Inhibition of PI-3K restores nuclear p27^{Kip1} expression in a mouse model of K-ras driven lung cancer

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1

Abstract

Reduced expression of the CDK inhibitor p27^{Kip1} (p27) in human lung cancer correlates with tumour aggressiveness and poor prognosis. However, the regulation of p27 expression and the role of p27 during lung cancer are poorly understood. Urethane-induced lung tumours in mice frequently harbor mutations in the Kras oncogene and here we use this model to address the regulation of p27 during tumorigenesis. The Ras effector Akt is known to regulate p27 mRNA abundance by phosphorylating and inactivating the FOXO transcription factors. Phosphorylated Akt and FOXO proteins were both increased in lung tumours, correlating with a reduction in p27 mRNA transcript. Akt also directly phosphorylates p27 and regulates its nuclear/cytoplasmic localization. Tumours showed a reduced nuclear/cytoplasmic ratio of p27 protein, together with an increase in phosphorylated Thr198 p27 in the cytoplasmic pool. Treatment of lung tumour-bearing mice with the phosphoinositol-3 kinase inhibitor LY294002 induced a rapid decrease in phosphorylated Akt and phosphorylated p27, concomitant with an increase in nuclear p27. Germline p27 deficiency accelerated both the growth and malignant progression of urethane-induced lung tumours, and did so in a cell autonomous manner, confirming a causal role of p27 in tumour suppression. These results demonstrate that p27 is a potent barrier to the growth and malignant progression of Kras-initiated lung tumours. Further, the reduction of nuclear p27 in tumours is mediated by oncogene signaling pathways, which can be reversed by pharmacologic agents.

Introduction

One approach to cancer treatment is to enhance tumour suppressor activity in tumours. The effectiveness of this strategy has been recently demonstrated using genetically engineered mouse models, where restoration of expression of a tumour suppressor, in this case p53, led to complete tumour regression (Ventura et al. 2007; Martins et al. 2006; Xue et al. 2007). This approach is currently not feasible in humans, the primary limitation being that most known tumour suppressors are mutated or deleted in tumours. The Cdk inhibitor p27[Kip1] (p27) is a notable exception to the classic tumour suppressor gene paradigm in that the *CDKN1B* gene that encodes p27 is rarely mutated in tumours (Kawamata et al. 1995; Ponce-Castaneda et al. 1995). Instead, the levels of p27 protein are reduced or mislocalized and this correlates with tumour aggressiveness and poor prognosis (Chu et al. 2008). A causal role for p27 in tumour suppression has been shown by the increased susceptibility of p27 deficient mice to tumour development (Nakayama et al. 1996; Fero et al. 1996; Kiyokawa et al. 1996; Fero et al. 1998). The fact that the gene encoding p27 remains intact in most human cancers, provides an opportunity to design therapies that might enhance expression of p27. Key elements to the success of this strategy are to understand the mechanism(s) leading to misexpression of p27 and determine whether manipulating these pathways can enhance p27 expression in tumours.

p27 abundance is regulated by a number of different pathways throughout the cell cycle. The levels of p27 are high in quiescent cells and markedly reduced as cells transit through the cell cycle. In S phase, p27 is phosphorylated at Thr187 by CDK2, targeting p27 for ubiquitination by SKP2 and subsequent degradation by the proteasome (Sheaff et al. 1997). The KPC ubiquitin ligase was recently identified as a second mechanism to target p27 for degradation during G1 (Kamura et al. 2004). Microinjection studies showed that active Ras can reduce p27 by three pathways: MAPK in G1, PI3K in G1 and S, and via increased SKP2 in S and G2 (Sa and Stacey 2004). Colorectal cancer cell lines exhibit proteasome-dependent degradation of p27 (Loda et al. 1997), and many tumours express high levels of its ubiquitin ligase, SKP2, suggesting that the levels of p27 in tumours are regulated by proteasomal degradation (Bloom and Pagano 2003). Recent studies revealed that nonreceptor tyrosine kinases, such as Src, phosphorylate p27, consequently decreasing its stability (Chu et al.

2007; Grimmler et al. 2007), which may be yet another mechanism to reduce p27 abundance in tumours.

In addition to decreasing overall p27 levels, exclusion of p27 from the nucleus and retention in the cytoplasm can effectively reduce its CDK inhibitory activity. Cancers of the breast, thyroid, esophagus, and colon show specific reduction in nuclear p27, suggesting p27 mislocalization may be important for tumour progression (Blain and Massague 2002). At least two pathways have been proposed for nuclear export of p27 during G1 and S phase; association with Jab1, a component of the COP9-signalosome complex (Tomoda et al. 1999), or direct binding to the transporter CRM1 via the nuclear export signal of p27 (Connor et al. 2003). Nuclear export is regulated by the phosphorylation of p27 at specific residues. Ser10 phosphorylation of p27 by human kinase-interacting stathmin (hKIS or KIST) increases the binding of p27 to the nuclear exporter CRM1 (Boehm et al. 2002). Experiments utilizing knock-in mice carrying a Ser to Ala mutation at position 10 of p27 showed reduced levels of p27 in cultured MEFs (Kotake et al. 2005) and impaired nuclear export of p27 (Besson et al. 2006). Phosphorylation of Thr198, by either Akt or RSK1/2, results in binding to the protein 14-3-3 and retention in the cytoplasm (Fujita et al. 2003). This increases the stability of p27 by preventing proteasomal turnover (Kossatz et al. 2006). Cytoplasmic retention of p27 in human breast cancer cells was shown to be regulated by phosphorylation of Thr157 by the Akt kinase (Shin et al. 2005; Viglietto et al. 2002; Liang et al. 2002). However, Thr157 is not present in rodent p27, so this pathway is not evolutionarily conserved.

p27 is also regulated at the transcriptional levels. In quiescent cells, FOXO proteins are located within the nucleus where they bind to the p27 promoter and initiate transcription (Dijkers et al. 2000). In response to growth factors or oncogenic signals, FOXO proteins are phosphorylated by Akt, resulting in cytoplasmic sequestration, decreased p27 transcription, and cell proliferation (Medema et al. 2000). As Akt is active in many tumours, this may represent an additional mechanism to reduce p27 during tumour growth.

Lung cancer is among the leading causes of death worldwide and non-small cell lung cancer (NSCLC) accounts for ~87% of these cases (Singhal et al. 2005). Reduced expression of p27 is seen in ~30% of NSCLC cases, and five independent studies have shown a significant correlation of reduced p27 with disease recurrence or death (Chu et al. 2008). Mutations in

Kras and EGFR are frequently seen in lung cancer (Ding et al. 2008) and signaling from both can downregulate p27 in cell lines (Sa and Stacey 2004; Busse et al. 2000). Inui et al. reported that p27 mRNA levels were not reduced in lung tumors, although these were not stratified by Ras mutational status or Akt activity (Inui et al. 2003). Catzavelos *et al.* reported that Ras mutation and p27 expression seem to occur independently in adenocarcinoma of the lung (Catzavelos et al. 1999). Thus, the mechanism(s) contributing to altered regulation of p27 during lung tumourigenesis are unclear.

Urethane treatment of mice induces alveolar/bronchiolar adenomas and adenocarcinomas, which closely resemble human NSCLC adenocarcinoma in morphologic and molecular characteristics (Malkinson 2001). Here we use this model to examine the role of p27 in lung tumour suppression and the mechanisms leading to its misregulation.

Results

p27-deficient mice are susceptible to non-small cell lung cancer   To establish an autochthonous tumour model to examine the role of p27 in lung cancer, cohorts of *p27+/+, +/-, and -/-* littermates were injected with urethane. The lung tumour incidence for all three genotypes was 100% at both 30 and 50 weeks. At 30 weeks, both tumour multiplicity and tumour size were significantly greater in *p27-/-* and *+/-* mice compared to wildtype littermates (Figure 1A). The average number of tumours greater than 1mm in diameter was 1.4 $\pm$ 1.87, 4.2 $\pm$ 3.5, and 7.0 $\pm$ 4.5 for *p27+/+, +/-* and *-/-* mice, respectively (p $_{het-wt}$ = 0.026; p $_{null-wt}$ = 0.0012; two-tailed t test). Only four *p27-/-* mice survived beyond 40 weeks of age (median age 46 weeks), due to complications arising from excess lung tumour burden and pituitary tumours, and data from these four mice are included in the 50 week graph (Figure 1B). Tumour burden was greater at 50 versus 30 weeks for all genotypes. Again, tumour multiplicity and size were both increased in p27 deficient mice compared to wildtype littermates (Figure 1B, 1D). The average number of tumours per mouse at 50 weeks of age in p27 intact mice was similar to the number in *p27-/-* mice at 30 weeks of age (Figure 1C), suggesting that the primary effect of p27 is to inhibit tumour growth as opposed to tumour initiation.

Since the tumour burden of *p27+/-* mice was increased relative to *p27+/+* mice, we examined the *p27* locus for loss of heterozygosity. Analysis of tumours from *p27+/-* mice showed retention of the wildtype *p27* allele in all 24 tumours examined (Figure 1E), consistent with previous findings that p27 is haploinsufficient for tumour suppression (Fero et al. 1998).

Histological evaluation revealed that the majority of tumours were alveolar/bronchiolar adenomas that displayed papillary, solid, or mixed morphologies. In p27 intact mice, adenocarcinomas were not seen until 50 weeks of age, while both p27 null and heterozygous mice developed an average of one malignant tumour per mouse by 30 weeks of age (Figure 1F). These tumours where characterized by marked cellular atypia and local invasion indicating that p27 deficiency increased both tumour growth and malignant tumour progression.

Tumour suppression by p27 is cell autonomous Detection of mutations in cancer genes in tumours is evidence for a cell autonomous mechanism of action. However, mutations in the *CDKN1B* gene encoding p27 are rarely observed in tumours. Forced expression of p27 can inhibit tumour cell proliferation, and mice lacking p27 are tumour prone. However, this evidence is insufficient to prove that p27 suppresses tumours through a cell autonomous mechanism and other approaches are required to establish this conclusively. We used a neonatal lung tissue transplantation model to address this. Lung tissue from neonatal p27 deficient or wildtype mice was transplanted to the ear of adult wildtype recipients. After engraftment, mice were treated with urethane and transplant size was measured up to 30 weeks. Tumours developed from ~50% of transplants from *p27-/-* (10/19) or *p27+/-* mice (19/39), compared to only 25% (5/20) from *p27+/+* mice (Figure 2A). Tumours were larger from both *p27-/-* and *p27+/-* transplants (Figure 2B). Histologic evaluation indicated that the majority of tumours were bronchioalveolar adenomas and carcinomas. Thus, cells lacking p27 are more susceptible to tumourigenic outgrowth than are p27 intact cells, indicating a cell autonomous mechanism of tumour suppression by p27 .

Mutation of *Kras* in urethane-induced lung tumours Sequence analysis of lung tumour DNA revealed that 80% of urethane-induced tumours had a CAA to CTA transversion mutation at codon 61 of the *Kras* gene, resulting in a Q61L amino acid change (Figure 3A). The frequency of this mutation was similar in tumours from A/J, B6, and B6/129 strains. The

frequencies of *Kras* mutations were also similar in tumours from *p27+/+, +/-* and *-/-* mice (Figure 3B).

Nuclear exclusion of p27 protein in lung tumours Since p27 deficient mice showed increased susceptibility to urethane-induced lung tumours, we examined the status of p27 protein in lung tumours from p27 intact mice. Staining of tissue sections with a monoclonal anti- p27 antibody revealed prominent nuclear p27 staining in normal lung parenchymal cells but reduced nuclear p27 in tumour cells (Figure 4A). Western blot analysis of nuclear and cytoplasmic fractions prepared from frozen tissues revealed that tumours exhibit reduced nuclear p27 while retaining the cytoplasmic pool (Figure 4B). Quantitation with densitometry confirmed the ratio of nuclear to cytoplasmic p27 was markedly reduced in tumours compared to normal lung (Figure 4C). Therefore, both mutation of *Kras* and aberrant subcellular localization of p27 protein are cardinal features of this tumour model.

As both Ras mutation and altered p27 expression are consistently observed together, we asked whether oncogenic signals emanating from mutant Ras protein actively drive p27 mislocalization. We compared the phosphorylation status of specific residues of p27 (Ser10, Thr187, and Thr197) shown to be involved in regulation of cellular localization, as well as upstream candidate regulators between normal lung and tumour lysates.

Ras signaling is expected to induce cyclin D1, activate Cdk2 and drive cell cycle progression. Cdk2, in turn, is known to phosphorylate residue Thr187, targeting p27 for Jab1-mediated export and Skp2 degradation (Sheaff et al. 1997). We found an increase in both cyclin D1 and Cdk2 kinase activity in tumour extracts, consistent with the enhanced proliferation of lung tumour cells (Figure 5A). We also observed an increase in Jab1 and Thr187 phospho- p27 in cytoplasmic fractions of lung tumours (Figure 5B), leading to the conclusion that Cdk2-dependent phospho-Thr187 p27 is exported and accumulates in the cytoplasm. The cytoplasmic levels of two ubiquitin ligases for p27, Skp2 and Kpc, were similar between tumour and normal tissue, suggesting that proteasome degradation via upregulation of Skp2 or Kpc may not be the major pathway to regulate p27 abundance in this tumour model.

Ras also signals through PI3K to activate Akt, which in turn can phosphorylate p27 at multiple sites including Ser10, Thr157 (in human but not in mouse), and Thr198 (Thr197 in the mouse), each of which has been shown to promote nuclear to cytoplasmic shuttling (Susaki

and Nakayama 2007).   Phosphorylated Ser10 p27 was found in nuclear and cytoplasmic fractions and did not appear to differ between normal and tumour extracts (Figure 5C). However, as total nuclear p27 is markedly reduced in nuclear fractions from tumours, the proportion of p27 that is phosphorylated within tumour nuclear pools could be increased.

Levels of phospho-Thr198 p27 were markedly increased in lung tumours, notably in the cytoplasmic fraction (Figure 5C).   The levels of 14-3-3, which binds to cytoplasmic p27 phosphorylated at Thr198 (Fujita et al. 2003) were also modestly elevated, suggesting a plausible scenario of Akt dependent phosphorylation of Thr198 p27 and cytoplasmic retention of p27 by 14-3-3. These data together indicate p27 is phosphorylated at multiple sites in lung tumours, one or more of which could signal nuclear export and cytoplasmic retention.

Reduction of p27 transcript in lung tumours   While most studies have focused on post translational regulation of p27 abundance, transcriptional control is also documented (Philipp-Staheli et al. 2004).   Quantitative real time PCR revealed a five to ten fold reduction in p27 mRNA transcript in 13 out of 13 lung tumours examined, as compared to normal lung tissue (Figure 6A).   Signaling from Ras has been linked to p27 transcription via Akt dependent phosphorylation of Forkhead transcription factors (Medema et al. 2000).   Lung tumour extracts showed an increase in active phospho-Akt (Figure 5C), as well as phosphorylated cytoplasmic FOXO1 and FOXO3a. Phosphorylated FOXO4a, by contrast, shows equivalent expression in tumour and normal lung (Figure 6B). These data are consistent with the idea that oncogenic Kras signaling through PI3K activates Akt, which phosphorylates FOXO transcription factors, resulting in their retention in the cytoplasm, thereby reducing p27 transcription.

In summary, p27 expression in lung tumours is altered in at least two ways: increased phosphorylation leading to nuclear exclusion, and reduced transcription. Both mechanisms could be due to Ras signaling through Akt.

Inhibition of PI3K and Akt restores p27 levels in tumours Since the gene encoding p27 is intact in tumours, we asked whether its expression could be restored by pharmacologically blocking pathways leading to its misregulation. As Akt plays a role in both transcriptional and posttranslational regulation of p27, we used the PI3K inhibitor LY294002 to inhibit this pathway.   Treatment of the mouse lung tumour cell line SP10 with LY294002 led to a

reduction of phospho-Akt, a reduction in phospho-FOXO, and an increase in both p27 transcript and protein levels (Figure 7A, B). Therefore, inhibition of the PI-3 Kinase pathway can enhance p27 levels in tumour cells by increasing *p27* transcription.

We next treated tumour-bearing A/J mice with LY294002 and analyzed tumours for short term changes in Akt signaling and p27 expression. Western blot analysis of tumour lysates showed a marked reduction of phospho-Akt and phosphorylated FOXO in inhibitor treated mice compared to tumours from vehicle treated mice (Figure 7C), while total cellular levels of Akt remained unchanged. This shows that LY294002 treatment effectively inhibited the PI-3K to Akt to FOXO signaling cascade in lung tumours *in vivo*. Next, we examined p27 expression and phosphorylation. LY294002 treatment reversed the nuclear exclusion of p27 in tumours, as revealed by both Western blots of tumour lysates (Figure 7C) and immunohistochemistry of lung tumour sections (Figure 7D). Relative to untreated tumours, tumours from LY294002 treated mice showed equal nuclear/cytoplasmic distribution of p27 and more prominent nuclear staining for p27. Since LY294002 inhibits Akt, and Akt can phosphorylate p27 directly, we examined the phosphorylation status of p27. Western blotting of lysates from inhibitor treated tumours showed a reduction in both cytoplasmic phospho-Thr198 and phospho-Ser10 p27 (Figure 7E). Together, these findings indicate that aberrant expression of p27 in tumours is an active processes regulated by the PI3K-Akt pathway, and that the mislocalization of p27 in tumours can be reversed through the use of pathway specific inhibitors.

Discussion

These studies demonstrate that p27 acts as a barrier to the growth and malignant progression of Kras driven NSCLC. Both homozygous and hemizygous germline deletion of p27 led to an increase in both the size and malignancy of tumours. The observation that the average number of tumours in wild type mice at 50 weeks was similar to the number of tumours in p27 deficient mice at 30 weeks, together with the observation that tumour suppression by p27 is cell autonomous, argues that the primary effect of p27 is to inhibit the clonal expansion of initiated tumour cells. Tumours from all p27 genotypes harbored mutations in the Kras oncogene, establishing that p27 specifically inhibits Kras-driven tumour

growth. The ubiquitous reduction of nuclear p27 in tumours further implies that cell cycle inhibition by p27 is a critical barrier to be overcome for such clonal expansion.

p27 and malignant progression In addition to inhibiting tumour growth, p27 suppressed malignant lung tumour progression. By 30 weeks of age, 67% of p27 deficient mice had malignant disease, averaging one adenocarcinoma per mouse, while no carcinomas were observed in p27 intact mice. It is at present not clear whether this is due to increased tumour growth, leading to an increase in rate of tumour evolution toward malignancy, or is a more direct consequence of p27 functions; e.g. due to loss of differentiation or increased invasion. p27 can induce cell differentiation *in vitro* (reviewed in (Philipp-Staheli et al. 2001)), and p27 deficiency leads to impaired differentiation of several cell types *in vivo* (Casaccia-Bonnefil et al. 1997; Nakayama et al. 1996; Zindy et al. 1999; Tong et al. 1998). Although the mechanisms by which p27 can trigger differentiation are poorly understood, p27 induced cell cycle exit appears to be tightly linked to terminal differentiation. p27 also plays a role in cell migration, independent of its Cdk inhibitory function (Besson et al. 2004a). p27 has been shown to inhibit cancer cell invasion (Yuan et al. 2007; Schiappacassi et al. 2008) by binding to microtubule destabilizing protein, stathmin (Baldassarre et al. 2005). On the other hand, a promigratory role for cytoplasmic p27, through interaction with the Rho family of GTPases and the cytoskeleton, has also been described (McAllister et al. 2003; Besson et al. 2004b). Hence, tumours with reduced or mislocalized p27 may have impaired capacity to differentiate and/or enhanced migratory ability thereby increasing their malignant behavior.

Malignant tumour progression due to reduced p27 has been documented in other mouse cancer models (Philipp et al. 1999; Philipp-Staheli et al. 2002; Shaffer et al. 2005), indicating this is a characteristic trait of tumour suppression by p27. In human cancer, including NSCLC, reduced p27 expression correlates with higher tumour grade and stage, features which incorporate differentiation, invasion and metastasis (Chu et al. 2008). Together, this indicates that p27 is a barrier to malignant progression and the misregulation of p27 in tumours is causally linked to tumour aggressiveness.

p27 misregulation in tumours Having established that p27 inhibits both the growth and malignant progression of NSCLC, we next examined the mechanism by which tumours circumvent p27. Lung tumours showed a consistent reduction in the nuclear pool of p27,

while largely retaining cytoplasmic expression, as well as a marked reduction of mRNA transcript. Thus, transcriptional control as well as subcellular localization contribute to a reduction of nuclear p27 in tumours, which can lead to increased CDK activity. Activating mutations in Kras were also cardinal features of this tumour model, suggesting a mechanistic link between Kras signaling and p27 loss. Indeed, an emerging model to explain the basis of cooperation between oncogene activation and tumour suppressor gene loss posits that oncogenes directly signal to tumour suppressor genes. The Ras effector, Akt, has been shown to regulate p27 at multiple levels, so we asked whether p27 misregulation in tumours is a direct consequence of Akt signaling. p27 can be barred from the nucleus by pathways involving phosphorylation at Ser10, Thr157 (human but not mouse), and Thr198, all of which have been reported as Akt targets. We did not observe significant changes in abundance of phospho-Ser10 p27 in lung extracts. However, as the total nuclear p27 levels were decreased in tumours, it is possible that the ratio of phospho-Ser10 p27 to total p27 is increased in these tumours. Akt can also phosphorylate p27 on Thr198 and promote binding to 14-3-3 in the cytoplasm (Fujita et al. 2003; Motti et al. 2005; Motti et al. 2004). Lung tumours showed a marked increase in phospho-Thr198 p27, specifically in the cytoplasmic pool, together with a modest increase in 14-3-3, suggesting phospho-Thr198 may direct nuclear export **a**nd increase stability of p27 in the cytoplasm. Indeed, inhibition of Akt in both mouse lung tumour cell lines and in tumour-bearing mice led to a reduction in phospho-Ser10 and phospho-Thr198 p27 and redistribution of p27 to the nucleus.

Akt is also a key player in mediating signaling from Ras through the FOXO transcription factors to regulate p27 transcription (Medema et al. 2000). Increased phosphorylation of FOXO proteins was seen in cytoplasmic fractions from lung tumours, correlating with reduced p27 mRNA levels. LY294002 reduced phosphorylation of both Akt and FOXO and increased p27 transcript and nuclear p27 protein levels. Together these findings demonstrate that Ras signaling through the PI3K/Akt pathway regulates p27 abundance and localization at both the transcriptional and post-translational levels.

In summary, p27 acts as a potent barrier to Kras initiated NSCLC that must be overcome for efficient clonal expansion and malignant progression. However, in contrast to most known tumour suppressors which are inactivated by mutation, p27 reduction is an active and reversible process; in this case, mediated by oncogenic signaling. If a similar mechanism

occurs in human lung cancer, this would create a window of opportunity for therapeutic intervention.

Materials and methods

Mice Experiments were approved by the FHCRC Institutional Animal Care and Use Committee. Two cohorts of 129 x C57BL/6J F_1 *p27-/-, p27+/-,* and *p27+/+* mice (Fero et al. 1996) as well as C57BL/6J and A/J mice were treated with urethane (1mg/g bodyweight, i.p.) at 12 days of age. Cohorts were sacrificed at 30 or 50 weeks of age. Tumours visible on the surface of all lung lobes were counted and measured using a dissecting microscope. Normal and tumour tissues were either snap frozen in liquid nitrogen for DNA, RNA, and protein extraction or fixed in neutral buffered formalin. Fixed tissue was processed and stained with H&E for histological examination. DNA for genotyping was obtained by digestion of tissue with proteinase K in InstaGene Matrix solution (Bio-Rad, Hercules, CA, USA). Each mouse was genotyped by PCR using primers specific for the wild-type or *neo* interrupted *p27* locus (Fero et al. 1996).

Kras sequence analysis Genomic DNA was isolated from lung tumours using the QIAGEN QIAamp DNA mini kit. The *Kras* locus at codon 61 was amplified using primers 5'-GACTCCTACAGGAAACAAGT-3' and 5'–GAAGATATTCACCATTATAG-3' (You et al. 1989) by PCR and sequenced using the Big Dye Sequencing protocol (PE-Biosystems).

Western blot analysis Nuclear and cytoplasmic protein extracts were prepared as described (Schreiber et al. 1989), with modifications (Philipp-Staheli et al. 2002). Protein concentrations were standardized using the Bradford assay (BioRad), and equal loading was confirmed by Ponceau S staining of PVDF membranes after blotting (Immun-Blot™ PVDF membrane, BioRad). The following antibodies were used for Western blotting: p27 (sc-528), phospho- Thr187 p27 (sc-16324), phospho- Ser10 p27 (sc-12939), cyclinD1 (sc-755), cdk2 (sc-163), Jab1 (sc-9074), 14-3-3 θ (sc-732), β-tubulin (sc-9104) and lamin B1 (sc-20682) were from Santa Cruz Biotechnology (Santa Cruz, CA). p45/Skp2 antibody (ab19877) was purchased from Abcam and the phospho-Thr198 p27 purchased from R&D Systems (AF3994). The anti-KPC1 antibody (H00063891-A01) was obtained from Novus Biologicals.

Akt phospho-specific (9271, 9275) and FOXO family (9464, 9466, 9471) antibodies were purchased from Cell Signaling (Beverly, MA).

Histopathology and immunohistochemistry   Lung lobes with embedded tumours were removed and fixed in 10% neutral buffered formalin for 4-6 h, processed and embedded in paraffin. Step sections were made, with every fifth section stained with H&E for the most accurate histologic quantification of tumour multiplicity and malignancy. All slides were reviewed by a pathologist (SK) blind to the genotype. Diagnosis of adenoma was based on discrete, well demarcated foci consisting of a uniform population of epithelial cells with round nuclei and a moderate amount of eosinophilic cytoplasm. Compared with adenomas, adenocarcinomas showed greater cellular atypia, regional variation of growth patterns, increased mitotic activity, and local invasion into adjacent bronchioles or vessels. Immunohistochemistry for p27 was performed as described (Philipp-Staheli et al. 2002) using a mouse monoclonal antibody from Neomarkers (Fremont, CA). Controls included no primary antibody and/or normal rabbit serum, and tissues from p27 null mice.

Histone kinase assay Protein extracts were prepared as described above and stored in 50% glycerol. 400 µg of protein were incubated on ice in 500 µl RIPA buffer containing Cdk2 antibody. Then 30 µl of washed Protein A-Sepharose beads were added, and the reaction was rotated for 1 h at 4° C. Beads were washed twice in RIPA and once in a buffer containing 25 mM Tris HCl (pH 7.5), 70 mM NaCl, 10mM $MgCl_2$, and 1 mM DTT. The beads were then incubated with Histone H1 as a substrate and ^{32}P-γATP for 30 min at 37° C. The reaction was stopped by the addition of sample buffer, denatured for 5 min at 95° C, and the protein separated on a 12% SDS acrylamide gel run for 45 min at 150 volts. The gel was washed, fixed and exposed to X-ray film to visualize bands.

Real-time PCR assay The relative levels of p27 mRNA were assessed using RTPCR as described (Philipp-Staheli et al. 2004), using the forward primer 5'-AGGCTGGGTTAGCGGAGC-3'; reverse primer 5'-GAACCGTCTGAAACATTTTCTTCTGT-3'; and probe 5' FAM-ACCTTGCTGCAGAAGATTCTTCTTCGCAA-TAMRA 3'.

Tumour DNA isolation and LOH analysis DNA was extracted from frozen tumours using BioRad InstaGene Matrix and p27 PCR conditions are available on request. PCR products were run on a 1% agarose gel stained with ethidium bromide.

Ear engraftment   Lungs were removed from neonatal mice derived from NIH *p27+/-* x B6/129 *p27+/-* crosses and cut into $2mm^3$ sections and kept on ice. Recipient NIH wild type adult mice were anesthetized with avertin. A small pouch was made in both ears using a scalpel and scissors. A $2 mm^3$ section of lung was inserted, and the pouch sealed with super glue. For a given recipient, transplant of a different *p27* genotype in each ear served as a control. Mice were injected with urethane once a week for 6 weeks (0.25 mg/g body weight i.p.). The ear grafts were measured every other week and mice were sacrificed at 30-38 weeks, and tissues processed as described above.

PI3K inhibition   SP10 lung tumour cells (ATCC), derived from a spontaneous lung tumour from an A/J strain mouse, were grown to 70% confluence, serum starved overnight, then serum containing media with 50 µg/ml LY294002 (Sigma) was added. Cells were harvested at 1, 2, 4, 6, and 18 h post-treatment and whole cell lysates were made using buffer containing 150 mM NaCl, 50 mM Tris pH 8, and 1% NP-40. Cells not subjected to serum starvation or inhibitor treatment were used as controls in Western analysis. For *in vivo* studies, nine month old lung tumour-bearing A/J mice were injected with LY294002 (100 mg/kg body weight, i.p.) or DMSO three times over a six hour period.

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Titles and legends to figures

Figure 1. *p27* is a lung tumour suppressor Histogram comparing the number and sizes of urethane-induced lung tumours in *p27+/+, +/-,* and *-/-* mice at (A) 30 weeks and (B) 50 weeks post-treatment.   Only four p27 null mice survived to a median age of 46 weeks and the tumour counts from these mice are graphed in (B).   (C)   Table showing mean number of tumours per mouse according to genotype and age. (D) Photograph of lungs from *p27+/+* and *p27-/-* mice showing increased tumour burden in *p27-/-* lungs. (E) Loss of heterozygosity analysis of DNA from tumours from *p27+/-* mice, n= 24 tumours examined in total. DNA from *p27+/+* and *-/-* tumours are shown as controls. wt= wild type *p27* allele. mu= knockout *p27* allele. (F) Histogram showing mean number of adenocarcinomas per mouse at 30 and 50 weeks of age. Insufficient numbers of p27-/- mice survived to 50 weeks, so these are not shown. Shown are p values from unpaired t-test for comparisons between genotypes. (G) Photomicrographs of lung sections with an alveolar/bronchiolar adenoma from a *p27+/+* mouse (left) and an adenocarcinoma from a *p27-/-* mouse (right). Note the discrete boundary between adenoma and normal lung (clear arrow) and monomorphic, orderly tumour cells (clear arrowhead), compared to invasive tumour cells of the adenocarcinoma into lung parenchyma and local airway (black arrows) and pleomorphic appearance of tumour cells (black arrowheads).

Figure 2. Tumour suppression by *p27* is cell autonomous Lung tissue from *p27+/+, +/-,* or *-/-* mice was transplanted to the ears of wild type recipients. After engraftment, mice were then treated with urethane and transplant size was measured up to 30 weeks *(p27+/+* n=14, *p27+/-* n=23, *p27-/-* n=14). (A) Bar graph shows percent transplants that developed tumours. (B) Size of transplanted tumours at 30 weeks. Each symbol represents one transplant.

Figure 3. *K-ras* mutations are frequent in lung tumours (A) The chromatograms show the *Kras* wildtype sequence (left) and an CAA>CTA mutation in codon 61 (right) from a tumour. (B) Table shows frequency of *K-ras* mutations in tumours from different genetic backgrounds and *p27* status.

Figure 4. Nuclear p27 is reduced in lung tumours   (A) Photomicrograph of a lung adenoma section from a wild type mouse stained with a p27 antibody. Note strong nuclear staining in normal lung parenchymal cells and reduced nuclear staining in tumour cells. (B)

Western blot of extracts from lung tumours and normal lung from wild type mice probed with p27 antibody. n: nuclear extracts, c: cytoplasmic extracts. p27 antibody specificity is indicated by tissue from p27 null mouse (-/-) as a negative control. (C) Quantitation of nuclear (n) to cytoplasmic (c) p27 protein expression from Western blots of normal and tumour lysates. Numbers show median values of n/c ratios.
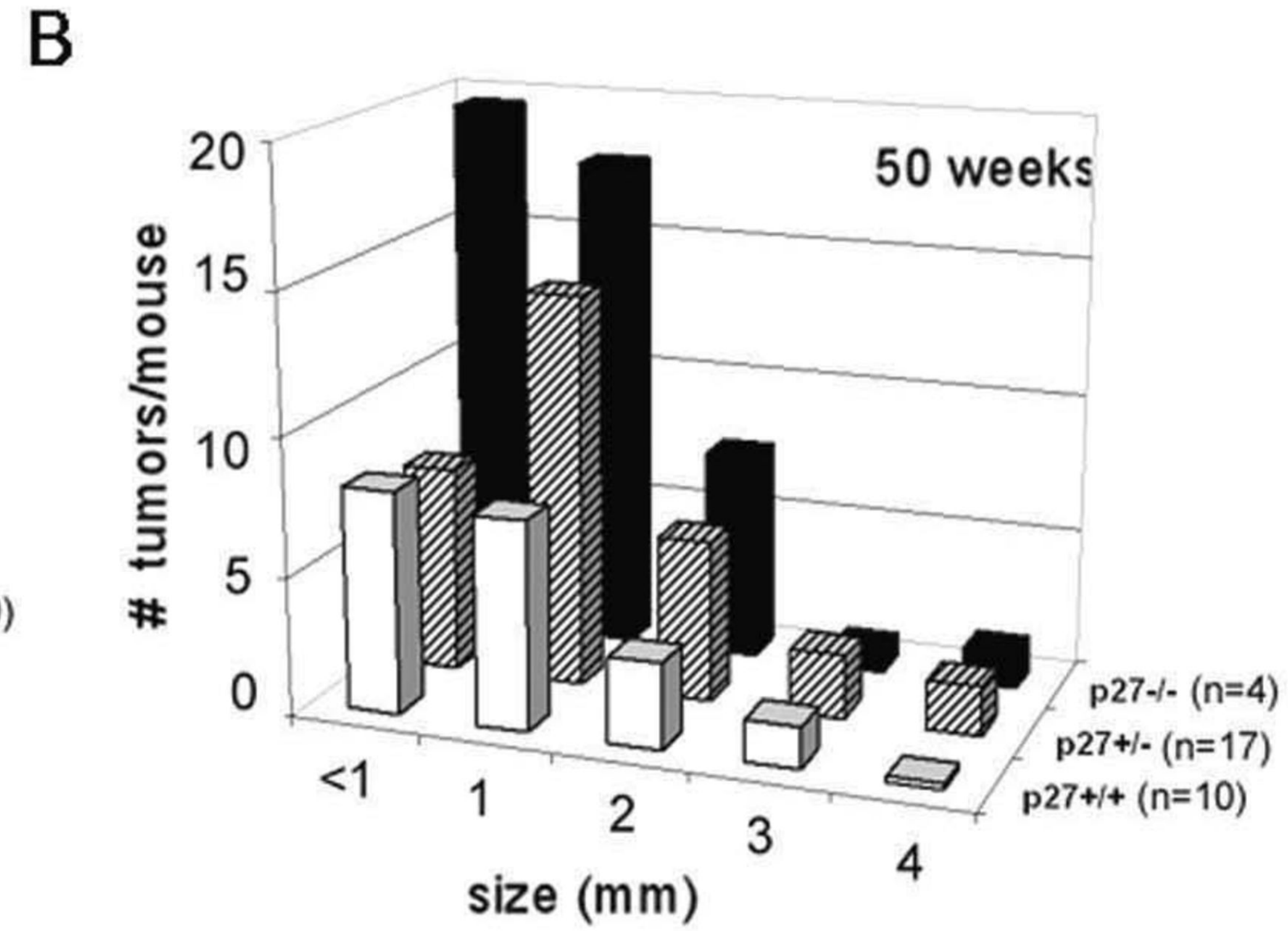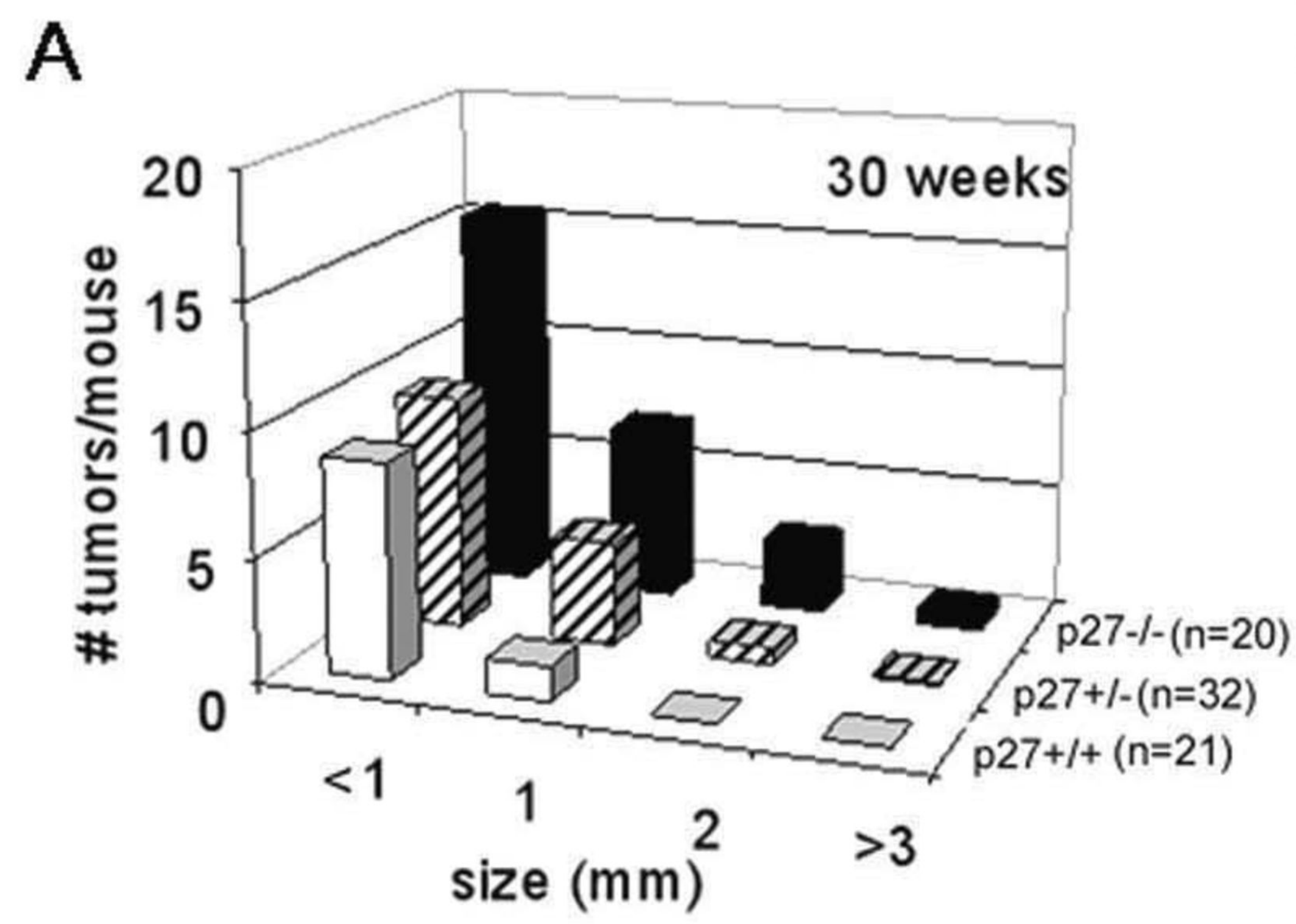
Figure 5. Posttranslational regulation of p27 in lung tumours (A) Increased Cdk2 activity in lung tumours from wild type mice versus normal lung using histone H1 as a substrate. Western blot of whole cell lysates shows Cdk2 protein levels unchanged and an increase in cyclin D1 in lung tumours compared to normal lung. (B) Western blot of total p27, phospho-T187 p27, Jab1, KPC, and Skp2 in tumours and normal lung, with lamin B1 and β-tubulin as nuclear and cytoplasmic fractionation controls. Note increase in p-T187 p27 in cytoplasmic fractions compared to normal lung. n: nuclear extracts, c: cytoplasmic fractions. (C) Western blot of p-T198 p27, p-S10 p27, and 14-3-3-θ. Note increased p-T198 p27 in cytoplasmic tumour fractions.

Figure 6. Reduced *p27* transcript and increased p-Akt and p-FOXO in lung tumours (A) Bar graph of *p27* mRNA expression levels derived from real-time PCR using total RNA isolated from lung tumours and normal lung (NL) from wild type mice. (B) Western blot shows expression levels of phosphorylated forkhead family members, p-FOXO1, p-FOXO3a, and p-FOXO4a in lung tumour extracts compared to normal lung. n: nuclear extracts, c: cytoplasmic fractions.

Figure 7. Inhibition of the PI3K pathway increases nuclear p27 in tumours (A) Time course analysis of effects of LY294002 (LY) on SP10 mouse lung tumour cell line. Shown are Western blots of cell lysates probed with antibodies to p-Akt, p-FOXO, and p27 at the indicated times after addition of serum and LY. The control (ctrl) lane is from cells not serum starved. (B) Real-time PCR of p27 mRNA from treated (+LY) and control (-LY) treated SP10 cells 8 and 24 h after start of PI3K inhibition. (C) Western blot of total and p-Akt, p-FOXO, and p27 in lung tumours from wild type mice 5 h after treatment with LY. n: nuclear extracts, c: cytoplasmic fractions. (D) Sections of representative lung tumours immunostained for p27 shows increase in nuclear p27 following LY294002 treatment. (E) Western blot of tumour

lysates shows decreased p-T198 p27 and p-S10 p27 in LY294002 treated lung tumours. Lamin B1 is nuclear fraction loading control. N.Lung = normal lung.
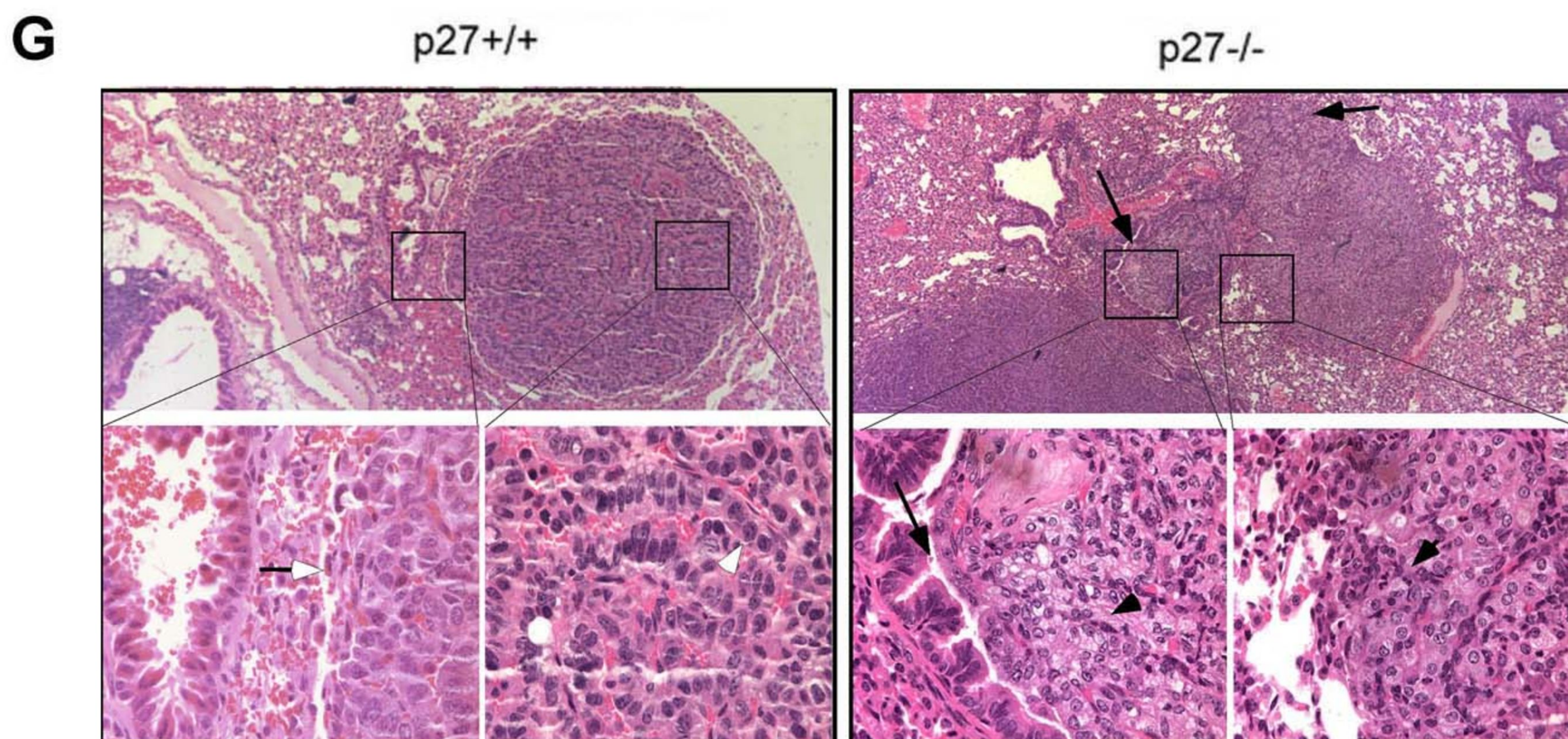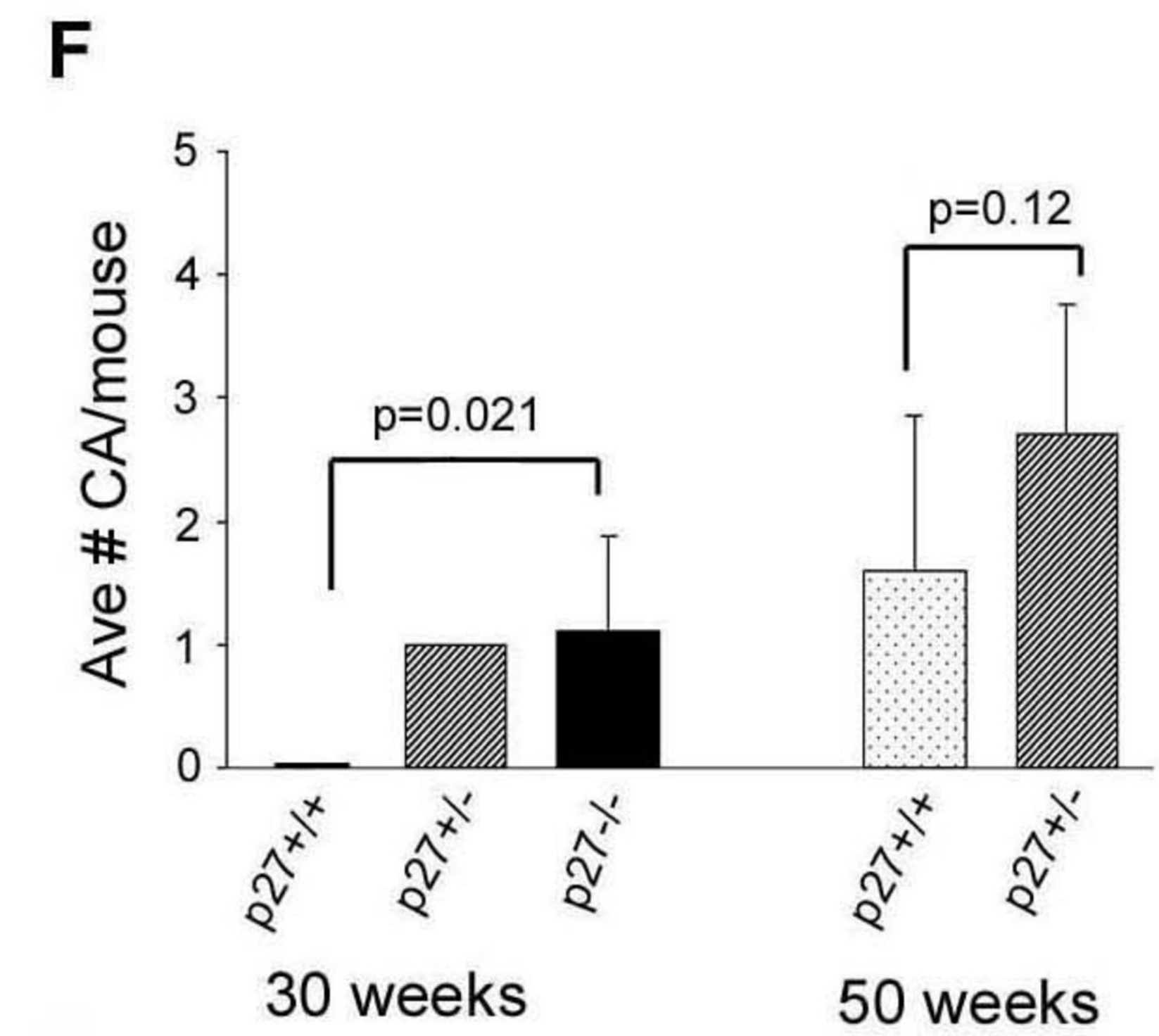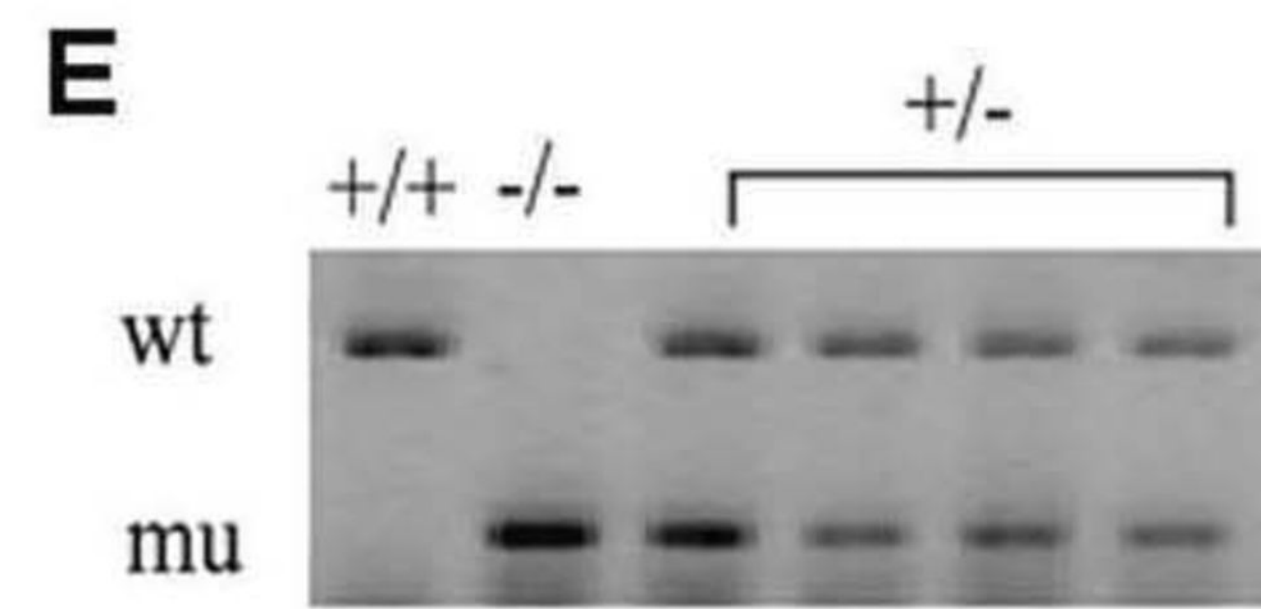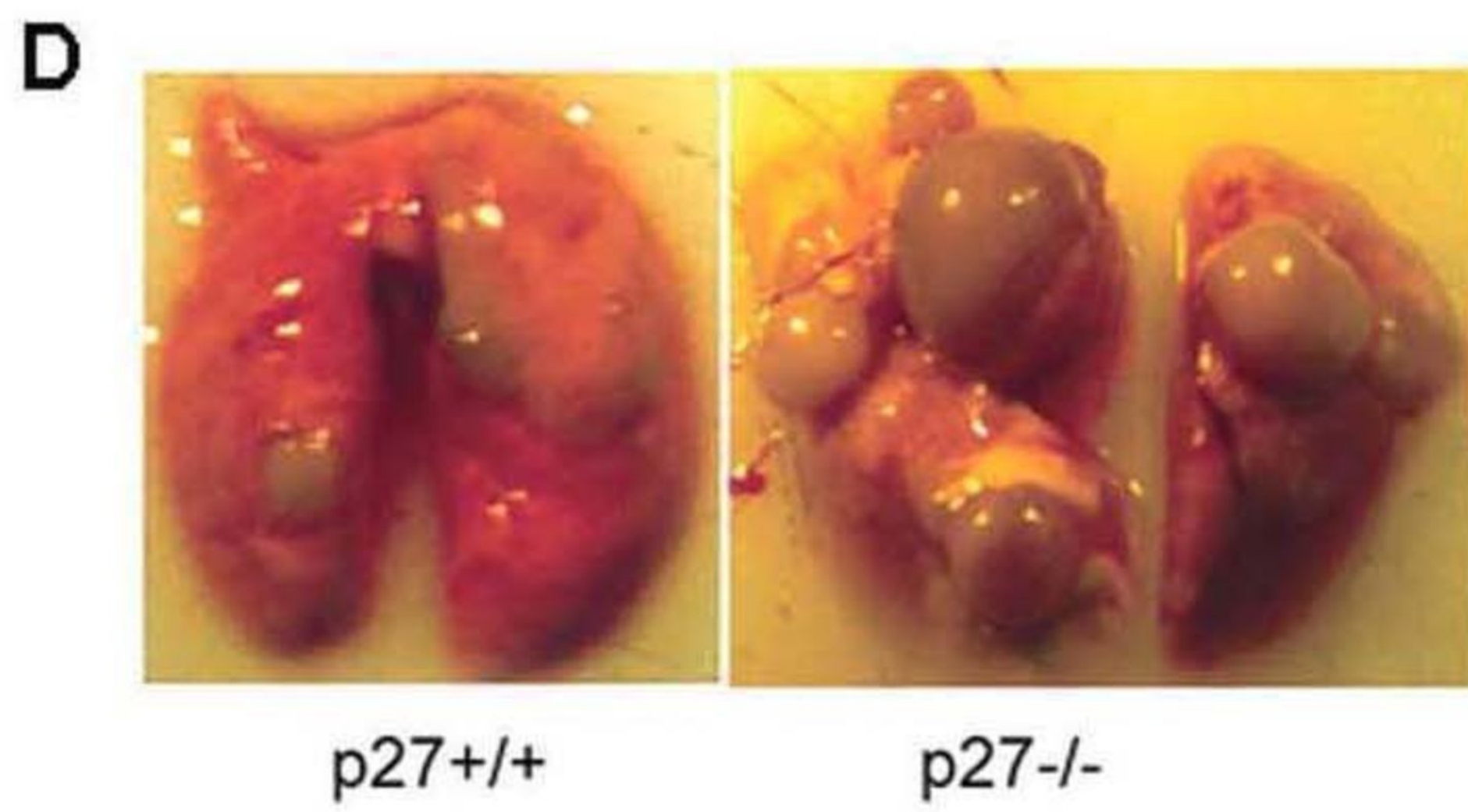
A 30 weeks

tumors/mouse — size (mm): <1, 1, 2, >3

p27-/- (n=20)
p27+/- (n=32)
p27+/+ (n=21)

B 50 weeks

tumors/mouse — size (mm): <1, 1, 2, 3, 4

p27-/- (n=4)
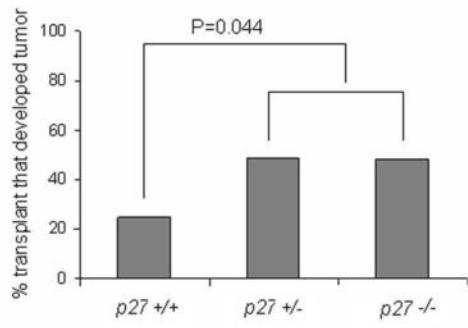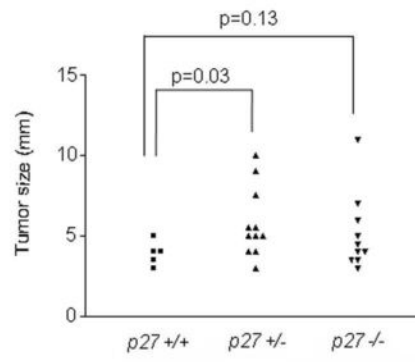p27+/- (n=17)
p27+/+ (n=10)

C Lung tumor multiplicity at 30 and 50 weeks post-urethane injection.

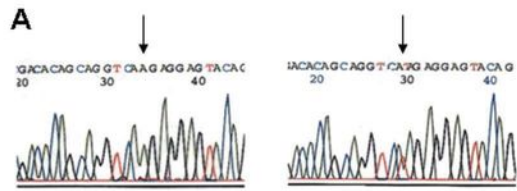| Weeks	p27 genotype	# mice	# tumors/mouse Mean ± SD	p value two-tailed t test
30	p27+/+	11	10.1 ± 3.0	
30	p27+/-	14	14.5 ± 6.7	p=0.054
30	p27-/-	16	25.9 ± 12.1	p=0.0004
50	p27+/+	10	20.4 + 3.8	
50	p27+/-	17	31.3 ± 12.9	p=0.038
50	p27-/-	4	35.5 ± 14.2	p=0.015

p value = p27+/- and -/- compared to +/+ ; SD = Standard deviation

D

p27+/+   p27-/-

E

+/+ -/-   +/-

wt
mu

F

Ave # CA/mouse

p=0.021   p=0.12

p27+/+ p27+/- p27-/-   p27+/+ p27+/-
30 weeks   50 weeks

G

p27+/+   p27-/-

A

% transplant that developed tumor

P=0.044

p27 +/+   p27 +/-   p27 -/-

B

Tumor size (mm)

p=0.13

p=0.03

p27 +/+   p27 +/-   p27 -/-

A

B

Frequency of *Kras* mutation at codon 61.

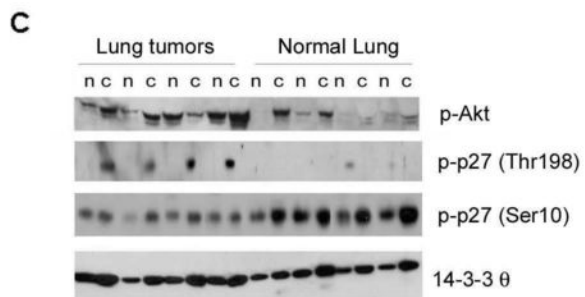| Mouse strain/ genotype	# tumors with K-ras^{Q61L}
A/J	4/6
B6	5/7
B6/129	4/4
p27+/+	8/10
p27+/−	23/30
p27−/−	12/13

A

Lung tumor   Normal lung

B

Lung tumors   -/-   Normal lung

n c n c n c n c n c n c n c n n c n c n c n c c

→ p27

C

n/c ratio

2

1.5

1

0.74

0.5

0.22

0

tumors   normal

A

Lung tumors | Normal lung

Cdk2 activity

Cdk2

CyclinD1

B

Lung tumors | Normal lung

n c n c n c n c n c | n c n c n c n c n c

p27

p-p27 (Thr187)

KPC1

Jab1

Skp2

Lamin B1

Tubulin

C

Lung tumors | Normal Lung

n c n c n c n c | n c n c n c n c

p-Akt

p-p27 (Thr198)

p-p27 (Ser10)

14-3-3 θ

A

B

A

+ LY   - LY
ctrl 1 2 4 6 18 1 2 4 6 18   hrs

Akt

p-Akt

p-FOXO

p27

B

p27mRNA (% of 0 h)

250%
200%
150%
100%
50%
0%

0 h   8 h   8 h   24 h   24 h
       -LY   +LY   -LY   +LY

C

- LY   + LY
n c n c n c n c   n c n c n c n c

Akt

p-Akt

p-FOXO

p27

D

-LY   +LY

E

N. Lung   - LY   +.LY
n c n c n c n c n c n c n c n c n c

p-p27 (Thr198)

p-p27 (Ser10)

Lamin B1