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A Synthetic Lethal Interaction between K-Ras Oncogenes and Cdk4 Unveils a Therapeutic Strategy for Non-small Cell Lung Carcinoma

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SUMMARY

We have unveiled a synthetic lethal interaction between K-*Ras* oncogenes and *Cdk4* in a mouse tumor model that closely recapitulates human non-small cell lung carcinoma (NSCLC). Ablation of *Cdk4*, but not *Cdk2* or *Cdk6*, induces an immediate senescence response only in lung cells that express an endogenous K-*Ras* oncogene. No such response occurs in lungs expressing a single *Cdk4* allele or in other K-*Ras*-expressing tissues. More importantly, targeting *Cdk4* alleles in advanced tumors detectable by computed tomography scanning also induces senescence and prevents tumor progression. These observations suggest that robust and selective pharmacological inhibition of Cdk4 may provide therapeutic benefit for NSCLC patients carrying K-*RAS* oncogenes.

INTRODUCTION

Genetic interrogation of the cell cycle in mice has revealed that most cell types proliferate well in the absence of the interphase Cdks, Cdk2, Cdk4, and Cdk6 (Santamaría et al., 2007). A fourth interphase Cdk, Cdk3, is inactivated by a naturally occurring mutation in most strains of laboratory mice, thus indicating that it is dispensable for normal homeostasis (Ye et al., 2001). However, interphase Cdks, either individually or in combination, are essential to drive proliferation of certain cell types during specific developmental stages (Malumbres and Barbacid, 2009). For instance, Cdk4 is essential for proliferation of insulin-producing beta cells only during postnatal development (Rane et al., 1999; Tsutsui et al., 1999). Likewise, mice lacking Cdk2 and Cdk4 complete embryonic development to die at birth due to a defect in the number of cardiomyocytes (Barrière et al., 2007). Yet, ablation of Cdk2 and Cdk4 in adult mice does not result in obvious pathological conditions except for hyperglycemia, a direct consequence of the essential role of Cdk4 for proliferation of postnatal pancreatic beta cells (Barrière et al., 2007). These findings have raised the possibility that interphase Cdks may also be selectively required by tumor cells depending on their cellular origin and, possibly, their pathogenetic profile (Malumbres and Barbacid, 2009).

Previous studies have provided evidence that Cdk4 and its cognate cyclin, Cyclin D1, are required for the development of

Significance

CDK inhibitors have failed as anti-cancer agents due to their limited activity and significant toxicity. Recent genetic evidence indicates that whereas Cdk1 is essential for the mammalian cell cycle, interphase Cdks, Cdk2, Cdk4, and Cdk6, are only essential for proliferation of highly specialized cells. Here, we provide genetic and pharmacological evidence indicating that Cdk4, but not Cdk2 or Cdk6, is essential for proliferation of lung cells providing they express a K-*Ras* oncogene. To date, a selective CDK4 inhibitor has shown no significant therapeutic benefit in clinical trials against leukemias and breast tumors. Our results suggest that this compound as well as more potent CDK4 inhibitors should be tested in clinical trials against K-*RAS*-driven lung adenocarcinomas.

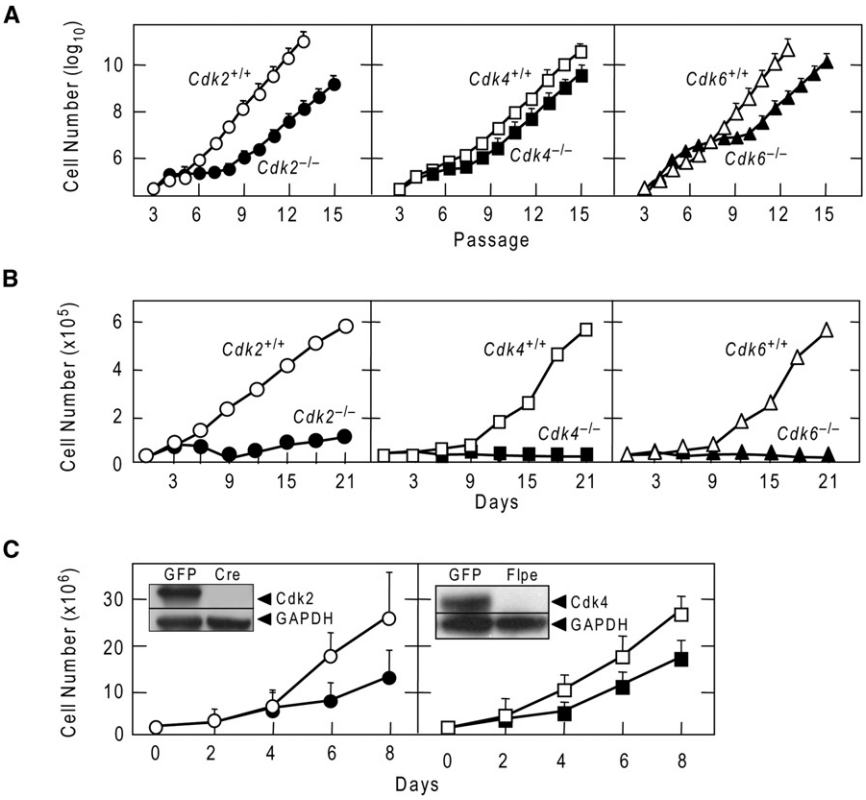

Figure 1. The Proliferative Advantage Conferred by an Endogenous K-*Ras*^{G12V} Oncogene Requires Expression of Interphase Cdks

(A) Immortalization of primary MEFs following a 3T3 protocol including those derived from *K-Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{+/+}$ (open circles) and *K-Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ (solid circles) littermates; *K-Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open squares) and *K-Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid squares) littermates; *K-Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{+/+}$ (open triangles) and *K-Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{-/-}$ (solid triangles) littermates. Cells were cultivated in DMEM media supplemented with 10% FBS and 600 nM 4OHT.

(B) Growth curves of the above primary MEFs cultivated in DMEM media supplemented with 2% FBS and 600 nM 4OHT. Symbols are as those described in (A).

(C) Growth curves of foci derived from (left) *Cdk2*$^{lox/lox}$ MEFs and (right) *Cdk4*$^{frt/frt}$ MEFs transformed by H-*Ras* and *E1a* oncogenes and infected with adenoviruses encoding (left) Cre and (right) Flpe recombinases (solid symbols) or GFP (empty symbols). Inserts show Western blot analysis of Cdk2 and Cdk4 protein levels at the conclusion of the experiment. Data shown represent mean ± SD, n = 3.

breast tumors induced by ErbB-2 (Landis et al., 2006; Yu et al., 2006). Likewise, mice *null* for Cdk4 are resistant to skin tumors induced by the two-stage DMBA+TPA carcinogenesis protocol or by expression of a K5-Myc transgene (Rodriguez-Puebla et al., 2002; Miliani de Marval et al., 2004). The fact that these experiments were carried out with mice lacking Cdk4 in their germ line makes it impossible to determine whether Cdk4 is required for initiation of tumorigenesis, for instance, by decreasing the number of susceptible cells, or for tumor progression by limiting the proliferative properties of cancer cells. On the contrary, ablation of Cdk2 in mice lacking its putative inhibitor, the p27^{Kip1} tumor suppressor, had no effect on tumor development (Martín et al., 2005). Thus, indicating that Cdk2 does not mediate the tumor suppressor properties of p27^{Kip1} in spite of the well-known interaction between these molecules in in vitro assays.

In this study, we have interrogated the role of the interphase Cdks in the development of non-small cell lung carcinomas (NSCLC) driven by an endogenous K-*Ras* oncogene (Guerra et al., 2003) with the ultimate goal of identifying potential synthetic lethal interactions between oncogenic K-*Ras* signaling and loss of Cdk activity. Identification of such synthetic interactions may serve to design better therapeutic strategies to treat K-*RAS*-driven NSCLC in human patients.

RESULTS

Loss of Individual Cdks Abrogate the Proliferative Advantage Induced by K-*Ras*^{G12V} in Mouse Cells

To evaluate the contribution of individual interphase Cdks to the various phenotypes triggered by endogenous K-*Ras* oncogenes, we first established primary MEFs derived from K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice (Guerra et al., 2003) deficient for Cdk2, Cdk4, or Cdk6 (Ortega et al., 2003; Malumbres et al., 2004; Barrière et al., 2007). As previously reported, expression of the resident K-*Ras*^{G12V} oncogene upon exposure to 4OHT bypassed the replicative senescence response displayed by MEFs during "culture shock" before they become adapted to grow in vitro (Guerra et al., 2003; Tuveson et al., 2004). Ablation of any of the interphase Cdks "restored" this senescence response, albeit they did not prevent cells from become immortal (Figure 1A). K-*Ras*^{G12V} expression also allowed these cells to proliferate under limiting serum availability (2% of FBS), a condition that does not support expansion of normal MEFs (Tuveson et al., 2004). Interestingly, elimination of any individual interphase Cdk eliminated the ability of these K-*Ras*^{G12V}-expressing MEFs to grow under limiting serum conditions (Figure 1B).

Fully transformed MEFs were also sensitive to loss of interphase Cdks. Conditional *Cdk2*$^{lox/lox}$ or *Cdk4*$^{frt/frt}$ MEFs (Ortega et al., 2003; Barrière et al., 2007) were transformed by ectopic expression of H-*Ras* and *E1a* oncogenes. Individual foci were subsequently infected with adenoviral particles expressing Cre or Flpe recombinases to eliminate the conditional *Cdk2* or *Cdk4* alleles, respectively (Figure 1C). Ablation of these *Cdk* alleles significantly reduced the proliferation of transformed MEFs. These observations are specific for transformed MEFs given that acute deletion of *Cdk2* or *Cdk4* in nontransformed *Cdk2*$^{lox/lox}$ or *Cdk4*$^{frt/frt}$ MEFs had no significant effect on their proliferative properties (Ortega et al., 2003; Barrière et al., 2007). These results suggest that the proliferative advantages conferred in vitro by *Ras* oncogenes require a full complement of interphase Cdks.

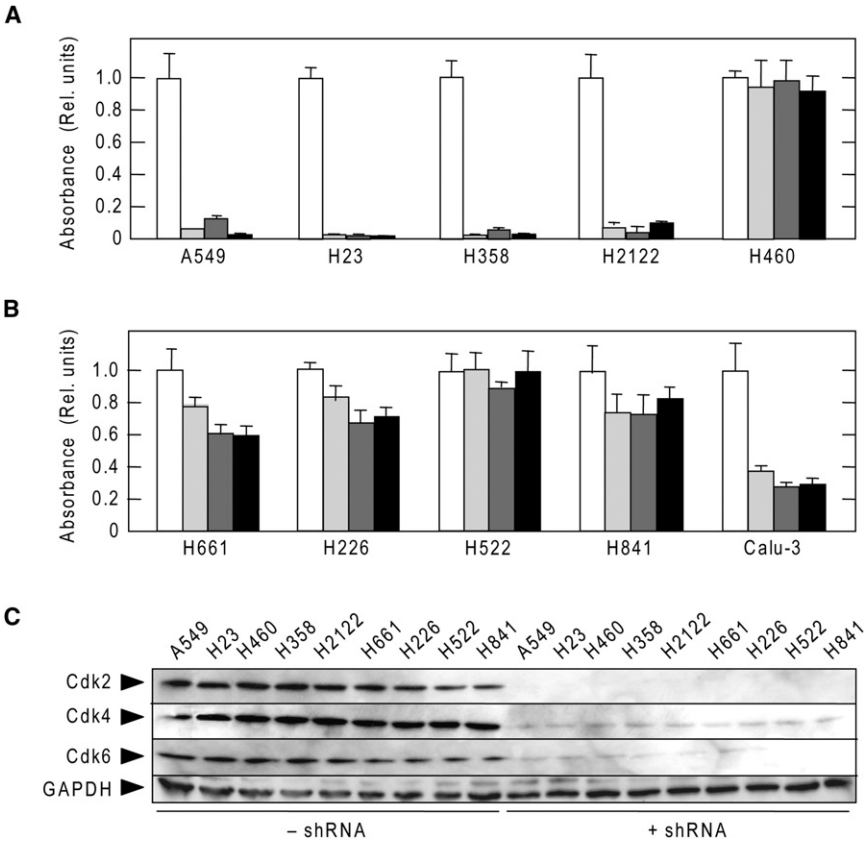

Figure 2. Knockdown of Interphase CDKs Hinders Proliferation of Human NSCLC Cell Lines Harboring K-*RAS* Oncogenes

(A) Human NSCLC cell lines carrying K-*RAS* oncogenes were infected with lentiviral particles expressing shRNAs against *CDK2* (light-gray boxes), *CDK4* (dark-gray boxes), or *CDK6* (black boxes) and allowed to proliferate in culture for 7 days. Total cell numbers were monitored and normalized compared to those of the corresponding tumor cell line infected with lentiviral particles expressing a control scramble shRNA (open boxes). Data shown represent mean ± SD, n = 3.

(B) Same as in (A) except that the human NSCLC cell lines carried wild-type K-*RAS* alleles.

(C) Western blot analysis of the infected cultures to assess the efficiency of shRNA-mediated knockdown. GAPDH is shown as a loading control.

Human NSCLC Cell Lines Containing K-*RAS* Oncogenes Require the Full Complement of Interphase CDKs

Next, we examined whether human tumor cells carrying K-*RAS* oncogenes may also require selective interphase CDKs for proliferation. Ten human NSCLC tumor cell lines, five of which carrying K-*RAS* oncogenes (Uchiyama et al., 2003; Blanco et al., 2009), were grown in the presence of shRNAs specific for *CDK2*, *CDK4*, or *CDK6* (Figures 2A and 2B). These shRNAs inhibited the expression of their cognate *CDK* alleles by at least 90% (Figure 2C). As illustrated in Figure 2A, four of the five NSCLC tumor cell lines carrying K-*RAS* oncogenes also required the full complement of interphase CDKs for proliferation since expression of any of the shRNAs was sufficient to block proliferation. The resistant H460 cell line is known to express very low levels of the oncogenic K-RAS protein (Uchiyama et al., 2003), a property that may explain its tolerance to proliferate in the absence of CDK2, CDK4, or CDK6 (Figure 2A). However, the five NSCLC cell lines lacking K-*RAS* oncogenes were unaffected by losing expression of single CDKs, with the possible exception of Calu-3 (Figure 2B). These observations resemble those obtained with mouse cells and suggest that human NSCLC cells carrying K-*RAS* oncogenes also become addicted to the full complement CDK activity, at least in culture.

Tumor Development in Mice Devoid of Individual Interphase Cdks

These results raised the possibility that decreasing overall Cdk activity by ablating or knocking down individual Cdks may attenuate the oncogenic properties of K-*Ras* oncogenes in vivo. To explore this possibility, we crossed K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice with animals *null* for each of the interphase *Cdk*s including *Cdk2*$^{-/-}$, *Cdk4*$^{-/-}$, and *Cdk6*$^{-/-}$ (Ortega et al., 2003; Malumbres et al., 2004; Barrière et al., 2007). These strains were also deficient for the additional interphase Cdk, Cdk3, because of a naturally occurring mutation that results in a premature stop codon (Ye et al., 2001). K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice treated at weaning with 4OHT to allow expression of the resident K-*Ras*^{G12V} oncogene develop adenomas and NSCLCs that closely recapitulate those of human origin. Compound K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice carrying either of the three *Cdk null* alleles were treated with limiting doses of 4OHT to prevent the induction of an excessive number of focal tumors that may obscure the natural development of NSCLCs in an effort to mimic those events that take place in human patients. As illustrated in Figure 3A, Cdk6 deficiency did not result in significant lifespan extension in K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{-/-}$ mice as compared with those expressing Cdk6 (50% survival at 40 weeks). Moreover, a cohort of K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{-/-}$ mice sacrificed 6 months after 4OHT administration revealed similar tumor number, size, and grade than those observed in control K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{+/+}$ animals (Figure 3B).

In the absence of Cdk2, K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ mice displayed an increased lifespan of ~8 weeks compared with those animals expressing Cdk2 (34 versus 42 weeks, a 23% increase). This effect was likely to be a consequence of the reduced tumor burden (~50%) observed in K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ mice compared with K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{+/+}$ littermate controls (Figure 3C). Tumor reduction was similar among low- and high-grade lesions, suggesting that ablation of *Cdk2* may have an inhibitory effect on K-*Ras*-induced tumor initiation rather than on tumor progression (Figure 3D). It could be argued that these tumors do not require expression of Cdk6 or Cdk2 because these knockout strains

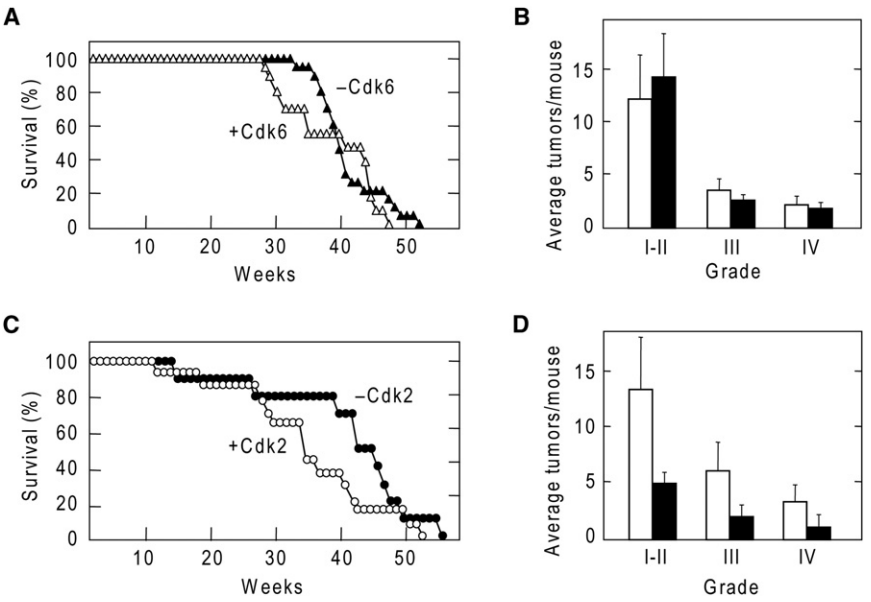

Figure 3. K-*Ras*^{G12V}-Induced NSCLC in Mice Lacking Cdk2 or Cdk6

(A) Survival curve of K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{+/+}$ (open triangles) and K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{-/-}$ (solid triangles) littermates treated intraperitoneally with 4OHT (1 mg twice a week for 2 weeks) at weaning.

(B) Number and grade of lung tumors present in K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{+/+}$ (open bars; n = 6) and K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk6*$^{-/-}$ (solid bars; n = 7) littermates sacrificed 6 months after 4OHT treatment. Data shown represent mean ± SD.

(C) Survival curve of K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{+/+}$ (open circles) and K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ (solid circles) littermates treated intraperitoneally with 4OHT at weaning.

(D) Number and grade of lung tumors present in K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{+/+}$ (open bars; n = 6) and K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ (solid bars; n = 6) littermates sacrificed 6 months after 4OHT treatment. Data shown represent mean ± SD. See also Figure S1.

have developed compensatory mechanisms during embryonic or postnatal development. Thus, we decided to interrogate whether NSCLC that developed in the presence of Cdk2 may be more sensitive to acute ablation of this kinase. As illustrated in Figure S1 (available online), exposure of K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{lox/lox}$ mice to 4OHT for 3 months resulted in limited increased survival that closely resembled that observed in mice lacking *Cdk2* in their germ line (Figure S1A). Likewise, the relative reduction in tumor burden and tumor grade in these mice was very similar to that observed in K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ animals (Figure S1B). These results were not due to limited Cre-mediated recombination (Figure S1C). Moreover, all tumors tested displayed complete ablation of the conditional *Cdk2*^{lox} alleles (Figure S1D).

Cdk4 null mice develop diabetes and die at approximately 8 months of age (Rane et al., 1999; Tsutsui et al., 1999). This defect prevented us from studying the impact of Cdk4 deficiency on the survival of K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice because we did not wish to bias the study by using the low percentage of mice (mostly female) that survive >1 year of age. Thus, we decided to examine a cohort of K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ mice along with their K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ littermate controls, 6 months after induction of K-*Ras*^{G12V} expression. As illustrated in Figure 4A, we observed a significant reduction in tumor burden regardless of the glycemic state of the mice (data not shown). Moreover, all tumors were benign lesions (grades I and II) (Figure 4B). We also observed a dramatic reduction in the number of K-*Ras*^{G12V}-expressing cells in the normal parenchyma, as determined by surrogate β-Geo expression (Figure 4A). These results indicate that ablation of Cdk4 selectively decreases the number of K-Ras^{G12V}-expressing cells and hinders the development of K-*Ras*^{G12V}-induced NSCLCs.

Lack of Cdk4 Prevents Development of Aggressive Lung Adenocarcinomas

It has been reported that the wild-type K-Ras protein has tumor suppressor properties (Zhang et al., 2001) and is frequently lost in advanced mouse and human lung tumors (Li et al., 2003; To et al., 2008). We have generated a mouse model to study this phenomenon by replacing the wild-type K-*Ras* allele by a conditional K-*Ras*^{lox} allele in K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice. The resulting K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice developed much more aggressive lung tumors than those carrying a wild-type K-*Ras* allele (50% survival at 25 versus 42 weeks) (C.G., D.S. and M.B., unpublished data). Thus, we decided to use this strain to test the effect of Cdk4 deficiency on tumor development. As illustrated in Figures 4C and 4D, 6 months after the administration of 4OHT, lungs of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ mice displayed NSCLCs of large size, most of which were diagnosed as high grade (mainly grades III and IV with some occasional grade V tumor). However, in K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ mice the number of lung tumors was reduced 6- to 8-fold (Figure 4D). Moreover, tumor progression was also affected by the absence of Cdk4 since K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ mice displayed some grade III adenocarcinomas but not grade IV or grade V (metastatic) tumors (Figure 4D). The overall number of K-Ras^{G12V}-expressing cells was also significantly decreased in these mice.

K-Ras^{G12V} Expression in *Cdk4*$^{-/-}$ Lungs Activates an Immediate Senescence Response

Next, we interrogated how loss of Cdk4 expression decreased the overall number of K-Ras^{G12V}-expressing lung cells. First, we examined the number of adult lung cells positive for the surrogate β-Geo marker 2 weeks after a single injection of 4OHT, a time when most β-Geo-positive cells appeared as individual cells. As illustrated in Figures 5A and 5B, at this time point lungs showed similar number of K-Ras^{G12V}-positive cells, regardless of whether they expressed Cdk4 or not. Two weeks later, however, the levels of K-Ras^{G12V}-positive cells in K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ lungs were 8- to 10-fold higher than those observed in lungs of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ mice. Moreover, most β-Geo-positive cells in *Cdk4*$^{+/+}$ lungs appeared in clusters of 30–100 cells, suggesting that they had

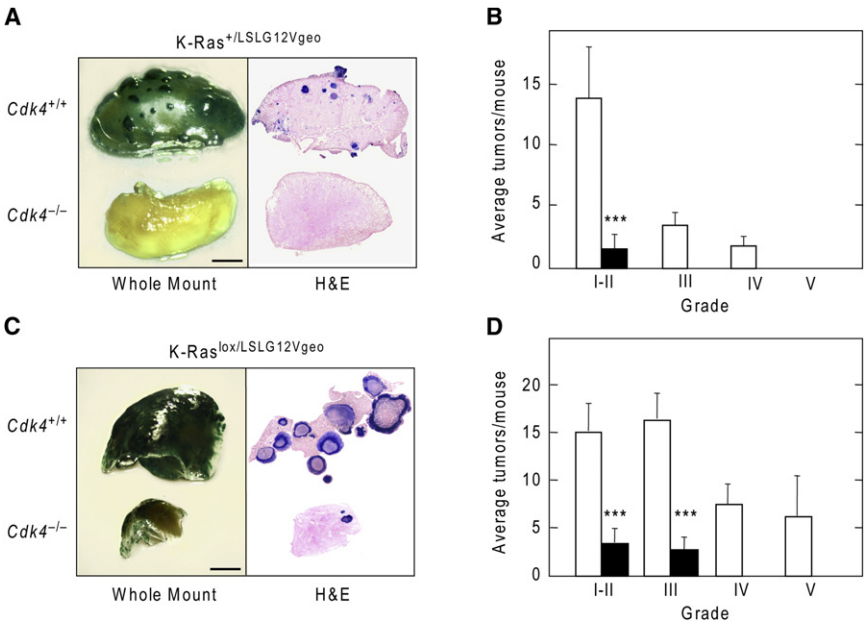

Figure 4. Effect of Cdk4 Deficiency on K-*Ras*^{G12V}-Induced NSCLC

(A) Whole-mount X-Gal (left) and H&E staining (right) of representative lung specimens of 7-month-old K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ and K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ littermates treated with 4OHT at weaning. Blue staining corresponds to K-*Ras*^{G12V}-expressing cells. The scale bar represents 2 mm.

(B) Number and grade of lung tumors present in K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open bars; n = 6) and K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid bars; n = 5) littermates sacrificed 6 months after 4OHT treatment. Data shown represent mean ± SD. ***p < 0.001.

(C) Whole-mount X-Gal (left) and H&E staining (right) of representative lung specimens of 7-month-old K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ and K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ littermates treated with 4OHT at weaning. The large size of the lobule obtained from K-*Ras*$^{+/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ is due to tumor burden. The scale bar represents 2.5 mm.

(D) Number and grade of lung tumors present in K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open bars; n = 5) and K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid bars; n = 5) littermates sacrificed 6 months after 4OHT treatment. Data shown represent mean ± SD. ***p < 0.001.

initiated unscheduled proliferation in response to K-Ras^{G12V} expression (Figure 5A). In contrast, β-Geo-positive cells in the *Cdk4*$^{-/-}$ lungs remained mostly as single cells or as very small clusters of two to four cells (Figure 5A). These results indicate that expression of K-Ras^{G12V} failed to induce sustained proliferation of lung cells in the absence of Cdk4.

These observations were confirmed by 5-bromo-2′-deoxyuridine (BrdU) labeling (Figure 5C). One week after 4OHT treatment, ~15% of K-Ras^{G12V}-positive cells were labeled with BrdU independently of their Cdk4 status (data not shown). A week later, the percentage of BrdU-positive cells in Cdk4$^{+/+}$ lungs increased to 37% (Figure 5D). However, only 3.5% of the K-Ras^{G12V}-expressing cells in lungs lacking *Cdk4* displayed BrdU staining. Thirty days after 4OHT treatment, these percentages became 46% and 2.2%, respectively (Figure 5D). These observations are unlikely to be due to apoptosis of *Cdk4*$^{-/-}$ lung cells given that we failed to detect any positive staining for active Caspase 3A (data not shown).

Next, we focused our attention on cellular senescence as a potential mechanism responsible for the lack of proliferation of K-Ras^{G12V}-expressing *Cdk4*$^{-/-}$ lung cells. Previous studies have shown that K-Ras^{G12V}-induced adenomas express senescence-associated (SA) endogenous β-Galactosidase activity (Collado et al., 2005). Yet, it is unknown when this senescence marker appears during adenoma formation and whether senescence plays a role in preventing cell proliferation during preadenoma stages. As illustrated in Figure 6A, only lung sections of *Cdk4*-deficient mice displayed senescent cells upon K-Ras^{G12V} expression. No SA-β-Gal staining could be observed in lung sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ mice (Figure 6A) or in *Cdk4*$^{-/-}$ animals lacking the K-*Ras*^{G12V} oncogene (data not shown). The percentage of senescent cells in *Cdk4*$^{-/-}$ lungs (~4%) was similar to the number of cells known to express the endogenous K-Ras^{G12V} before clonal expansion (compare Figures 5B with Figure 6B). Such a result suggests that most K-Ras^{G12V}-expressing cells in *Cdk4*$^{-/-}$ lungs undergo senescence instead of initiating a proliferative response.

Induction of senescence appeared to be exquisitely specific for lung cells. As illustrated in Figure 6C, no SA-β-Gal staining could be observed in colon sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ mice in spite of wide expression of K-Ras^{G12V}, as determined by surrogate β-Geo staining. Similar results were observed in other K-Ras^{G12V}-expressing tissues including pancreas, stomach, and thymus (data not shown). Interestingly, none of these tissues undergo tumor development, or even hyperplasia, as a consequence of K-Ras^{G12V} expression even in the presence of Cdk4. Finally, lungs from K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ mice treated in parallel with 4OHT also lacked senescent cells reinforcing the notion that senescence is a Cdk4-specific response (Figure 6D).

In vitro, oncogene-induced senescence is mediated by activation of the DNA damage response (Di Micco et al., 2006; Bartkova et al., 2006; Mallette et al., 2007) and is accompanied by loss of Cyclin D1 expression (Pontano et al., 2008). K-Ras^{G12V} expression in *Cdk4*$^{-/-}$ lungs also led to the appearance of γ-H2AX-positive cells with a frequency similar to those positive for SA-β-Gal (Figures 6E and 6F). Likewise, expression of Cyclin D1 selectively disappeared in K-*Ras*^{G12V};*Cdk4*$^{-/-}$ cells 1 month after exposure to 4OHT (Figures 6G and 6H). In summary, expression of an endogenous K-Ras^{G12V} oncoprotein in lung cells lacking Cdk4 induces an immediate senescence response that prevents cell proliferation, and as a consequence tumor development.

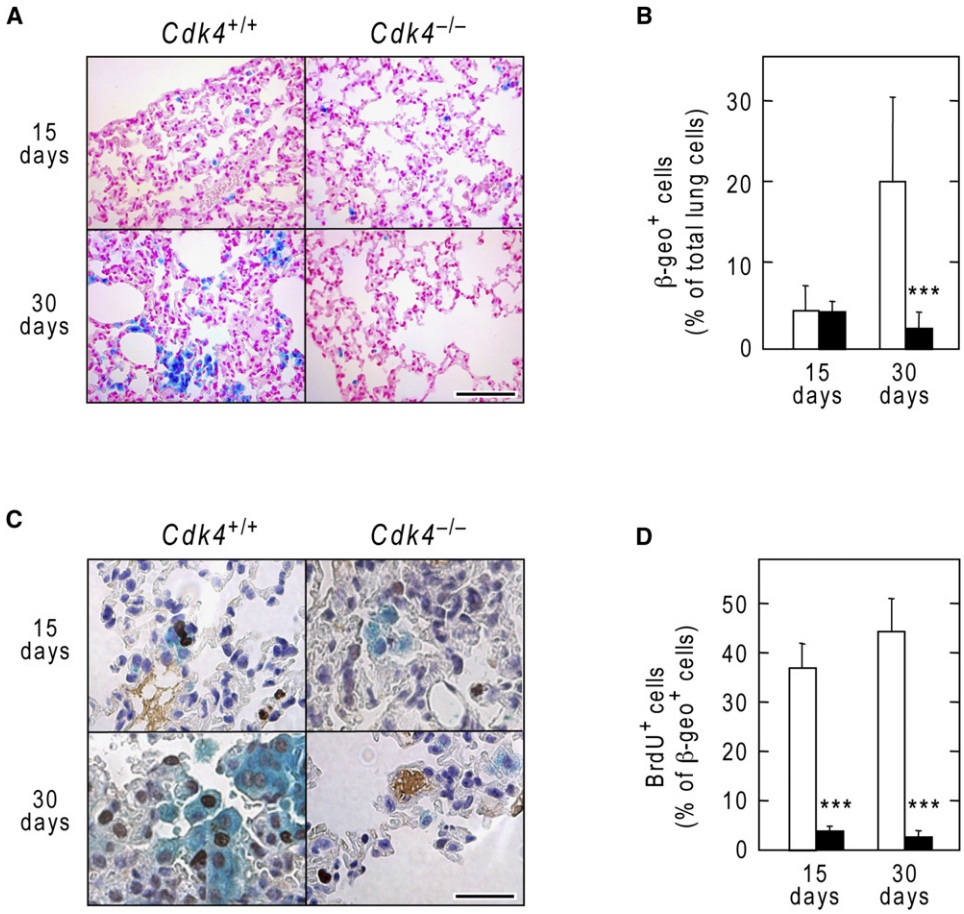

Figure 5. Lack of K-*Ras*^{G12V}-Induced Proliferation in Lung Cells Devoid of Cdk4

(A and B) Detection (A) and quantification (B) of K-Ras^{G12V}-expressing cells (based on the expression of the β-Geo surrogate marker, β-Geo+ cells) in lung sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open bars; n = 4) and K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid bars; n = 4) littermates, 15 and 30 days after exposure to a single dose of 4OHT (1 mg) applied at weaning. Data shown represent mean ± SD. ***p < 0.001. The scale bar represents 50 μm (A). (C and D) Detection (C) and quantification (D) of β-Geo+ cells positive for BrdU staining (BrdU+ cells) on X-Gal-stained whole-mount lung sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open bars; n = 4) and K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid bars; n = 4) littermates, 15 and 30 days after exposure to 4OHT. BrdU was injected 24 hr prior to the analysis. Data shown represent mean ± SD. ***p < 0.001. The scale bar represents 20 μm (C).

Cdk4 Is Essential for Progression of K-Ras^{G12V}-Driven NSCLC

To evaluate whether Cdk4 may also be required for progression of pre-existing tumors, we replaced a *Cdk4 null* allele by a conditional *Cdk4*^{frt} allele to allow *Cdk4* ablation in a temporally controlled manner (Barrière et al., 2007). The resulting K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice developed tumors with similar kinetics to those carrying wild-type *Cdk4* alleles, indicating that 50% inhibition of Cdk4 expression has no effect on tumor development (data not shown). Tumor-bearing mice, as determined by computed tomography (CT) scanning (tumor size >1 mm diameter) were inoculated with adenoviruses expressing Flpe recombinase (Ad-*Flpe*) or control green fluorescent protein (Ad-*GFP*). Three-dimensional reconstruction of the CT scans 3 months after Ad-*Flpe* infection revealed a reduced number of lesions with significantly smaller size as those present in mice inoculated with Ad-*GFP* (Figure 7A). The average volume of the tumors in mice exposed to Ad-*Flpe* was approximately one-fifth of those present in Ad-*GFP* treated mice (n = 5) (Figure 7B). In addition, the number of grade III–IV lesions decreased significantly (Figure 7C). Most importantly, all tested high-grade tumors that progressed after Ad-*Flpe* exposure retained Cdk4 expression (Figure 7D) because of lack of excision of the conditional allele (data not shown). These results indicate that Cdk4 expression is an essential requirement for progression of already established K-*Ras*^{G12V}-driven tumors.

Tumors in which the *Cdk4*^{frt} allele was efficiently excised displayed a remarkable reduction in BrdU incorporation just 2 weeks after being exposed to Ad-*Flpe* (Figure 7E, upper panels). Moreover, these tumors showed widespread SA-β-Gal staining indicating that most tumor cells have entered a senescent state upon loss of Cdk4 expression (Figure 7E, lower panels). Protein extracts from microdissected lung lesions showing significant reduction of Cdk4 expression displayed upregulation of senescence markers such as p19^{Arf} and p53$^{P\text{-}Ser15}$ (Palmero et al., 1998; Webley et al., 2000) (Figure 7F). None of these markers were observed in tumors exposed to Ad-*GFP* (Figure 7F). Onset of the senescence response was

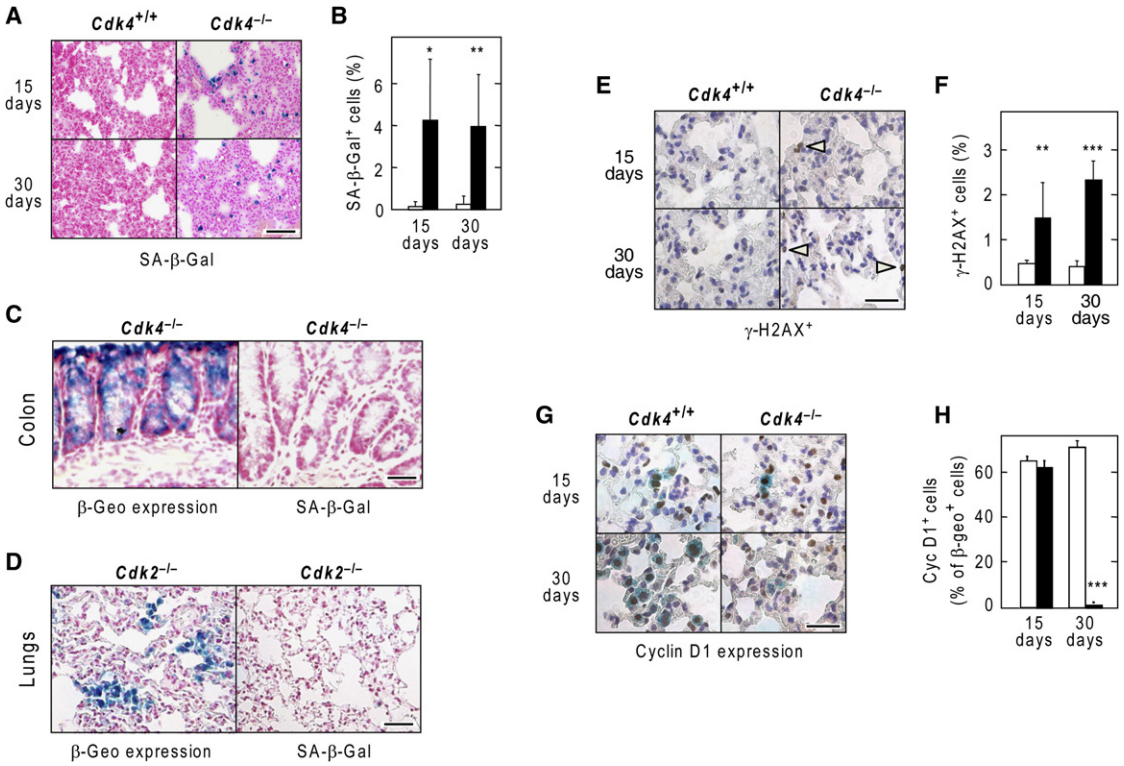

Figure 6. Cdk4 Selectively Inhibits K-*Ras*^{G12V}-Induced Senescence in Lung Cells

(A) Detection and (B) quantification of senescence-associated β-galactosidase (SA-β-Gal)-positive cells in lung sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open bars; n = 4) and K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid bars; n = 4) littermates, 15 and 30 days after exposure to 4OHT. Data shown represent mean ± SD. *p = 0.012; **p = 0.0017.

(C) Detection of K-Ras^{G12V} expression (based on its surrogate marker β-Geo) (left) and absence of senescence-associated β-galactosidase (SA-β-Gal) (right) in colonic crypts of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{/}$ mice.

(D) Detection of K-Ras^{G12V} expression (based on its surrogate marker β-Geo) (left) and absence of senescence-associated β-galactosidase (SA-β-Gal) (right) in lungs of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk2*$^{-/-}$ mice.

(E) Detection and (F) quantification of γ-H2AX-positive cells in lung sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open bars; n = 4) and K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid bars; n = 4) littermates, 15 and 30 days after exposure to 4OHT. Arrowheads mark cells with positive staining. Data shown represent mean ± SD. **p = 0.006; ***p < 0.001.

(G) Detection and (H) quantification of β-Geo+ cells positive for Cyclin D1 expression (CycD1+ cells) on X-Gal stained whole-mount lung sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ (open bars; n = 4) and K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ (solid bars; n = 4) littermates, 15 and 30 days after exposure to 4OHT. Data shown represent mean ± SD. *p < 0.001. Scale bars represent 50 μm (A, C, and D) and 20 μm (E and G).

accompanied by infiltration of the lesions by T lymphocytes (CD3$^+$ cells) and cells of neutrophil-granulocyte lineage (MPO$^+$ cells) (Figure S2) but not by B-lymphocytes (data not shown). In summary, advanced K-*Ras*^{G12V}-driven lung tumors require expression of Cdk4 to avoid the onset of senescence followed by activation of an immune response, two events that ultimately result in effective inhibition of tumor progression.

Cdk4 Inhibitors

Cdk inhibitors have been used in clinical trials with limited success (Malumbres et al., 2008; Lapenna and Giordano, 2009). Among these compounds, only PD0332991, and possibly P1446A-05 (www.clinicaltrials.gov), are selective for Cdk4 and Cdk6 with no significant activity on other Cdks (Fry et al., 2004). Preclinical studies have illustrated that PD0332991 has inhibitory activity against some human tumor xenografts that retained pRb expression (Fry et al., 2004). Since K-Ras^{G12V}-driven NSCLC is sensitive to Cdk4 ablation (Figure 7A), we decided to validate the therapeutic efficacy of PD0332991 in these mice. K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ animals were treated at weaning with 4OHT and monitored by CT 4 months later. Mice that did not show tumors by CT at this time (CT– mice) were treated for 30 days (o.i., daily) either with vehicle (n = 4) or with two doses of the inhibitor, 100 mg/kg (n = 6) and 150 mg/kg (n = 6), a dose previously shown to be the maximal tolerated dose (MTD) (Fry et al., 2004). Whereas three out of the four animals (75%) treated with vehicle developed lesions easily detectable by CT at the end of the treatment, only two out of 12 mice treated with PD0332991 (17%) developed such lesions (data not shown).

K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$ animals carrying lung tumors as determined by CT imaging (CT+ mice) were randomized and treated either with vehicle or with the above indicated doses of PD0332991 for 30 days. Mice were monitored for tumor development by CT at 15 days and at the end of the treatment (30 days). As illustrated in Figure 8A, animals that received

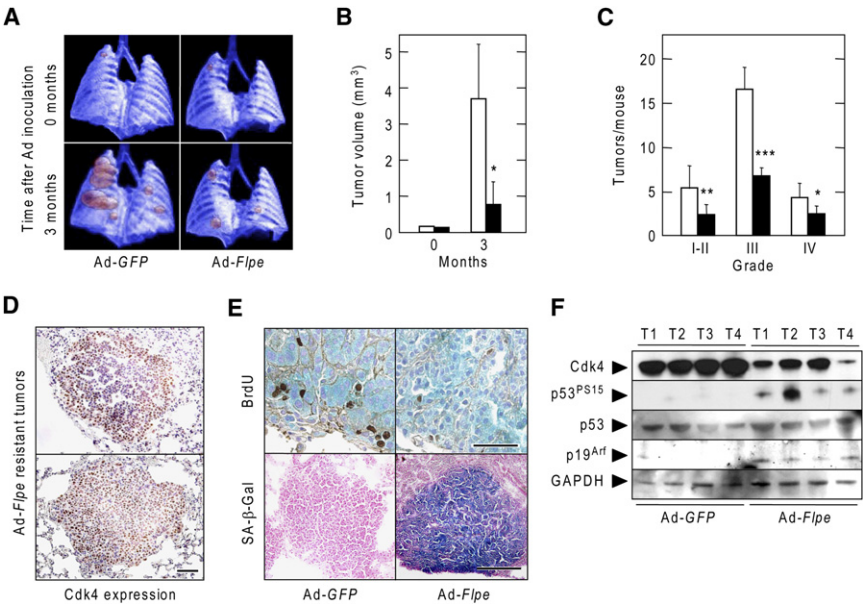

Figure 7. Conditional Ablation of Cdk4 Expression Inhibits Progression of K-*Ras*^{G12V}-Driven NSCLC

(A) Three-dimensional reconstruction of CT scans from representative K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice treated with adenoviral particles expressing control GFP (Ad-*GFP*) or Flpe (Ad-*Flpe*) at the time of treatment and 3 months after treatment.

(B) Quantification of the average tumor volume of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice treated with control Ad-*GFP* (open bars; n = 5) or with Ad-*Flpe* to excise the *Cdk4*^{frt} conditional allele (solid bars; n = 5). Data shown represent mean ± SD. *p = 0.014.

(C) Quantification of tumor burden present in K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice 3 months after exposure to Ad-*GFP* (open bars; n = 5) or Ad-*Flpe* (solid bars; n = 5). Data shown represent mean ± SD. *p = 0.049; **p = 0.005; ***p < 0.001.

(D) Partial (top) and uniform (bottom) Cdk4 expression, as determined by IHC analysis with anti-Cdk4 polyclonal antibodies, in residual tumors of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice treated with Ad-*Flpe*. The scale bar represents 100 μm.

(E) The top panels show BrdU incorporation in whole-mount X-Gal-stained lung sections and the bottom panels show senescence-associated β-galactosidase (SA-β-Gal) staining of lung sections obtained from K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice 2 weeks after exposure to Ad-*GFP* or Ad-*Flpe*. Scale bars represent 20 μm (top) and 200 μm (bottom).

(F) Western blot analysis of individual tumors (T1 to T4) microdissected from lungs of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice 3 months after treatment with Ad-*GFP* or Ad-*Flpe* with antibodies specific for Cdk4, p53 phosphorylwithated at residue Ser15, p53, and p19^{Arf}. Migration of the corresponding proteins is indicated by arrowheads. Antibodies against GAPDH were used as loading controls. See also Figure S2.

vehicle increased the number of CT+ tumors by 3-fold at the 15 day time point and by 4-fold at the end of the experiment. In contrast, mice treated with the Cdk4 inhibitor only increased the number of tumors by 1.5-fold (Figure 8A). These differences became more significant when we measured tumor burden. As shown in Figure 8B, mice exposed to vehicle increased their tumor burden by 10-fold after 15 days of observation and 25-fold by the end of the experiment. In contrast, mice exposed to PD0332991 only increased their tumor burden by 3- to 4-fold at 15 days and 5- to 6-fold at 30 days (Figure 8B). No significant differences were observed between the two doses of inhibitor, suggesting that we may have reached saturating levels even at the lower dose of 100 mg/kg. We also compared the metabolic activity of these tumors as criteria for advanced tumor development. Whereas 59% of the lesions in the vehicle-treated cohort were positive for ^{18}F-glucose uptake by positron emission tomography (PET), only 29% of the mice treated with the inhibitor displayed ^{18}F-glucose uptake (data not shown).

Finally, we investigated whether exposure to PD0332991 blocked phosphorylation of pRB, the primary substrate for Cdk4 and elicited a senescence response. As illustrated in Figures 8C and 8D, PD0332991 efficiently diminished the phosphorylation of pRb in the Cdk4-specific Ser807 and Ser811 residues. However, tumors exposed to PD0332991 did not express SA-β-Gal. Likewise, none of the tumors tested had increased phosphorylation on p53$^{P\text{-}Ser15}$ residues or increased p19^{Arf} expression (data not shown). These results suggest that induction of a senescence response must require a more robust and possibly sustained inhibition of Cdk4 activity that could be achieved with the PD0332991 inhibitor.

DISCUSSION

We have assessed the relative contribution of individual interphase Cdks, Cdk2, Cdk4, and Cdk6, to the proliferative and oncogenic properties conveyed by expression of an endogenous K-*Ras*^{G12V} oncogene. In culture, the absence of any individual interphase Cdk cancelled the ability of K-*Ras*^{G12V}-expressing MEFs to proliferate in limiting serum conditions and to overcome senescence induced by adaptation to culture conditions. We obtained similar results using human tumor cell lines derived from NSCLCs providing that these tumor cells also carry K-*RAS* oncogenes. The lack of specificity for any particular CDK suggests that K-RAS signaling in these tumor cells demands increased overall CDK activity, rather than phosphorylation of selective substrates.

In principle, these findings raised the possibility that limited inhibition of overall interphase CDK activity may have selective therapeutic effect on K-*RAS*-driven lung tumors. Yet, these in vitro studies could not be fully paralleled in vivo. Expression of a resident K-*Ras*^{G12V} oncogene in mice *null* for *Cdk6* resulted in efficient induction of NSCLCs. Likewise, absence of Cdk2 did not prevent tumor formation. In this case, however, there was a limited reduction in tumor burden that resulted in increased life span. These findings are similar to those currently observed with a number of targeted therapies in clinical trials. However,

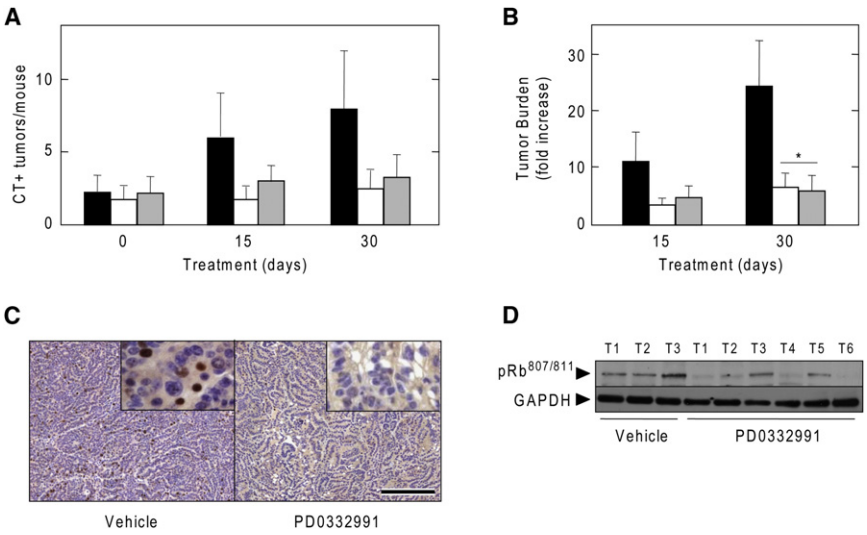

Figure 8. A Selective Cdk4 Inhibitor, PD0332991, Inhibits K-*Ras*^{G12V}-Driven NSCLC

(A) Number of CT+ tumors in 5-month-old tumor-bearing K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ mice (exposed to 4OHT at weaning) treated daily for 30 days with vehicle (solid bars; n = 7), or with an oral dose of 100 mg/kg (open bars; n = 6) or 150 mg/kg (gray bars; n = 6) of PD0332991. Data shown represent mean ± SD.

(B) Increased tumor burden, determined by CT analysis, during a 30 day period in 5-month-old tumor-bearing K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ mice treated as indicated above. Total tumor burden at the beginning of the experiment was 9.68 mm^3, 5.35 mm^3, and 9.71 mm^3 for mice treated with either vehicle (solid bars; n = 7), 100 mg/kg (open bars; n = 6), and 150 mg/kg (gray bars; n = 6) of PD0332991, respectively. Data shown represent mean ± SD. *p = 0.045.

(C) Immunohistochemical analysis of phosphorylated pRb at Cdk4-specific residues Ser807/811 in lung sections of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ mice 1 month after treatment with vehicle (left) or 150 mg/kg of PD0332991 (right). Scale bar: 200 μm. Insert shows a 10× magnification of a representative area.

(D) Western blot analysis of individual tumors microdissected from lungs of K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{+/+}$ mice 1 month after treatment with either vehicle (T1–T3) or 150 mg/kg of PD0332991 (T1–T6) with antibodies specific for pRb phosphorylated at residues Ser807/811. Antibodies against GAPDH were used as loading controls. Migration of the corresponding proteins is indicated by arrowheads.

the lower complexity of these K-*Ras*^{G12V}-driven mouse tumors suggests that inhibition of CDK2 may not yield significant therapeutic benefit for NSCLC patients.

On the contrary, absence of *Cdk4* had major consequences for tumor growth. K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ mice developed very few tumors, all of them of benign nature. These observations were a consequence of an unexpected synthetic lethal interaction between the K-*Ras*^{G12V} oncogene and the absence of Cdk4 expression in lung cells that resulted in the immediate onset of senescence. This response was observed during the initial rounds of K-Ras^{G12V}-induced cell division, well before the appearance of hyperplastic lesions or adenomas. Moreover, this response was exquisitely selective for tissue type (lung) and interphase Cdk (Cdk4). Expression of K-*Ras*^{G12V} in other tissues devoid of Cdk4, including colon and pancreas, two tissues known to yield K-*Ras* oncogene-driven tumors in both mice and humans, did not induce senescence. Likewise, we did not observe a K-*Ras*^{G12V}-driven senescence response in lungs of mice lacking Cdk2. The reason why lung cells are so dependent on Cdk4 for K-Ras^{G12V}-driven proliferation is unknown, given that normal lung development does not require Cdk4 expression (Rane et al., 1999; Tsutsui et al., 1999). Recently, Campaner et al. (2010) have demonstrated that lack of Cdk2 promotes senescence of c-Myc-driven tumors. Thus, individual interphase Cdks may be specifically required for development of certain tumors depending on the tissue of origin and the driving oncogenic insults (Malumbres and Barbacid, 2009).

Given that Cdk4 *null* mice occasionally developed benign adenomas, lung cells must have alternative mechanisms to bypass K-Ras^{G12V}-induced senescence. It is possible that tumor initiating cells in the lung may not be a homogeneous population and respond differently to K-Ras^{G12V} expression. Thus, tumor-initiating cells may include a small subpopulation resistant to K-Ras^{G12V}-induced senescence regardless of the expression of Cdk4. These K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/-}$ cells that escape initial senescence and form adenomas eventually expressed senescence markers as previously described in lesions with a full Cdk complement (Collado et al., 2005; Prieur and Peeper, 2008). Interestingly, the absence of Cdk4 must help to re-enforce this antitumor barrier since *Cdk4*$^{-/-}$ adenomas do not progress to malignant adenocarcinomas.

The induction of a senescence response in CT+ tumors upon cleavage of the conditional *Cdk4*^{frt} allele may have important therapeutic implications for human NSCLC. Conditional ablation of Cdk4 expression in CT+ tumors induces widespread senescence that significantly reduces cell proliferation, leading in most cases to disappearance of the tumor. Indeed, all actively growing tumors in Adeno-*Flpe*-treated K-*Ras*$^{lox/LSLG12Vgeo}$;*RERT*$^{ert/ert}$;*Cdk4*$^{-/frt}$ mice invariably carried an intact *Cdk4*^{frt} allele and expressed Cdk4. Hence, indicating that tumor progression required continuous Cdk4 expression. Loss of Cdk4 in CT+ tumors also triggers an immune response likely to contribute to the observed antitumoral effect. Ablation of *Cdk4* in mouse models carrying additional mutations such as loss of p53 should provide clinically relevant information as whether tumor cells that have accumulated other genetic alterations retain the capacity of entering senescence upon ablation of Cdk4 expression.

Recent studies have used high-throughput RNAi libraries to identify synthetic lethal interactions between the TBK1 and STK33 kinases with K-*RAS* oncogenes (Barbie et al., 2009; Scholl et al., 2009). One of these studies also identified synthetic lethal interactions with several Cdks including CDK1, CDK2, CDK6, CDK7, and CDK8, but not CDK4 (Barbie et al., 2009). It is possible that the RNAi library may not contain optimal sequences against CDK4. Based on our own findings,

identification of synthetic lethal interactions in cell lines may not always be validated in vivo (e.g., loss of Cdk2 and Cdk6 in K-RAS-expressing NSCLC cell lines). Use of genetically modified mouse models that closely recapitulate the natural history of human tumors should strengthen the value of potential synthetic lethal interactions with regard of their potential use in clinical trials.

It could be argued that ablating expression of a target may not mimic inhibition of its biological activity. Conditional knockin strategies may eventually circumvent this problem. Ultimately, however, the use of selective inhibitors, when available, may be the most direct approach. Here, we have shown that a Cdk4/Cdk6 selective inhibitor, PD0332991, had significant therapeutic activity in K-Ras^{G12V}-induced NSCLC. As expected, this inhibitor blocked Cdk4-mediated phosphorylation of pRb. Yet, it failed to induce the robust senescence response observed upon genetic ablation of the *Cdk4* locus. Thus, it is probable that in order to induce a senescence response, Cdk4 inhibitors must induce a more robust and durable Cdk4 inhibition. Indeed, it is possible that induction of senescence might be used as a biomarker for adequate Cdk4 inhibition in future clinical trials. So far, PD0332991 has failed to show activity in phase I clinical trials against undefined advanced solid tumors, non-Hodgkin's lymphoma, and mantle cell lymphoma. Additional clinical trials on multiple myeloma and breast cancer in combination with other drugs are currently ongoing (http://www.cancer.gov/). The results described here suggest that PD0332991, as well as other potential CDK4 selective inhibitors should be tested in NSCLC positive for K-*RAS* oncogenes, either alone or in combination with other drugs. Our findings should also encourage renewed efforts to design more potent and specific Cdk4 inhibitors.

EXPERIMENTAL PROCEDURES

Cell Culture Assays

MEFs were isolated from E13.5 embryos and propagated according to standard 3T3 protocols. For proliferation assays, 5×10^4 cells were plated on six-well plates in duplicate in DMEM supplemented with 2% fetal calf serum. Human NSCLC cell lines were purchased from the ATCC. For proliferation assays 1×10^3 cells were plated in 96-well plates and their growth rate determined by the MTT cell proliferation kit (Roche).

Mice

The *K-Ras*$^{+/LSLG12Vgeo}$;RERT$^{ert/ert}$, *Cdk2*$^{lox/lox}$, *Cdk2*$^{-/-}$, *Cdk4*$^{frt/frt}$, *Cdk4*$^{-/-}$, and *Cdk6*$^{-/-}$ strains have been previously described (Guerra et al., 2003; Ortega et al., 2003; Malumbres et al., 2004; Barrière et al., 2007). K-*Ras*$^{lox/lox}$ mice will be described elsewhere. Compound strains were generated by standard crosses. CreERT2-mediated recombination was induced at weaning by intraperitoneal administration of 4-OHT (1 mg/dose). Elimination of the conditional *Cdk4*^{frt} allele was achieved by tail vein inoculation of adenoviral particles (5×10^9 plaque-forming unit [pfu]) expressing the Flpe recombinase. Adenoviruses expressing GFP were used as controls at the same dose. All animal experiments were approved by the CNIO Ethical Committee and performed in accordance with the guidelines stated in The International Guiding Principles for Biomedical Research involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS).

Histopathology and Immunohistochemistry

Mice were sacrificed at the time points specified in the text. For routine histological inspection lung lobes were fixed in 10% buffered formalin (Sigma) and embedded in paraffin. For quantification and classification of tumor lesions, lung lobes were processed for whole-mount X-Gal staining (Guerra et al., 2003). Whole-mount stained lung lobes were serially sectioned and tumors counted classified according to histopathological grading (Jackson et al., 2005). For endogenous SA-β-galactosidase detection, lung lobes were snap-frozen in O.C.T. (Sakura) and processed on 10 μM cryostat sections with a Senescence β-Galactosidase staining kit (Cell Signaling) in accordance with the manufacturer's recommendations. Counterstaining of cryostat sections was performed with nuclear fast red. Antibodies used for immunohistochemistry analysis included anti Cdk2, Cdk4, and Cdk6 rabbit polyclonal antibodies generated in our own laboratory as well anti-Cyclin D1 (Neomarkers), γ-H2AX (Millipore) and BrdU (GE Healthcare).

Western Blot Analysis

Tissue samples and cell pellets were processed as previously described (Barrière et al., 2007). The following primary antibodies were used: anti-Cdk2 and Cdk4, p19^{Arf} (Upstate), p53 (Novocastra), Ser15-p53 (Cell Signaling), pRb-S807/811 (Cell Signaling), and GAPDH (Sigma).

PD0332991 Treatment

The Cdk4 inhibitor was dissolved in a stock solution of sodium lactate buffer (50 mM [pH 4]) and was administered daily during 1 month at 150 mg/kg or 100 mg/kg by gavage. The vehicle control group received an equivalent volume of sodium lactate buffer solution.

Computed Tomography

Mice were anesthetized with a continuous flow of 1% to 3% isoflurane/oxygen mixture (2 liters/min), and the chest area was able to be imaged at one time with the GE eXplore Locus micro-CT scanner (GE Healthcare). The isotropic resolution of this instrument is 45 μm. The micro-CT image acquisition consisted of 400 projections collected in one full rotation of the gantry in ~10 min. The image acquisition was not respiratory-gated. The X-ray tube settings were 80 kV and 450 μA. The resulting raw data were reconstructed to a final image volume of 875 × 875 × 465 slices at 93 μm^3 voxel dimensions. The reconstructed slices were output in the CT manufacturer's raw format and were corrected equal to Hounsfield units. The reconstructed images were viewed and analyzed with MicroView analysis software (GE Healthcare).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.ccr.2010.05.025.

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