung cancer cells A549 or H522. 293T human embryonic kidney cells were transfected with OpenBiosystems lentiviral packaging mix with nonsilencing, Cul3 2702, Cul3 32413, or Cul3 351781 shRNA plasmids to produce lentivirus that harbors Cul3-specific shRNA sequence. Cells were transfected according to the Open Biosystems’ lentivirus production protocol. To make stable Cul3 knockdown cells, Cul3-specific shRNA encoding lentivirus was used to transduce A549 or H522 cells. The cells were then grown under puromycin selection in RPMI. A549 cells with stable Cul3 knockdown, or expressing the nonsilencing control, were then transfected with SABiosciences SureSilencing shRNA plasmids for human PTEN (catalog number KH003271H) or with the negative shRNA encoding plasmid control. The cells were maintained in RPMI with 1x penicillin, streptomycin, 10% FBS, 1μg/mL puromycin, and 32 μg/mL hygromycin at 37°C, and 5% CO2.

Histopathology and IHC

Formalin-fixed tissues were embedded in paraffin and stained with H&E. IHC for CC10 was performed using a goat anti-mouse C-terminus peptide CC10 polyclonal antibody (Santa Cruz Biotechnology) with detection by a horseradish peroxidase (HRP) Polymer Kit (Biocare). IHC for TGF-β (Dako) as the chromogen. IHC for SPC was performed using a rabbit anti-proSP-C polyclonal antibody (Millipore); detection was with a rabbit EnVision+ HRP-polymer kit (Dako) with DAB as the chromogen. IHC for SB was performed using goat polyclonal anti-SB (R&D Systems). More details are provided in Supplementary Methods.

Transposon insertion analysis

Detailed methods are available in Supplementary Materials. Briefly, LM-PCR was performed on DNA isolated from tumors. PCR amplicons were sequenced using the Illumina GALX sequencing platform. Sequences were mapped to the genome and CIs were identified using the TAPDANCE bioinformatics pipeline.

Western blotting

Cells were lysed in standard RIPA buffer and loaded onto 12.5% SDS–PAGE gels. Lysates were transferred to PVDF membranes and blocked in 5% milk TBS-T. The following primary antibodies were used: anti-CUL3 (Bethyl Labs A301-109A), anti-PTEN (138G6; Cell Signaling Technology #9559S), anti-NRF2 (D1C9; Cell Signaling Technology #8882S), anti-KEAP1 (D1G10; Cell Signaling Technology #7705S), and anti-GAPDH (14C10; Cell Signaling Technology #2118L). Goat–anti-rabbit IgG–HRP was used to detect primary antibodies (Santa Cruz Biotechnology SC-2004). HRP was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, product #34096) and visualized using a FluoroChemE imager (Protein Simple). Densitometry was measured using ImageJ software.

Cell proliferation assay

Cell proliferation was measured using an MTS assay for 5 to 6 days (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega). Cells (3,500) were plated in 96-well plates and OD490/650 levels were measured after 1 hour using a spectrophotometer (Epoch BioTek). Data were analyzed using GraphPad Prism.

Colony formation assay

Cells were grown in between layers of 0.5% Sea Plaque Low Melt Agarose (Lonza Cat. # 50101) in RPMI supplemented with 10% FBS, penicillin, streptomycin, and antibiotic selection for 3 weeks. Colonies were fixed, stained with crystal violet, and imaged. Colony counts were analyzed using ImageJ software.
Bead array
RNA was isolated from A549, A549-CUL3-KD, A549-PTEN-KD, and A549 CUL3-PTEN-DKD cells using TRIzol (Life Technologies; cat. no. 15996-026) following manufacturer’s protocol. RNA was analyzed using the Human HT-12v4 bead array (Illumina; cat. no. BD-103-0204). Differentially expressed genes were defined by pair-wise comparisons using rank invariant normalization following the gene expression workflow from Partek Genomics Suite software. All Bead Array data have been deposited in the Gene Expression Omnibus database (GSE68869).

Superoxide detection assay
Cells were seeded at equal numbers in 6-well plates and incubated for 24 hours. Cells were washed in PBS and then incubated with media containing 5 μM/L MitoSOX reagent (Molecular Probes, Cat. #M36008) for 3 hours. A minimum of 20 brightfield and red fluorescent images were taken for each well at ×20 magnification using an inverted fluorescence microscope (Nikon Eclipse TE200). Images were analyzed using ImageJ. Cells were counted in each brightfield image. Red fluorescence was measured using the ImageJ integrated density measurement function after setting the maximum and minimum pixel intensities to 30 and 90, respectively. Average integrated density/cell was calculated for each well.

Results
SB Mutagenesis drives lung tumor formation
We performed four forward genetic screens in mice to identify genes involved in lung cancer. Our screen in wild-type mice consisted of the following three alleles: (i) a conditional SB11 transposase allele (Rosa26-LsL-SB11; refs. 15, 19); (ii) a Cre-recombinase cDNA driven by the Surfaceant Protein-C promoter (Sp-Cre; ref. 16); and (iii) a concatamer of oncogenic transposons (T2/Onc; refs. 17, 18; Supplementary Fig. S1A–S1C). In these mice, SB mutagenesis occurs in lung epithelial cells because SB11 transposase enzyme is activated by the lung-specific Cre recombinase (Supplementary Fig. S2). Control mice consisted of littermates with two of the three alleles. We also conducted three additional screens using the same three alleles introgressed into mice with the following predisposing alleles: (i) p19ARFfl/fl (Supplementary Fig. S1D–S1F). These mice are referred to as p19, p53, and Pten. The p19ARFfl/fl allele is a germline knockout. The initial report describing these mice found that roughly 30% of these mice develop tumors within 6 months and the majority of these tumors are blood tumors or sarcomas (20). The p53fl/fl−R270H allele is a dominant negative p53 allele that is not expressed in cells unless Cre recombinase is present. Therefore, these mice are heterozygous for p53, except in Cre-expressing cells. In the absence of Cre, these mice develop tumors due to p53 heterozygosity; while in animals expressing a germline Cre, the tumor spectrum changes slightly to favor carcinomas (21). Ptenfl/fl−R270H mice lose expression of Pten in cells expressing Cre recombinase and this allele has been used to study brain and prostate cancer (14, 22).

The experimental genotype, number of mice, lung tumor penetrance, and median survival in the four screens are displayed in Table 1. Potential neoplasms in other tissues were also detected at necropsy (Supplementary Table S1), which may have resulted from leaky or non-lung-specific expression of the Spc-Cre transgene. Lung tumor penetrance in the wild-type, p19 and p53 screens, was not substantially higher in the experimental groups compared to the control groups, so we did not analyze these tumors. Instead, we focused on tumors arising in the Pten predisposed background, as there was a large difference in penetrance between all controls (6%) and experimental animals (29%).

The Pten cohort consisted of five different genotypes (Table 2). All animals were homozygous for the Ptenfl−/− knock-in allele and contained the conditional SB11 transposase. Because SB transposon mobilization in some cases is biased toward reinsertion into the donor chromosome, we used two strains of T2/Onc transgenic mice. In one strain, there were roughly 25 copies of the transposon linked as a concatamer and resident on chromosome 15 (T2/Onc(15); ref. 17), whereas the second strain contained approximately 215 copies of the transposon as a concatamer on chromosome 4 (T2/Onc(4); ref. 18). The difference in lung tumor penetrance between these two experimental lines was 23% for T2(Onc(4)) and 37% for T2(Onc(15)). Of the three control groups, the two with wild-type Pten had a tumor penetrance of 3% for T2(Onc(4)) and 4% for T2(Onc(15)), whereas the control group with loss of Pten had an intermediate penetrance of 11% (Table 2). Interestingly, we expected a higher penetrance in the experimental group with the larger number of transposons (T2/Onc(4)), but this group actually had a lower penetrance than the low-copy group (T2(Onc(15))). It is possible that the higher number of transposon insertions was detrimental to cell survival, and thus resulted in a lower tumor penetrance. Survival correlated with the presence or absence of Pten, as the Pten−/− control group became moribund at the same rate as the experimental group (Pten−/− and SB mutagenesis; Fig. 1). Upon histologic examination, both the Pten−/− control group and the experimental group had extensive bronchiolar epithelial hyperplasia, which may be the reason why moribidity was similar between these two groups.

There was no difference in survival between the mice carrying the different transposon alleles (Supplementary Fig. S3).

Because of the small size of the tumors that developed (Supplementary Fig. S4), in most cases, the entire tumor was excised and used for extracting DNA. In those cases where microscopic examination of lung lesions was performed, a spectrum of lesions was observed, including bronchiolar and alveolar epithelial

### Table 1. Lung tumor penetrance and median survival in four SB screens

<table>
<thead>
<tr>
<th>Screen</th>
<th>Genotype</th>
<th>Exp mice with lung tumors, % (n)</th>
<th>Median survival of exp mice (d)</th>
<th>Control mice with lung tumors, % (n)</th>
<th>Median survival of ctrl mice (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Rosa26-LsL-SB11 × T2/Onc × Spc-Cre</td>
<td>17 (55)</td>
<td>NA</td>
<td>12 (80)</td>
<td>NA</td>
</tr>
<tr>
<td>p19</td>
<td>Rosa26-LsL-SB11 × T2/Onc × Spc-Cre × p19ARFfl/fl−/−</td>
<td>6 (99)</td>
<td>303</td>
<td>8 (100)</td>
<td>326</td>
</tr>
<tr>
<td>p53</td>
<td>Rosa26-LsL-SB11 × T2/Onc × Spc-Cre × p53fl/fl−R270H</td>
<td>22 (116)</td>
<td>419</td>
<td>15 (87)</td>
<td>443</td>
</tr>
<tr>
<td>Pten</td>
<td>Rosa26-LsL-SB11 × T2/Onc × Spc-Cre × Ptenfl−/−</td>
<td>29 (62)</td>
<td>320</td>
<td>6 (94)</td>
<td>415</td>
</tr>
</tbody>
</table>

*Median survival of wild-type mice was not determined because the majority lived longer than the endpoint of 1.5 years.
Table 2. Lung tumor penetrance in Pten screen

<table>
<thead>
<tr>
<th>Group Genotype</th>
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<tbody>
<tr>
<td>Ctrl 1 Ptenloxp/loxp × Rosa26-LsL-SB11 × T2/Onc(4)</td>
<td>3 (3)</td>
</tr>
<tr>
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<td>4 (26)</td>
</tr>
<tr>
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<td>11 (37)</td>
</tr>
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Candidate cancer genes are dysregulated in human lung cancers

To determine the relevance to human cancer, we analyzed the overlap of the 75 human CIS orthologs with various lists of human cancer genes. The current version of the Cancer Gene Census lists 522 functionally validated cancer genes (26). Nine of the CIS human orthologs overlapped with these documented cancer genes, an overlap that would not be expected by chance ($P < 0.0001$; Supplementary Table S4). The majority of cancer genes reported in the census are linked to hematopoietic cancers. To determine the specific relevance to lung cancer, we compared our CIS list to two major sources documenting somatic lung cancer mutations, The Cancer Genome Atlas (TCGA) and the Catalog of Somatic Mutations in Cancer (COSMIC). The vast majority of all annotated genes have a documented somatic mutation in human lung cancer based on these two sources (>18,000 genes in COSMIC and >17,000 genes in TCGA). If we limit our analysis to genes with at least 10 reported mutations (COSMIC > 7,209 and TCGA = 5,513), we find a significant overlap with our CIS genes [COSMIC overlap = 52 (P value 5.3e-8), TCGA overlap = 44 (P value 3.5e-11), Supplementary Table S4 and Supplementary Methods].

Figure 1.
Kaplan–Meier survival curves for two control groups (Pten+/− and Pten+/−) and the experimental group (Pten−/− and SB). (Log-rank Mantel-Cox test P values < 0.0001 for Pten−/− vs. Pten+/− and SB vs. Pten+/−).

hypermethylated, attributable to proliferation of club cells, primary pulmonary adenoma and adenocarcinomas (Fig. 2). The diagnosis of pulmonary adenomas and adenocarcinomas was based on the criteria recommended by the Mouse Models of Human Cancers Consortium (23), and expression of prosurfactant protein C (SPC) and/or club cell 10-kDa protein (CC10) in the tumors, %

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Lung cancer has one of the highest mutation rates among all types of cancer making it difficult to discern cancer-associated genetic mutations from background mutations. Factors including heterogeneity between cancer patients and differences in mutation rates within the genome due to transcription-coupled repair (27) and DNA replication timing (28) also confound analyses. Two recent large-scale genomic analyses of human lung squamous cell carcinoma (SCC; ref. 3) and adenocarcinoma (AC; ref. 2) published lists of significant amplifications/deletions, mutations, and genomic rearrangements based on exome and whole-genome sequencing. These analyses used algorithms that took into account some of the confounding factors. Our list of CIS human orthologs was significantly enriched in all three categories of genes determined in those studies to be possible drivers based on ampls/dels, mutations, and rearrangements (Supplementary Table S4; P 0.001–0.018).

Pathway analysis

We used Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) to analyze canonical pathways enriched in our list of 75 CIS genes. As might be expected, the canonical pathway identified with the lowest P value was Molecular Mechanisms of Cancer (Supplementary Table S5). This association was based on seven genes (GBP2, TAB2, CREBBP, CTNNAL1, RBP1, SMAD4, and GSK3B). Other canonical signaling pathways that were associated with these genes included the NF-κB, RANK, Integrin, and NGF pathways.

Our mouse model generates cancers by creating multiple random mutations in a single cell, as every cell contains either approximately 25 (T2/Onc(15)) or ~215 (T2/Onc(4)) transposons that can be mobilized to create mutations. We reasoned that the cancers arising in these mice are likely generated from multiple independent transposon insertions, but the frequency of each of these mutations in the different mice might not reach the level of significance required for classification as a CIS. To find combinations of insertions that may be cooperating to cause tumorigenesis, we adapted an algorithm called frequent itemset mining (29, 30). The algorithm identifies combinations of insertions that frequently co-occur in multiple tumors. These groups of genes can reach statistical significance, even though they do not individually reach significance as a CIS. All transposon insertions were associated with the nearest annotated gene and frequent itemset mining was used to determine sets of genes co-occurring in tumors from at least three different mice (Supplementary Methods). The 10 sets of genes with -Log (P value) >5 are listed in Supplementary Table S6. All of the sets were coordinately mutated in 3 separate mice and half of the sets consist of three genes, whereas the other half consisted of four genes. There were 23 unique genes included in the 10 gene sets, and only six of these reached significance as an individual CIS. The majority of these genes are associated with cancer and we propose that the mutations caused by the transposon insertions in or near these sets work together to cause tumorigenesis.

Cul3 functions as a tumor suppressor gene in human lung cancer cells

In addition to demonstrating a strong correlation between our CIS list of candidate driver genes and genes frequently disrupted in human lung cancer, it is important to demonstrate that these genes functionally act as driver genes. To this end, we choose to analyze one of the candidate drivers discovered in our screen, Cul3 (Cul3). We selected Cul3 for several reasons. First, TCGA analysis of lung SCCs identified the oxidative response pathway, specifically KEAP1/CUL3/NFE2L2, as a potential driver in this cancer (3). Second, CUL3 was mutated in 10 human lung adenocarcinomas as reported in the COSMIC database (31). Finally, we wanted to validate a “middle of the list” gene and Cul3 was identified in 25% of the mice in our screen. Approximately half of our CIS genes were identified in a larger percentage, and half in a smaller percentage of mice. On the basis of the location and orientation of the transposon insertions in the Cul3-mutant tumors (Supplementary Fig. S6), we predict that the transposon is causing a loss-of-function mutation. In humans, the protein product of CUL3 is a core scaffolding protein in an E3 ubiquitin ligase complex that includes KEAP1 and RBX1. Known substrates of this complex include NRF2 (gene name NFE2L2), IKKβ, and other proteins (32, 33). This complex is important in regulating oxidative stress and the recent TCGA analysis of lung SCC indicates that members of the complex may be disrupted in a significant percentage of these lung tumors (3).

In support of our hypothesis that CUL3 is a tumor suppressor, a trend toward reduced levels of CUL3 protein was seen in a series of cancer cell lines compared with a normal human bronchiolar epithelial cell line (HBEC; ref. 34; Fig. 3). Two of the cell lines analyzed were from patients with stage II cancer (A549 and H522) and one line was from a patient with stage IV cancer (H2030).

**Figure 2.** Representative photomicrographs demonstrating histologic findings. Lung sections show bronchiolar and alveolar epithelial hyperplasia (A, H&E attributable to club cell hyperplasia (B, hyperplastic cells express CC10). C, (H&E) an adenoma that expresses CC10 (D). Lung section shows an adenocarcinoma (E, H&E) that expresses prosurfactant protein C (F). IgG serum control staining for CC10 and proSP-C is shown in G and H, respectively (see Supplementary Materials and Methods for more details). Bar sizes in A–H are 100, 100, 250, 50, 500, 50, 50, and 50 microns, respectively.
The mutational status of A549 and H522 has been analyzed by whole-genome sequencing (35). CUL3, PTEN, and RBX1 were wild-type in both cell lines, whereas A549 had a nonsynonymous mutation in KEAP1. Surprisingly, we found that further reduction of CUL3 levels in these lines using shRNA resulted in an increased growth rate compared with control lines (Figs. 4 and 5, P value day 5 < 0.05 both graphs). Knockdown of CUL3 in the stage IV line, H2030, did not have a significant effect on growth (data not shown). Because our mouse model was predisposed to cancer using a conditional Pten knockout allele, we generated A549 cell lines stably expressing shRNA targeting CUL3 alone, PTEN alone, or both CUL3 and PTEN (Supplementary Fig. S7). Consistent with our hypothesis that loss of CUL3 cooperates with loss of PTEN, the double knockdown cells grew faster than the control line or either of the single knockdown lines (Fig. 5, P value day 5 < 0.01). To test the role of CUL3 and PTEN in anchorage-independent growth, we grew the cell lines in soft agar. Similar to the proliferation assay, the CUL3/PTEN double knockdown line formed the most colonies in soft agar compared with the single knockdown lines and the control line, although the difference between the CUL3 single and the double knockdown was not significant (Fig. 5).

Loss of CUL3 and PTEN activates the NRF2 signaling pathway

We measured changes in gene expression in the A549 cell strains with reduced levels of CUL3, PTEN, or both CUL3 and PTEN using the Illumina BeadArray platform. Interestingly, knockdown of PTEN resulted in changes in expression of only 11 genes (fold change > 2.0, FDR < 0.05), whereas knockdown of CUL3 caused changes in 87 genes, with the majority (59) being downregulated (Supplementary Table S7). Knockdown of both CUL3 and PTEN resulted in changes in expression of 120 genes, also with a majority (76) being downregulated.

Knockdown of CUL3 could affect many proteins regulated by ubiquitination because CUL3 is a scaffolding component of BTB–CUL3–RBX1 ubiquitin ligase complexes. The BTB protein functions as the targeting protein in this complex and CUL3 can
the same group identified 232 NRF2 target genes based on ChIP-Seq analysis of cells treated with sulforaphane, which induces NRF2-mediated anti-oxidative stress response (39, 40).

To understand the mechanism of synergy between CUL3 and PTEN, we identified genes that were discordantly regulated when CUL3, PTEN, or CUL3/PTEN were knocked down compared with control cells. Only one gene had a greater than 2-fold change in all three comparisons that was discordant. The gene, DKK1, is a secreted inhibitor of Wnt signaling and DKK1 down-regulation is associated with poor prognosis in lung cancer (41, 42). DKK1 was increased over 3-fold when PTEN was knocked down, but in CUL3 or CUL3/PTEN knockdown cells, DKK1 mRNA was decreased over 3-fold.

To measure the functional effect of CUL3 and PTEN in regulating oxidative stress, we incubated A549 control and knockdown cells with MitoSOX Red, a dye that fluoresces in response to levels of superoxide. As predicted, A549 cells with reduced levels of CUL3 and/or PTEN had significantly less fluorescence than control cells (Fig. 6, P < 0.005). Although there was no significant difference between the three knockdown lines, in three replicate experiments, the CUL3 single knockdown A549 cells consistently had the lowest amount of fluorescence. These results suggest that reduced levels of PTEN and/or CUL3 result in decreased production of superoxide, which could be protective for cancer cells.

Discussion

It is likely that multiple agents targeting several pathways will be required to extend the survival of lung cancer patients. For this strategy to work, new targets must be identified and validated. To this end, we have developed a method of identifying novel drivers of lung cancer using SB mutagenesis in forward genetic screens in mice. Using this method, we were able to identify 76 potential lung cancer drivers. A caveat to any mouse study is that the lung tumors generated may not be genetically or physiologically equivalent to human tumors. Unfortunately, identifying driver genes in human lung cancer is particularly problematic due to the large number of background passenger mutations. If our forward genetic screen in mice were identifying bona fide drivers of human lung cancer, we would expect to find the orthologous genes mutated or altered to a significant extent in human lung cancer. Analysis of large-scale genomic datasets on human lung cancer indicates that almost 90% of the orthologous human genes we identified in our mouse screen are mutated or present in genomic regions of amplification, deletion, or rearrangement in a significant portion of human lung cancers (Supplementary Table S4). This suggests that these genes are likely drivers of human lung cancer and warrant further investigation as potential targets for therapy.

The first screen we performed was on a wild-type background, and although we saw a slight increase in lung tumor penetration...
Knockdown of CUL3 and/or PTEN in A549 cells results in reduced superoxide production. Representative brightfield (left) and red fluorescent (right, converted to grayscale) images of (A and B) A549 control cells and (C and D) A549 CUL3/PTEN knockdown cells treated with MitoSOX. E, graph of average MitoSOX fluorescence/field of view/cell comparing control cells with PTEN, CUL3, and CUL3/PTEN knockdown A549 cells. **, t test P < 0.01; ***, t test P < 0.001.
stress pathway is activated in CUL3/PTEN double knockdown cells, which could explain how these cancer cells are able to withstand the strong oxidative stress generated in cancer. We also measured the effect of knocking down CUL3 and PTEN in the human lung cancer cell line and noted a significant decrease in the production of reactive oxygen species in cells with reduced CUL3 and PTEN. Our results indicate that loss of CUL3 and PTEN may synergize to produce some phenotypes, including proliferation (Fig. 5C) and oxidative stress gene expression regulation, while other phenotypes, including anchorage-independent growth (Fig. 5B) and superoxide production (Fig. 6) do not demonstrate a synergistic effect in the double knockdown.

In conclusion, this study has identified a large number of potential genetic drivers of lung cancer. Furthermore, we have shown that loss of CUL3 and PTEN may be driving tumorigenesis by activating the NRF2 oxidative stress pathway.

Disclosure of Potential Conflicts of Interest

D.Largeaspaeda has ownership interest (including patents) and is a consultant/advisory board member for Discovery Genomics, Inc. No potential conflicts of interest were disclosed by the other authors.

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Transposon Mutagenesis Screen Identifies Potential Lung Cancer Drivers and CUL3 as a Tumor Suppressor

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