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Nullifying the CDKN2A/B Locus Promotes Mutant K-ras Lung Tumorigenesis

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Abstract

Lung cancer commonly displays a number of recurrent genetic abnormalities and about 30% of lung adenocarcinomas carry activating mutations in the *K-ras* gene; often concomitantly with inactivation of tumor suppressor genes *p16INK4A* and *p14ARF* of the *CDKN2A/B* locus. However, little is known regarding the function of *p15INK4B* translated from the same locus. To determine the frequency of *CDKN2A/B* loss in human mutant *K-ras* lung cancer, The Cancer Genome Atlas (TCGA) database was interrogated. Two-hit inactivation of *CDKN2A* and *CDKN2B* occurs frequently in mutant *K-ras* lung adenocarcinoma patients. Moreover, *p15INK4B* loss occurs in the presence of biallelic inactivation of *p16INK4A* and *p14ARF* suggesting that *p15INK4B* loss confers a selective advantage to mutant *K-ras* lung cancers that are *p16INK4A* and *p14ARF* deficient. To determine the significance of *CDKN2A/B* loss in vivo, genetically engineered lung cancer mouse models that express mutant *K-ras* in the respiratory epithelium were utilized. Importantly, complete loss of *CDKN2A/B* strikingly accelerated mutant K-ras driven lung tumorigenesis, leading to loss of differentiation, increased metastatic disease and decreased overall survival. Primary mutant *K-ras* lung epithelial cells lacking *CDKN2A/B* had increased clonogenic potential. Furthermore, comparative analysis of mutant *K-ras;CDKN2A* null with *K-ras;CDKN2A/B* null mice and experiments with mutant *K-ras;CDKN2A/B* deficient human lung cancer cells indicated that *p15INK4B* is a critical tumor suppressor. Thus, the loss of *CDKN2A/B*

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is of biological significance in mutant *K-ras* lung tumorigenesis by fostering cellular proliferation, cancer cell differentiation and metastatic behavior.

Implications—Implications: These findings indicate that mutant *K-ras;CDKN2A/B* null mice provide a platform for accurately modeling aggressive lung adenocarcinoma and testing therapeutic modalities.

Keywords

CDKN2AB; mutant KRAS; lung cancer; mouse lung cancer models

Introduction

Lung adenocarcinoma, a leading source of cancer related mortality world-wide (1), harbors recurrent genomic abnormalities that provide a framework for the selection of targeted therapeutic agents (2-4). For these reasons, intense research efforts are under way to characterize the biological and therapeutic significance of recurrent genomic abnormalities in NSCLC. Activating mutations of *KRAS* occur in ∼30% of lung adenocarcinomas. KRAS is a small GTPase that regulates several oncogenic networks (5). Mutant KRAS plays a causative role in lung tumorigenesis, however it is not sufficient for the induction of high-grade lung adenocarcinomas in the absence of co-operating mutations that often involve the *CDKN2AB* locus (6-9).

The *CDKN2AB* locus encodes for several tumor suppressors. *CDKN2A* contains $p16^{INK4A}$ and $p14^{ARF}$ ($p19^{Arf}$ in mice), while *CDKN2B* contains $p15^{INK4B}$. Both, $p16^{INK4A}$ and $p15^{INK4B}$ promote G1 cell cycle arrest by inhibiting the CDK4/CDK6-retinoblastoma family of tumor suppressors, while $p14^{ARF}$ upregulates TP53 by inactivating its negative regulator MDM2 (10, 11). Both $p16^{INK4A}$ and $p15^{INK4B}$ are highly similar and appear to have originated from gene duplication (12-14), while $p14^{ARF}$ gene expression is initiated from an exon intercalated between $p16^{INK4A}$ and $p15^{INK4B}$ and an alternative reading frame of exon 2 and 3 of $p16^{INK4A}$ (14, 15) (Figure 1A). Several studies established that $p16^{INK4A}$ and $p14^{ARF}$ are bona fide tumor suppressors *in vivo* including in mutant *KRAS* lung adenocarcinomas (6, 9, 15-17).

Despite convincing biochemical evidence that $p15^{INK4B}$ is part of the TGF beta signaling pathway, much less is known regarding its tumor suppressor function *in vivo* (18). For example, $p15^{INK4B}$ is rarely mutated independently of the other *CDKN2AB* genes (14). Furthermore, in the absence of other mutations, $p15^{INK4B}$ null mice have only a mild tumor predisposition (19). However, the group of Anton Berns demonstrated that *Cdkn2ab* null mice are tumor-prone and develop an expanded tumor spectrum as compared to *Cdkn2a* null mice (20). This study led to the conclusion that $p15^{INK4B}$ provides a tumor suppressive function that is critical in the absence of $p16^{INK4A}$ and $p19^{ARF}$ (20). It is unknown whether $p15^{INK4B}$ has a tumor suppressive role in other tumor models or whether its status influences tumorigenesis driven by activating mutations of proto-oncogenes commonly occurring in human cancer. For instance, even though $p15^{INK4B}$ loss has been reported to occur in lung adenocarcinoma, its biological significance has yet to be established in this context (2, 21, 22).

In this manuscript, we study the significance of *Cdkn2ab* deficiency in the biology of mutant *Kras* lung adenocarcinoma with two genetically engineered mouse models that express mutant KRAS in the respiratory epithelium, and human lung cancer cell lines. Furthermore, we determined the mutation frequency and pattern of the *CDKN2A* and *CDKN2B* loci by analyzing *The Tumor Cancer Genome Atlas* database. Our data indicate that $p15^{INK4B}$ provides a tumor suppressive function in mutant KRAS lung tumorigenesis.

Materials and Methods

Plasmids and lentiviral particle production

The cDNA of murine *Cdkn2b* (clone ID 3495097) was obtained from Open Biosystems (Thermo) and cloned into pLVX-tight-puro (Clontech Laboratories, Mountain View, CA). Recombinant lentiviral particles were generated in 293T cells according to manufactures procedures.

Mouse models and tumor burden assessment

Tet-op-*Kras*;CCSP-*rtTA* were obtained from H. E. Varmus (6), *HIST1H2BJ-GFP* mice were from the Jackson Laboratory (23), $Ink4ab^{-/-}$ mice (hereafter named $Cdkn2ab^{-/-}$) were provided by Dr. Anton Berns (The Netherlands Cancer Institute Amsterdam, Netherlands), $LSL\text{-}Kras^{G12D}$ mice from the NCI mouse repository (24) and $Ink4a/Arf^{flox/flox}$ mice from the Jackson Laboratory (25). Mice were maintained in a mixed background (FVB/N/CD-1). Experiments were performed with F3 generation progeny or later progenies, and comparisons were made with littermates. We obtained lung specific KRAS expression at 4 weeks of age either by feeding mice with doxy implemented food pellets (Harlan Laboratories) or by intratracheal administration of Adenovirus-Cre at 8 weeks of age (University of Iowa, Gene transfer Vector Core) (6, 26). We administered doxycycline at 4 weeks of age to attempt to develop lung tumors prior to the development of tumors involving other organs, a common occurrence in *Cdkn2ab* null mice. All animal studies were completed according to the policies of the UT Southwestern Institutional Animal Care and Use Committee. We used digital quantification of the area occupied by tumors to the area of total lung using the NIH ImageJ (v1.42q) software.

Primary cultures and colony formation assays

Single cell suspensions of primary respiratory cells were sorted with a MoFlo cell sorter to select and plate GFP fluorescent cells on either irradiated or mitomycin C treated mouse embryonic fibroblasts (MEFs).

Cell lines

Human NSCLC cell lines H460 and A549 were from the Hamon Center cell line repository (UT Southwestern Medical Center).

Quantitative real-time PCR

We extracted RNA using the RNeasy kit (Qiagen) and generated cDNA using iScript™ cDNA kit (Biorad). For quantitative real time PCR, we used iTaq SYBR green supermix with ROX (Biorad).

Western blotting

We used the following antibodies: anti-PARP (Cell Signaling), anti-HSP90 (BD Bioscience), anti-p15 (Sigma) and anti-phospho-H3 (Millipore).

Immunohistochemistry

We used the following antibodies: GFP (Chemicon), phospho-S6 ribosomal protein, HMG2A and phospho-Erk (Cell Signaling), ALDH1A1 (Abcam), phospho-H3 (Millipore), NKX2-1 (Santa Cruz), SP-C (Chemicon) and CCSP (Santa Cruz). An HMG2A staining scoring system was used as follows: tumors were considered negative if no stain was visible, positive in few cells, positive in clusters of cells and positive if >50% of tumor cells scored HMG2a positive.

Analyses of the TCGA database

Information regarding mutation, segmented copy number, methylation, and clinical data of primary lung adenocarcinomas were obtained from TCGA data portal (https://tcga-data.nci.nih.gov/tcga) on June 8th, 2013. Only non-silent mutations were considered as described (27). Kaplan-Meier was used to estimate the survival curves and comparisons were performed using the log-rank test using SPSS Statistics 17.0. One-way ANOVA followed by Tukey *post hoc* test was used to compare the pathologic stage among groups.

Copy number and microarray analysis of lung cancer cell lines

Most of the KRAS and CDKN2A mutations data were obtained from the COSMIC database (Sanger Institute, UK), the literature or from our unpublished whole-exome sequencing data (28). mRNA expression microarrays were performed by us with Illumina HumanWG-6 V3 as previously described (29). They were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession GSE32036. Whole-genome SNP profiling was done as previously described (30).

Results

Cdkn2ab deficiency accelerates mutant KRAS lung tumorigenesis in mice

To characterize the effect of deficiency of genes encoded by *Cdkn2ab* on lung tumorigenesis (Fig. 1A), we generated compound mutant mice expressing mutant KRAS in the respiratory epithelium. We crossed *LSL-Kras*^{G12D} mice (*LSL-Kras* mice), which encode a latent mutant *Kras* gene, with mice harboring conditional alleles of *Cdkn2a* or *Cdk2ab* (20, 24, 25). We achieved lung specific tumor development by intratracheal delivery of adenovirus encoding for the Cre recombinase.

The overall median survival of *LSL-Kras;Cdkn2a*$^{-/-}$ was significantly decreased as compared to their littermates with a wild type *Cdkn2a* locus, while *LSL-Kras;Cdkn2a*$^{+/-}$ had a trend toward an intermediate survival between *Cdkn2a* wild type and null genotypes (Figure 1B). The survival of *LSL-Kras;Cdkn2ab*$^{-/-}$ mice was significantly decreased as compared to *LSL-Kras*, *LSL-Kras;Cdkn2ab*$^{+/-}$ or *LSL-Kras;Cdkn2a*$^{-/-}$ mice (Figure 1C). These findings demonstrate that complete loss of *Cdkn2ab* significantly promotes mutant KRAS lung tumorigenesis.

Mutant KRAS induces high-grade and metastatic lung adenocarcinomas in *Cdkn2ab* null mice

At the time of death, the majority of lung parenchyma was occupied by lung tumors in every genotype (Supplemental Figure S1A-C). This finding suggests that death is caused by respiratory compromise.

We determined genotype-specific histological features: lungs of *LSL-Kras* and *LSL-Kras;Cdkn2a*$^{-/-}$ mice contained predominantly lung adenomas (Figure 1D and E, panel 1 and 2) and occasional carcinomas with nuclear atypia (not shown). In contrast, *Cdkn2ab* null mice carried primarily high-grade lung adenocarcinomas consisting of atypical nuclei arranged in papillary structures (Figure 1E, panel 3). Moreover, we detected tumors arising from the bronchial epithelia in all three genotypes (Figure 1D and E, panel 4).

We observed that 44% of *LSL-Kras;Cdkn2a*$^{-/-}$ mice carried metastatic lesions in mediastinal lymph nodes, or the thoracic wall (Figure 1F and Supplemental table S1). This metastatic behavior was even more pronounced in *LSL-Kras;Cdkn2ab*$^{-/-}$ mice, where 89% of the mice carried lung tumors that metastasized to mediastinal lymph nodes and the myocardium (Figure 1F and Supplemental table S1). These finding indicate that *p15*^{Ink4b} deficiency leads to high-grade lung adenocarcinomas with aggressive behavior including the property to generate loco-regional metastasis.

Kras;Cdkn2ab null lung tumors display upregulation of proliferative markers

We determined the level of activity of targets of KRAS, which have been implicated in tumorigenesis, and of the mitotic marker phospho-Histone 3 (p-H3). *LSL-Kras;Cdkn2a* null and *LSL-Kras;Cdkn2ab* null tumors were diffusely positive for p-S6 and p-Erk1/2, with more intense staining in tumor segments with atypical nuclei and papillary morphology, while *LSL-Kras;Cdkn2ab*$^{+/+}$ lung tumors were faintly positive for p-S6 and p-Erk1/2 (Figure 2A).

The staining pattern of p-H3 was genotype dependent: *LSL-Kras;Cdkn2a*$^{-/-}$ tumors stained positive for p-H3 mostly at the tumor edge. In contrast, *LSL-Kras;Cdkn2ab*$^{-/-}$ tumors stained positive throughout the tumor mass. Furthermore, the number of mitotic cancer cells increases significantly in *LSL-Kras;Cdkn2ab*$^{-/-}$ tumors as compared to *Cdkn2a*$^{-/-}$ and *Cdkn2ab*$^{+/+}$ lung tumors (Figure 2A and B). We conclude that the loss of *Cdkn2ab* promotes cellular proliferation and progression of KRAS driven lung tumors.

Cdkn2ab deficiency leads to reduction of differentiation of KRAS^{G12D} driven lung tumors

To further characterize the lung tumors that develop in *LSL-Kras;Cdkn2ab* null mice, we evaluated the lung epithelial marker NK2.1 homeobox 1 (NKX2-1) and chromosomal high motility group protein HMGA2, which define the status of differentiation of lung adenocarcinoma driven by mutant KRAS in the mouse (31, 32). NKX2-1 is a master regulator of lung differentiation, which is expressed in the majority of human lung adenocarcinomas (33). Human lung tumors that are negative for NKX2-1 are poorly differentiated, display high-grade histological features and an aggressive behavior (33-35). NKX2-1 restrains lung cancer progression by enforcing a lung epithelial differentiation program by repressing the chromatin regulator HMGA2 (31, 32).

We found that lung tumors of *LSL-Kras;Cdkn2ab*$^{+/+}$ and *LSL-Kras;Cdkn2a*$^{-/-}$ mice are predominately NKX2-1 positive and HMGA2 negative (Figure 2C, D and Supplementary Figure S2A). In contrast, about 21% of *LSL-Kras;Cdkn2ab*$^{-/-}$ tumors lose NKX2-1 completely (Supplemental Figure S2A). In addition, about 73% of *LSL-Kras;Cdkn2ab*$^{-/-}$ tumors increase their number of HMGA2 positive cells (Figure 2C-E and Supplementary Figure S2B). Finally, we also determined that the metastases that developed in *Cdkn2a*$^{-/-}$ and in *Cdkn2ab*$^{-/-}$ lung tumors stain positive for HMGA2 (Supplemental Figure S2C). This observation strongly suggests that HMGA2 positive lung tumors have metastatic potential.

These data indicate that complete loss of the *Cdkn2ab* locus significantly promotes lung cancer tumor progression in the mouse. These observations imply that p15^{INK4B} provides an important tumor suppressor function in opposing mutant KRAS tumorigenesis.

Transgenic mouse model of genetically labeled cancer initiating cells

To characterize in better detail the biological consequence of *Cdkn2ab* deficiency in oncogenic *Kras* lung adenocarcinoma, we generated mice that allow the tagging and isolation of lung cancer cells. We took advantage of *CCSP-rtTA;Tet-op-Kras* mice, which carry a transgene encoding *Kras*^{G12D} under the control of the tetracycline operator (*Tet-op-Kras)*, and a transgene expressing the reverse tetracycline transactivator in the respiratory epithelium under the control of the Clara cell secretory protein promoter *(CCSP-rtTA)*. *Tet-op-Kras;CCSP-rtTA* mice develop lung cancer with 100% penetrance when continuously exposed to doxycycline (doxy) (6).

To visualize mutant KRAS expressing lung epithelial cells, we crossed the *Tet-op-Kras;CCSP-rtTA* strain with mice that carry a transgene encoding histone H2B fused to green fluorescence protein (H2B-GFP) under the control of the tetracycline operator (tetO-*HIST1H2BJ-GFP*) (23). We determined that upon exposure to food pellets impregnated with doxy, Tet-op-*Kras,CCSP-rtTA*;tetO-*HIST1H2BJ-GFP* (*Kras-GFP* mice) transgenic mice express H2B-GFP in lung epithelial cells (Supplemental Figure 3A-C) with the characteristics of type II pneumocytes (Figure 3A).

When exposed continuously to doxy, *Kras-GFP* mice develop mutant KRAS lung tumors that are also positive for H2B-GFP (Figure 3B). Lung tumor cells showed predominantly nuclear NKX2-1 staining; and cytoplasmic SP-C staining (Figure 3C). Tumor cells were predominantly negative for CCSP but we detected faint CCSP expression in a portion of

tumor cells as compared to the strong staining in the bronchiolar epithelial lining (Figure 3C). We conclude that this mouse model allows successful H2B-GFP tagging of lung cancer cells.

Cdkn2ab deficiency promotes the development of high-grade non-small cell lung carcinomas

We determined the effect of *Cdkn2a* or *Cdkn2ab* deficiency on lung tumorigenesis in *Kras-GFP* mice. Significantly, *Kras-GFP ;Cdkn2ab*$^{-/-}$ mice developed an increased number of tumors as well as an increased tumor burden 4.5 months after induction of mutant KRAS (Figure 4A, B and C). In this mouse model, loss of *Cdkn2a* resulted in an intermediate number of tumors and tumor burden at 4.5 months (Supplemental Figure S4A and B). We also observed an increase in tumor burden in *Kras-GFP;Cdkn2ab*$^{-/-}$ mice at time of death (Supplemental Figure S4C). These results indicate that *Cdkn2ab* loss promotes both mutant KRAS tumor initiation and progression.

Tumors arose within the lung parenchyma and alveoli in all three genotypes (Figure 4D); however, in this model only *Cdkn2ab* null mice generated tumors that arose from the bronchial epithelia (Figure 4D, panels 4 and 9 and Supplemental Figure S4D). This observation suggests that *Cdkn2ab* loss reduces the threshold to develop oncogenic KRAS induced tumors in distal bronchioles. In addition, *Cdkn2a* or *Cdkn2ab* null mice developed lung tumors characterized by nuclear atypia and papillary structures (Figure 4D panels 2, 7 and 3,8 and Supplemental Figure S4D) that closely resembles aggressive human NSCLC (36, 37). Others and we described similar morphologic changes in lung adenocarcinomas of *Cdkn2a* null mice induced by the doxy regulated mutant *Kras* transgene (6, 9, 38).

Lung tumors of *Cdkn2ab* deficient mice show an increase in the number of cells stained positive for targets of activated KRAS, such as p-S6 ribosomal protein and p-ERK1/2 (Supplemental Figure S4E). These findings suggest that *Cdkn2ab* deficiency leads to increased proliferative activity. These features reproduced the characteristics of lung tumors developed in *LSL-Kras;Cdkn2ab*$^{-/-}$ mice.

Kras-GFP;Cdkn2ab$^{+/+}$ mice had the longest survival, *Kras-GFP;Cdkn2a*$^{-/-}$ mice had an intermediate survival and *Kras-GFP;Cdkn2ab*$^{-/-}$ mice had the shortest survival among the three groups (Supplemental Figure S4G). These differences however were due mainly to sarcomas and lymphomas, which preclude the assessment of lung cancer-specific mortality (Supplemental Figure S4H and Supplemental Table S2).

H2B-GFP positive epithelial cells form colonies *in vitro*

To evaluate the properties of lung epithelial cells marked by H2B-GFP we obtained single cell suspensions from the lungs of *Kras-GFP* and CCSP-*rtTA*;tetO-*HIST1H2BJ-GFP* control transgenic mice (control-GFP mice). We found that one out of 85 H2B-GFP positive oncogenic KRAS expressing lung epithelial cells formed colonies whereas only 1 out of 150 GFP positive, but wild type *Kras* lung epithelial cells were able to do so (Supplemental Figure S5A and B). We did not detect any difference in the number of lung epithelial cells present in colonies of either control or mutant KRAS expressing GFP positive cells

(Supplemental Figure S5C). This finding suggests that mutant KRAS promotes clonogenicity, but not cell proliferation under these conditions.

We were able to passage these cells up to 5 times onto feeder cultures, suggesting that they can be propagated *in vitro* and have short-term self-renewal abilities. Of note, similar colony formation ratios were reported for bronchoalveolar stem cells (BASC), putative epithelial stem cells present at the bronchoalveolar junction (39).

We were unable to propagate H2B-GFP positive cells obtained from the respiratory epithelium beyond 5 passages due to loss of viability. In contrast, H2B-GFP positive primary lung epithelial cells obtained from *Kras-GFP;Cdkn2a*$^{-/-}$ or *Kras-GFP;Cdkn2ab*$^{-/-}$ mice readily give rise to cell lines when grown as monolayer cultures in conventional tissue cultures plates and retain a subpopulation of H2B-GFP positive cells for up to 20 passages (Supplemental Figure S5D and E). Moreover, H2B-GFP positive cells are positive for lung epithelial markers and the cancer stem cell marker ALDH1A1 (Supplemental Figure S5F). As expected, only H2B-GFP positive cells express mutant *Kras* (Supplemental Figure S5G). Moreover, mutant *Kras* expression is doxy dependent and cannot be reinduced (Supplemental Figure S5H). Taken together, these data suggest that deficiency of the genes of the *Cdkn2ab* locus promote immortalization of the respiratory epithelial cells.

Loss of *p15*^{INK4B} promotes mutant KRAS tumorigenesis in human lung cancer

To determine the frequency of the loss of genes of the *CDKN2AB* locus, we determined the mutation status of *p15*^{INK4B}, *p16*^{INK4A} and *p14*^{ARF} in a panel of 23 human NSCLC lines which express mutant KRAS. For this purpose we used direct sequencing to detect point mutations and SNP analysis the detect gene copy number alteration (Table 1). We established that 6 of the cell lines carried homozygous deletions of *CDKN2AB* and 15 cell lines carried at least heterozygous deletion of *CDKN2AB*. Only two cell lines (HCC44 and H1155) did not lose a single copy of the *CDKN2AB* allele. We validated nullyzygosity of the *CDKN2AB* locus in A549 and H460 by quantitative RT-PCR (Figure 5A). These findings indicate that loss of function of all three tumor suppressors of *CDKN2AB* is a common event in human lung adenocarcinoma.

We next analyzed patient data from *The Cancer Genome Atlas* (TCGA) database. Data with mutation, copy number, and methylation was available for 323 lung adenocarcinoma patients and showed that 30% (n=96) had oncogenic *KRAS* mutations. In addition, 51 out of the 96 patients (53%) with *KRAS* mutations also had two-hit *CDKN2A* inactivation by mutation, chromosomal deletion, and/or promoter methylation. Of these patients, 17 (18%) also showed inactivation in the *CDKN2B* locus by homozygous deletions. None of the patients in this database showed inactivation of *CDKN2B* but not of *CDKN2A*. These data indicate that the loss of the *CDKN2AB* locus is a common occurrence in human non-small cell lung cancer (Figure 5B). Notably, deficiency of *CDKN2A* is associated with higher pathologic stage (Supplemental table S3), poorer overall survival (Figure 5C) and progression-free survival (Figure 5D) in mutant *KRAS* lung adenocarcinoma patients. While there is a tendency to poorer progression-free survival with inactivation of *CDKN2B* in addition to *CDKN2A* (P=0.19), this difference was not significant. Anyway, inactivation of either *CDKN2A* or *CDKN2AB* is associated with poorer progression-free survival and

overall survival (Supplemental Figures S6A and B) independently of the *KRAS* mutation status.

p15^{INK4B} expression promotes growth arrest in human lung cancer cells

To determine the functional effect of p15^{INK4B} expression *in vitro*, we ectopically expressed 15^{INK4B} in mutant *KRAS* human lung cancer cell lines that also carry homozygous deletions of the *CDKN2AB* locus. We determined that reintroduction of 15^{INK4B} leads to an increase in the percentage of A549 and H460 cells in the G1 phase of the cell cycle (Figure 6A). Furthermore, we noticed a significant increase of the percentage of subdiploid cells in A549 cells, which indicate the presence of apoptosis. Western blot analysis confirmed that expression of p15^{INK4B} downregulates the phosphorylation of mitotic marker p-H3 (Ser 10) (Figure 6B). These results suggest that the expression of p15^{INK4B} promotes a tumor suppressor program that counteracts the proliferative effects of mutant KRAS in NSCLC cells.

Discussion

Recent technical advancements have allowed a systematic evaluation of statistically recurrent somatic mutations in NSCLC (2, 3). This data set holds the promise to better understand lung tumorigenesis and to identify novel treatments for lung cancer patients.

In this study we show that genetic inactivation of the *CDKN2A* and *CDKN2B* loci is a common event in mutant KRAS lung adenocarcinoma. By analyzing the TCGA database we determined that inactivation of *CDKN2A* by either somatic mutation, chromosomal deletion, and/or promoter hypermethylation, and inactivation of *CDKN2B* by homozygous deletion occur in about 50% and 20% of human lung adenocarcinomas, respectively. Previous reports did not have sufficient power to determine whether inactivation of the genes of *CDKN2AB* occurs with significant frequency in mutant *KRAS* lung cancer (2, 40). By analyzing the large TCGA sample, it is apparent that inactivation of *CDKN2AB* occurs at a significantly higher frequency than previously noted. Furthermore, we noticed that loss of *CDKN2B* rarely, if ever, occurs in the absence of *CDKN2A* somatic inactivation. Our analysis of the TCGA data set also indicates that loss of *CDKN2A* is associated with higher stage, higher incidence of metastasis, and consequently, decreased overall survival in mutant *KRAS* lung cancer patients. These findings have significant clinical implications since they suggest that mutant KRAS lung cancers with two-hit inactivation of CDKN2A identify a subset of patients with high-risk disease. In this regard it is of interest that these patients could benefit from treatment with focal adhesion kinase inhibitors (9).

We recently reported that expression of oncogenic KRAS in the respiratory epithelium significantly reduced the longevity of *p16^{Ink4a}/p19^{Arf}* null mice (9). We observed a further increase in lung cancer mediated mortality in mutant *Kras* mice conditionally deficient in the entire *Cdkn2ab* locus. This observation suggests a model whereby p15^{INK4B} loss confers a selective advantage to tumor cells that have lost p16^{INK4a} and p14^{ARF}. However, we did not detect a worse clinical outcome in patients with mutant *KRAS* lung cancers with loss of *CDKN2AB* as compared to patients with lung cancers mutant for *KRAS* and *CDKN2A*. We reason that this discrepancy could be due several variables including insufficient sample size

of the TCGA data set or the presence of concurrent mutations that affect the phenotype of human cancer that are not present in genetically engineered mice.

We determined histologically that *Cdkn2a* deficiency leads to a higher percentage of high-grade lung tumors in Tet-o-*Kras* mice than in *LSL-Kras* mice, while the lung tumor phenotype of mutant *Kras;Cdkn2ab* null mice is consistent between the two mouse models. It is likely that these differences are either due to the strength of the promoters used to express mutant *Kras* or to the fact that we exposed mice to doxy at 4 weeks of age to attempt to obtain lung tumors prior to the emergence of tumors in other sites. We reason that *LSL-Kras* mice, owing to the fact that mutant *Kras* is expressed from the endogenous promoter, model more closely mutant KRAS tumorigenesis.

Our *in vivo* cell-tracking experiments are consistent with a recent report indicating that a subset of alveolar type II cell co-expressing CCSP and SP-C give rise to lung adenocarcinomas upon mutant KRAS expression (41). The observation that loss of *Cdkn2ab* promotes the clonogenic ability of lung epithelial cells and their ability to readily give rise to cell lines supports the notion that *Cdkn2ab* regulates cell proliferation. This assertion is further supported by our observation that reintroduction of $p15^{INK4B}$ induces antiproliferative effects in human NSCLC lines. We also noticed that once mutant Kras was extinguished in *Cdkn2ab* null lung epithelial, it could no longer being induced. This observation leads to the speculation that mutant KRAS facilitates the maintenance of a cell state permissive the expression of the CC10 promoter driving rtTA or the accessibility of the Tet-op element driving mutant KRAS.

Overall, our data provide new genetic evidence that the loss of the three genes residing in *CDKN2AB* promotes mutant KRAS lung tumorigenesis by fostering cellular proliferation, cancer cell differentiation and metastatic behavior. We propose that mutant *Kras;Cdkn2ab* null mice provide a platform to accurately model aggressive mutant *Kras* lung adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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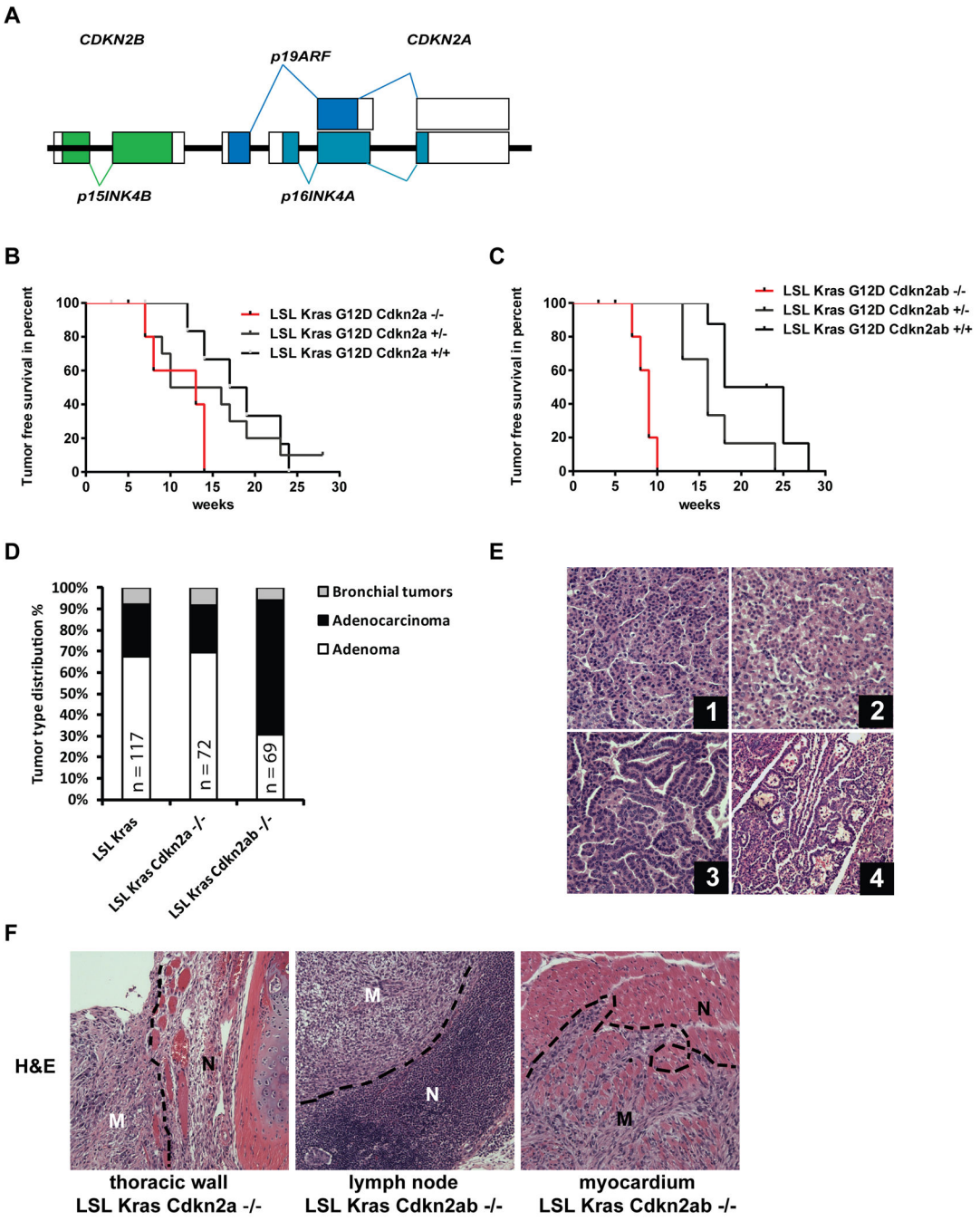


Figure 1. Loss of *Cdkn2ab* results in aggressive lung cancer in an oncogenic *Kras* conditional mouse

(A) Schematic of the *Cdkn2ab* locus in the mouse (not to scale). **(B)** Kaplan Meier survival curve of mice with the indicated genotypes (n=8, p=0.024, Log-Rank, Mantel-Cox, wild type versus *Cdkn2a* null). **(C)** Kaplan Meier Survival curve of mice with the indicated genotypes. Note that while *LSL-Kras;Cdkn2ab*$^{+/+}$ mice have a half-life of 21.5 weeks (n=8), *LSL-Kras;Cdkn2ab*$^{-/-}$ mice (n=7) have their half-life reduced to 9 weeks (p<0.0001, Log-Rank, Mantel-Cox). **(D)** Histogram of tumor type distribution of conditional mice with the

indicated genotypes. Numbers of tumors counted are indicated. **(E)** Representative images of lung cancers stained with Hematoxylin and Eosin (H&E). (1) Lung adenoma of *LSL-Kras* and (2) of *LSL-Kras;Cdkn2a*$^{-/-}$ mice. (3) Adenocarcinoma of *LSL-Kras;Cdkn2ab*$^{-/-}$ mice and (4) bronchial tumor of *LSL-Kras;Cdkn2ab*$^{-/-}$, magnification 200 fold. **(F)** Metastases in the indicated genotypes and anatomic location. M: metastatic tissue, N: normal tissue. H&E staining, magnification 100 fold.

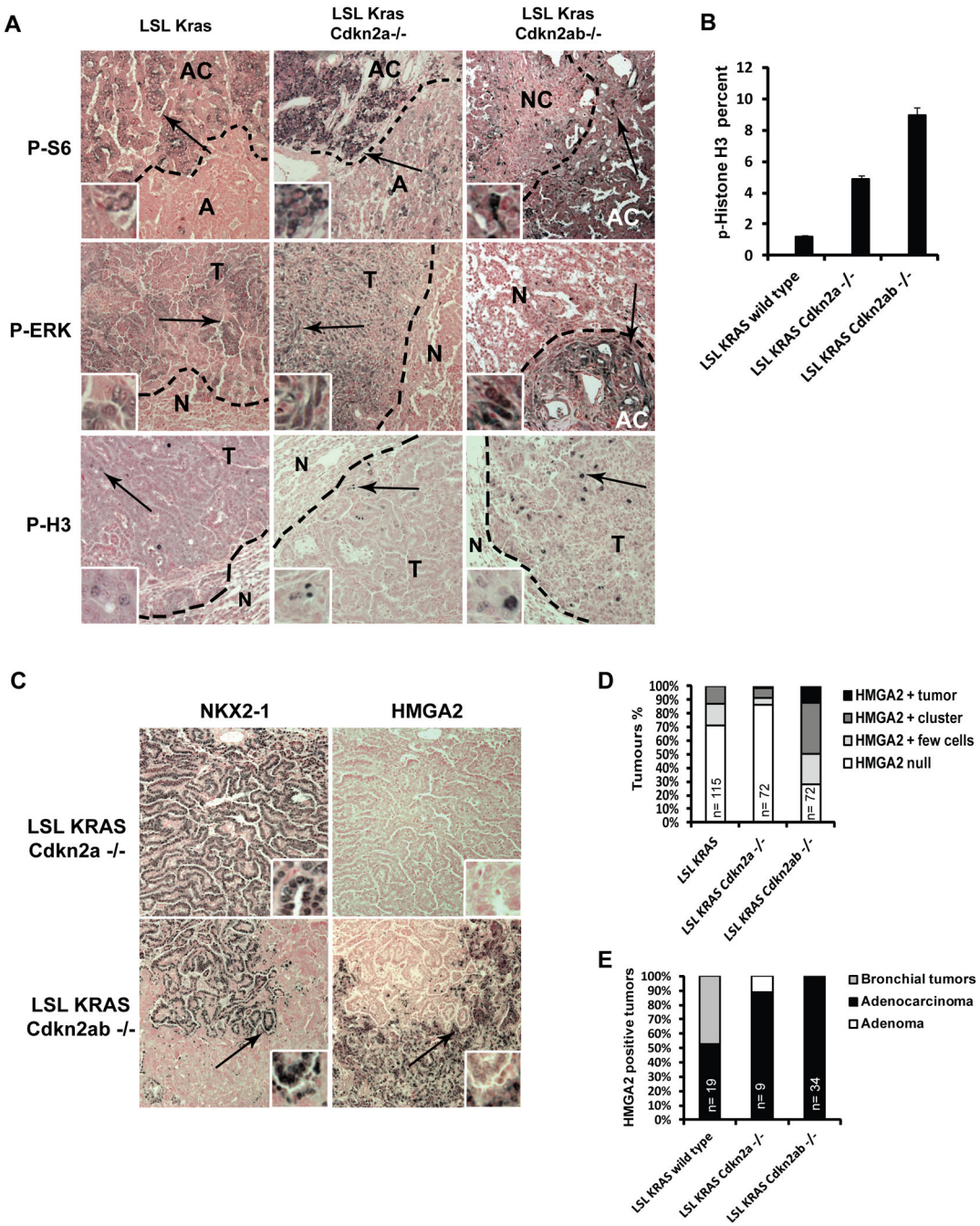


Figure 2. Loss of *Cdkn2ab* increases proliferation, and tumor progression in mutant *Kras* lung tumors

(A) Images of lung tumors stained with the indicated antibodies (100X). Antibody staining is shown in brown, nuclear fast red counter-stain is pink-red. Arrows show location of inlets. A – Adenoma, AC – Adenocarcinoma, T – tumor, N - normal tissue. **(B)** Histogram of the percentage of p-H3 positive cells lung tumors. **(C)** Sections of lung tumors stained as indicated (100X). Arrows indicate the location of inlets. **(D)** Histogram representing percentage of lung tumors with the indicated HMGA2 staining pattern (refer to methods for

scoring scale). **(E)** Anatomic location and classification of HMGA2 positive tumors. Number of tumors is indicated.

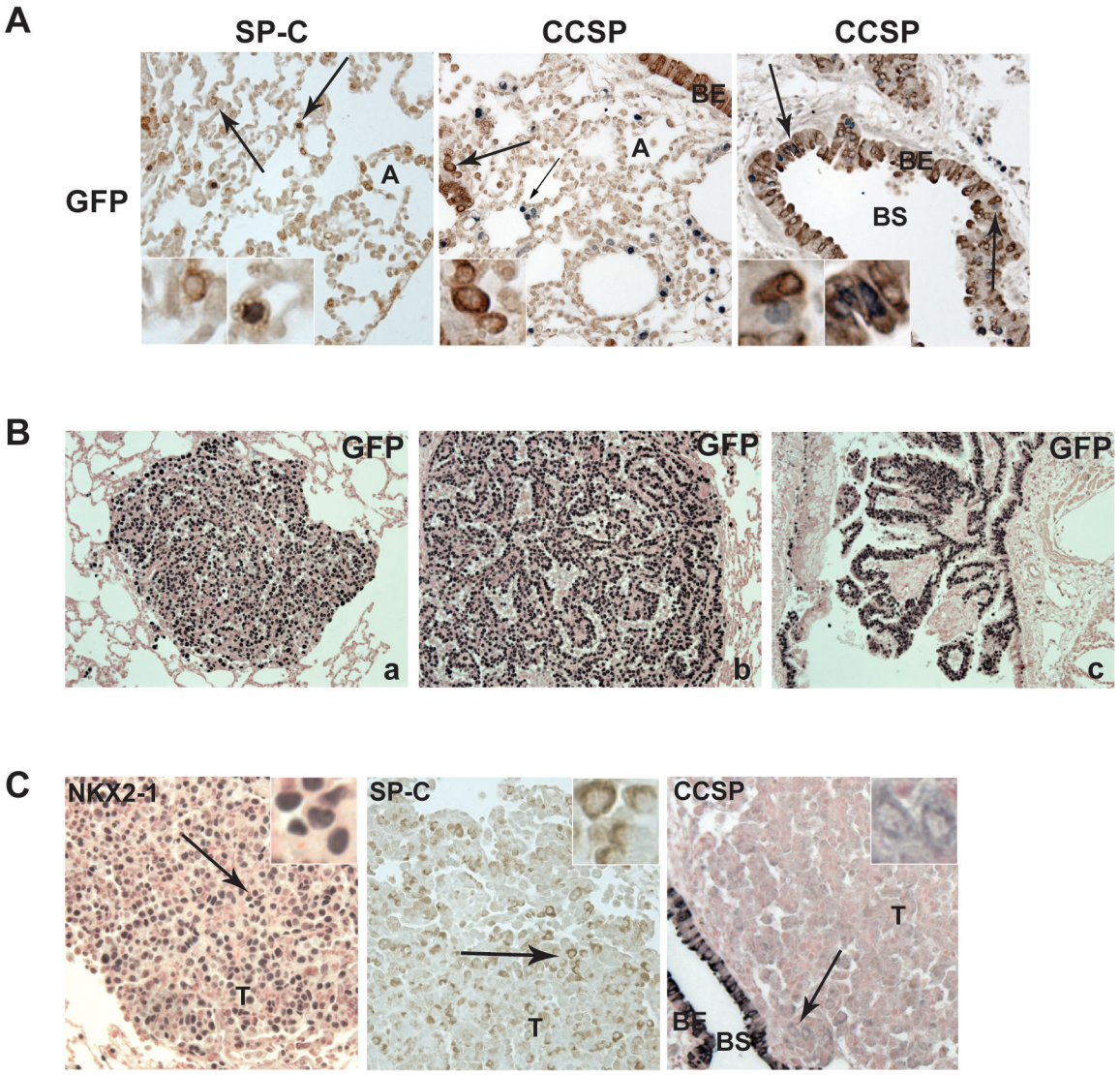


Figure 3. Lung epithelial cells and tumors genetically labeled by H2B-GFP

(A) Lung sections stained with the indicated antibodies. H2B-GFP positive cells in the alveoli stain positive for SP-C suggesting that they are alveolar type II pneumocytes. H2B-GFP staining: black-grey, SP-C and CCSP staining: brown. Arrows indicated the location of the inlets. Magnification 200X. A. **(B)** Immunohistochemistry of lung tumors from *Kras-GFP* mice stained as indicated: a) adenoma, b) adenocarcinoma, c) bronchial tumor (100X). **(C)** *Kras-GFP* tumors stained as indicated. Arrows indicate the location of the inlets. Images are enlarged 200 fold. CCSP-rtTA transgene is expressed, albeit at low level, in lung cancer cells. These data independently confirm the observations of Fisher et al. regarding the pattern of expression of CCSP-rtTA (6).

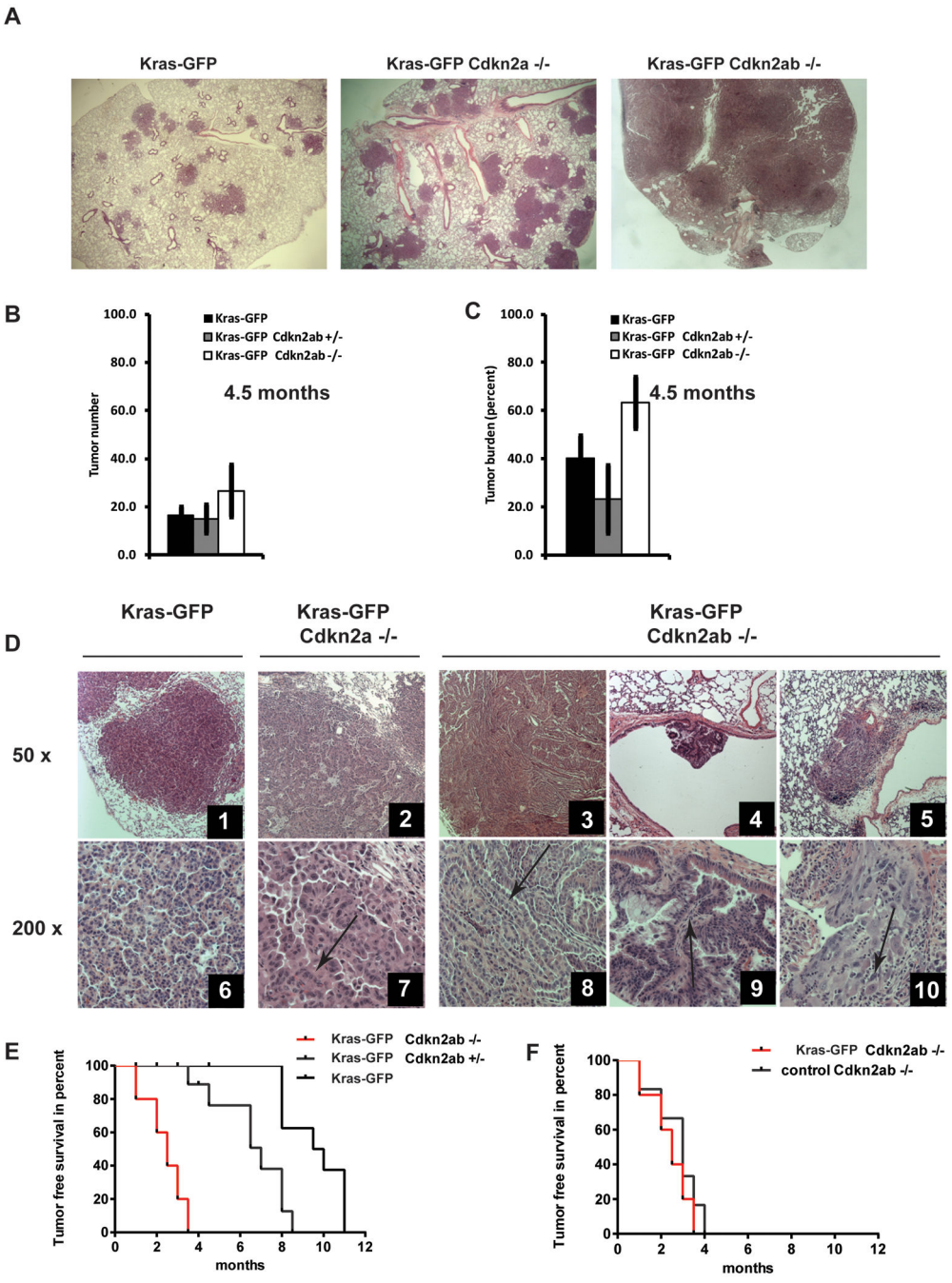


Figure 4. Loss of *Cdkn2ab* results in high-grade lung cancer and increased tumor burden

(A) Representative lung section of mice of the indicated genotypes 4.5 months after doxy exposure (15X magnification). **(B-C)** Histograms show tumor number or burden in mice of the indicated genotype after 4.5 months of doxy. Note increased tumor number/burden in mutant *Kras-GFP;Cdkn2ab*$^{-/-}$ mice. **(D)** Lung tumors of the indicated genotypes. Arrows indicate areas with papillar features and atypical nuclei (panels 6, 7, 8, 9) or with poor differentiation (panel 10).

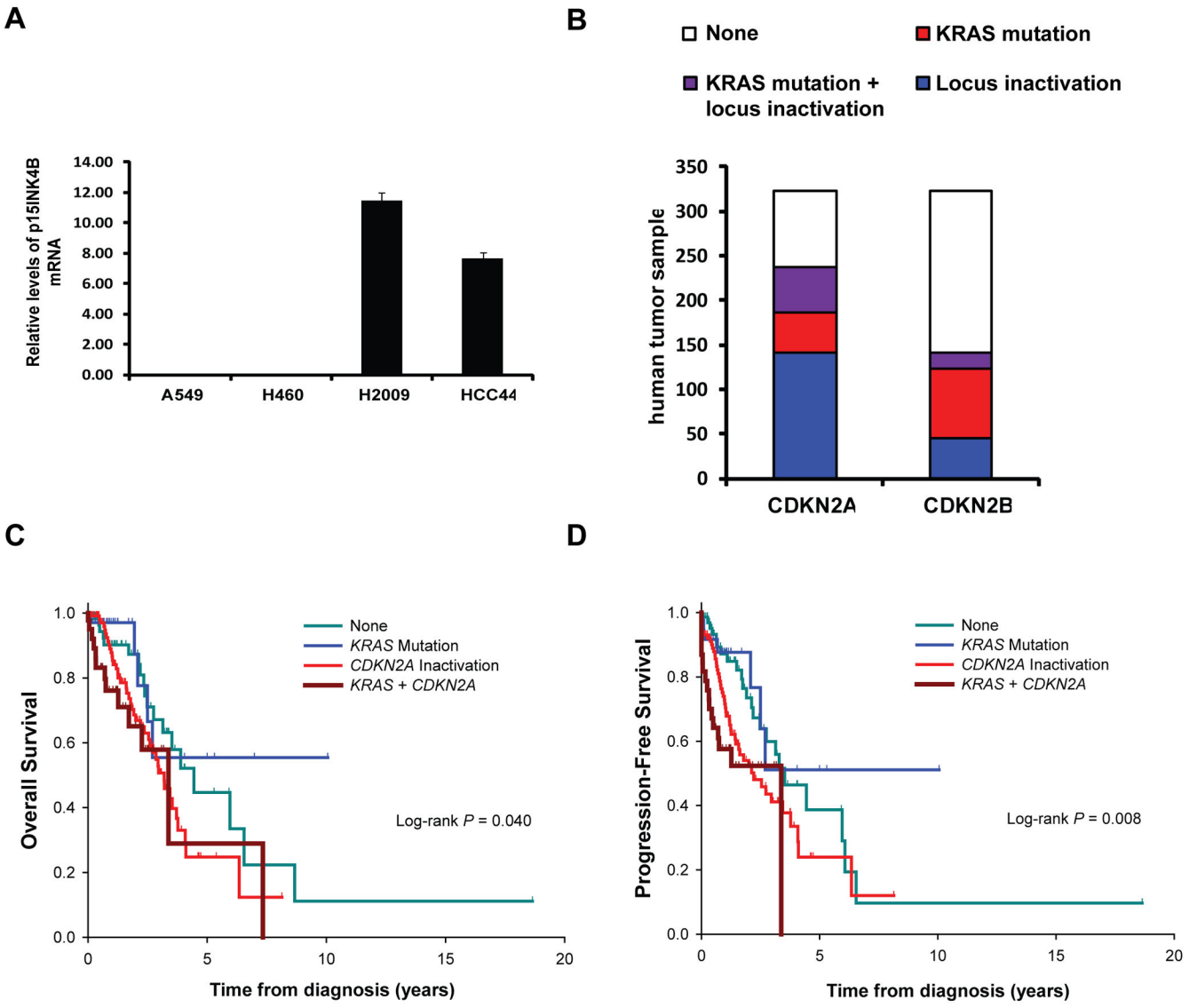
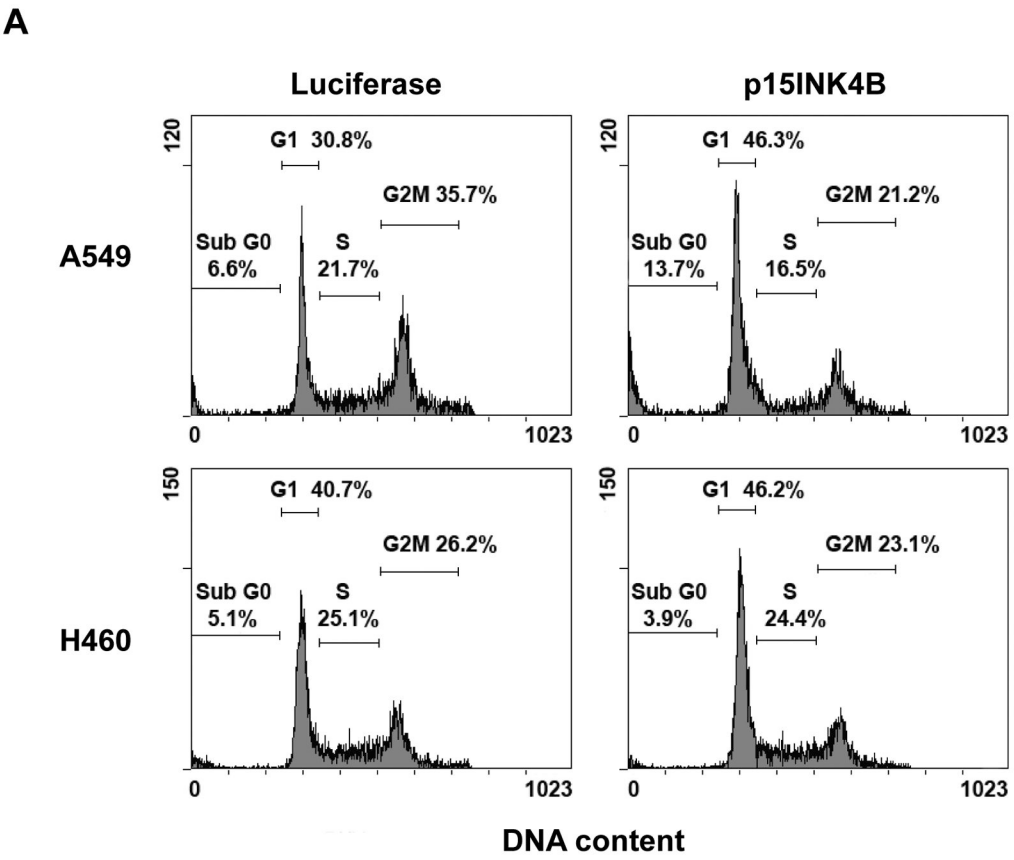


Figure 5. *CDKN2AB* status in human mutant *KRAS* lung adenocarcinoma

(A) Histogram of $p15^{INK4B}$ mRNA in NSCLC cell lines normalized to *GAPDH*. **(B)** Histogram shows the frequency of *KRAS* mutations and *CDKN2A* or *CDKN2AB* inactivation in lung adenocarcinoma in the TCGA data set. **(C)** Overall survival and **(D)** progression-free survival curves of patients with lung adenocarcinoma carrying mutant *KRAS* and/or inactivation of *CDKN2A*.



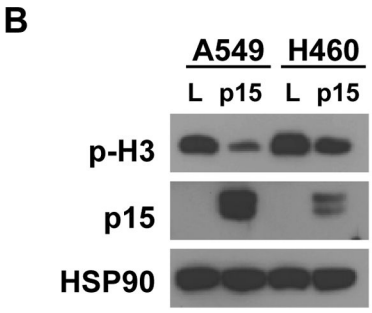


Figure 6. Reintroduction of p15^{INK4B} in *CDKN2AB* deficient lung cancer cell lines causes in growth arrest and apoptosis

(A) Cell cycle analysis of A549 and H460 cells after 72h of luciferase or p15^{INK4B} expression. Both cell lines show an increase in the G1 phase of the cell cycle. A549 cells also showed an increase in Sub-G1 fraction suggesting a portion of cells undergoes apoptosis. **(B)** Western blot analysis of A549 and H460 cells transduced as indicated. L: luciferase.

Table 1

CDKN2AB status of *KRAS* mutant non-small cell lung cancer cell lines

CDKN2AB mutation status is indicated as WT – wild type, HOMO – homozygous loss, HET – heterozygous loss, MUT – KRAS mutation. Pathologic classification of cell lines is indicated as it was given at the time the cell lines were raised.

Cell Line	Tumor Subtype	CDKN2AB Status	KRAS
A549	Adenocarcinoma	HOMO	MUT
H460	Large Cell	HOMO	MUT
H1944	Adenocarcinoma	HOMO	MUT
HCC1171	NSCLC	HOMO	MUT
HCC4017	Large Cell	HOMO	MUT
HOP-62	Adenocarcinoma	HOMO	MUT
H23	Adenocarcinoma	HET	MUT
H157	Squamous	HET	MUT
H358	Adenocarcinoma	HET	MUT
H441	Adenocarcinoma	HET	MUT
H1355	Adenocarcinoma	HET	MUT
H1373	Adenocarcinoma	HET	MUT
H1734	Adenocarcinoma	HET	MUT
H2009	Adenocarcinoma	HET	MUT
H2122	Adenocarcinoma	HET	MUT
H2347	Adenocarcinoma	HET	MUT
H2887	NSCLC	HET	MUT
HCC366	Adenosquamous	HET	MUT
HCC461	Adenocarcinoma	HET	MUT
HCC515	Adenocarcinoma	HET	MUT
Calu-6	Adenocarcinoma	HET	MUT
H1155	Large Cell	WT	MUT
HCC44	Adenocarcinoma	WT	MUT