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immerbeul, I., Garrett-Engele, C.M., Kossatz, U., Chen, X., Firpo, E.,
Grünwald, V., Kamino, K., Wilkens, L., Lehmann, U.,
Buer, J., Geffers, R., Kubicka, S., Manns, M.P., Porter, P.L., Roberts, J.M.,
Malek, N.P.
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murine models of lung and colon cancer
(2006) Proceedings of the National Academy of Sciences of the United
States of America, 103 (38), pp. 14009-14014.**

Testing the importance of p27 degradation by the SCF^{Skp2} pathway in murine models of lung and colon cancer

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Classification: Biological Sciences, Cell Biology

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Manuscript Information: 18 pages, 4 Figures

Abstract word count: 207

Paper character count: 45335

Abstract

Decreased expression of the CDK inhibitor p27^{kip1} in human tumors directly correlates with increased resistance to chemotherapies, increased rates of metastasis and an overall increased rate of patient mortality. It is thought that decreased p27 expression in tumors is caused by increased proteasomal turnover, in particular activation of the pathway governed by the SCF^{Skp2} E3 ubiquitin-protein ligase. We have directly tested the importance of the SCF^{Skp2}-mediated degradation of p27 in tumorigenesis by analyzing the tumor susceptibility of mice that express a form of p27 that can not be ubiquitinated and degraded by this pathway (p27T187A). In mouse models of both lung and colon cancer downregulation of p27 promotes tumorigenesis. However, we found that preventing p27 degradation by the SCF^{Skp2} pathway had no impact on tumor incidence or overall survival in either tumor model. Our study unveiled a previously unrecognized role for the control of p27 mRNA abundance in the development of non-small cell lung cancers. In the colon cancer model, the frequency of intestinal adenomas was similarly unaffected by the p27T187A mutation, but unexpectedly, we found it inhibited progression of intestinal adenomas to carcinomas. These studies may guide the choice of clinical settings in which pharmacologic inhibitors of the Skp2 pathway might be of therapeutic value.

Introduction

The CDK inhibitor p27^{kip1} controls the progression of cells through the G1 phase of the cell cycle by regulating the activities of Cyclin E and Cyclin A/Cdk2 complexes (1). Loss of p27 in the mouse leads to a significant increase in the proliferation of all tissues (2). It also predisposes the mouse to the spontaneous development of pituitary carcinomas and the occurrence of tumors in multiple tissues after irradiation or treatment with the carcinogen N-ethyl-N-nitrosourea (ENU). Importantly, reduction of p27 dosage by 50% in p27 heterozygous mice also leads to accelerated carcinogenesis making p27 a haploinsufficient tumor suppressor protein (3). By crossing p27 knockout mice to mouse strains with defects in other tumor suppressor proteins, cooperative functions of p27 with proteins like PTEN, CBP and Rb in preventing tumor formation were uncovered (4-6).

The results obtained in the p27 knockout mouse correlate closely with the observation that low expression levels of p27 alone or in combination with other defects predict an overall reduced survival of the human cancer patients. An important difference between p27 and other tumor suppressor proteins, like p53 or p16, is that the decrease in p27 expression in tumor tissue is not caused by mutations of the p27 gene or epigenetic silencing. It is generally believed that downregulation of p27 in cancers occurs through accelerated degradation of the p27 protein (7). Nevertheless, direct evidence in support of this hypothesis is limited to *in vitro* experiments in which extracts from increasingly aggressive primary human tumors had a correspondingly increased ability to degrade recombinant p27 (8).

These observations have suggested that pharmacologic stabilization of p27 in tumor tissues may be a useful therapeutic strategy (9). However such strategies require a detailed understanding of the molecular mechanisms involved in p27 turnover. It is now clear that the degradation of p27 during late G1 and S-phase depends upon the phosphorylation of p27 at Threonine 187 (T187). This phosphorylation leads to binding of p27 to the F-box protein Skp2 and subsequent polyubiquitylation by the Skp1-Cullin-F-box (SCF) E3 ligase (10-12).

Previously, we generated a mouse knock-in system in which T187 was replaced by alanine (T187A) and characterized how loss of this SCF^{skp2}-mediated pathway for p27 degradation affected normal development (13). Since that time other mechanisms for regulating p27 levels have been described. In addition to proteolytic degradation by the SCF^{skp2} E3 ligase, translational, transcriptional, mRNA stability, and alternative proteolytic mechanisms have been shown to regulate the abundance of the p27 protein (14-17). Furthermore the subcellular localisation of the p27 protein can be regulated through phosphorylation at different sites (18). The relative contributions of these many regulatory pathways to controlling p27 levels both in normal and tumor cells has not been investigated.

Results

Loss of p27 promotes tumorigenesis in the lung independent of SCF^{skp2} mediated p27 degradation

In this study we have focused on the importance of the SCF^{skp2}-mediated pathway for downregulation of p27 during tumorigenesis. Our strategy was to determine whether expression of p27T187A would affect tumor incidence, progression and overall survival in two different tumor models. The first model system was one in which spontaneous activation of a latent copy of the activated *K-ras* oncogene causes 100% of all transgenic animals (*K-ras**) to develop lung tumors (19). These tumors are reminiscent of human non-small cell lung carcinomas (NSCLC) with the earliest lesions developing after one week of age. We crossed the p27 null (p27^{-/-}) and p27T187A alleles into the *K-ras** background to generate homozygous p27^{-/-} /*K-ras**, or homozygous p27T187A/*K-ras** (p27T187A/*K-ras**) mice. To detect differences in survival we followed the entire colony of transgenic mice (*K-ras**, p27^{-/-} /*K-ras** and p27T187A/*K-ras**) and when an animal appeared moribund it was sacrificed and subjected to a focused necropsy. The tumors from all three genotypes were stage 1 moderately differentiated, non-invasive adenomas, and no difference in proliferation as measured by Ki67

staining was observed among the tumors (data not shown). As shown in Figure 1A, loss of p27 accelerated the course of the disease significantly leading to the death of 50% of all mice after 6 months ($P < 0.0001$). In contrast, most *K-ras** and p27T187A/*K-ras** mice were still alive at 6 months. This result indicated that loss of p27 cooperated with the activation of *K-ras* in the formation of lung tumors. Importantly the p27T187A/*K-ras** strain, expressing a potentially stabilized form of p27 protein, did not show a significant advantage in overall survival compared to wild-type p27 mice expressing the activated *K-ras* gene ($P > 0.3$).

p27 levels in NSCLC are regulated at the mRNA level

Next we compared the expression levels of p27 and p27T187A in lung cancer tissue and surrounding normal non-cancerous tissue by immunohistochemistry using an antibody specific to p27. This technique has been shown to accurately measure the amounts of p27 protein expressed in lung carcinomas (20). Specifically it distinguishes expression of p27 in tumor cells from tumor infiltrating cells like lymphocytes or blood vessels. As shown in Figure 1B, no differences in the expression of p27 were detectable between *K-ras** and p27T187A/*K-ras** mice. Both strains showed clear p27 staining in surrounding normal lung tissue while little or no p27 expression was detectable in the cancerous lesions.

Cks1 has previously been shown to be rate limiting for turnover of p27 by the SCF^{skp2} pathway (21, 22) in vitro and in vivo. Also, recent studies in human non-small cell lung cancer tissue suggested an inverse relation between cks1 and p27 expression (23). We therefore measured the protein expression levels of this critical component of the T187 and SCFskp2 dependent degradation mechanism in lung tumor and surrounding normal tissue of *K-ras** and p27T187A/*K-ras** mice. As shown in Figure 2A we found cks1 expression unchanged or even slightly decreased in lung tumor tissue in both wild-type and p27T187A mice. These experiments indicated that whereas p27 acted as a tumor suppressor protein in the development of NSCLC, its downregulation in tumor tissue was independent of the Skp2-

mediated T187 dependent degradation pathway.

We therefore wondered whether regulation at the RNA level rather than increased protein turnover might account for the observed changes in p27 expression. To test this hypothesis we measured p27 mRNA levels by quantitative real-time PCR in lung tumor tissue from *K-ras** and *K-ras**p27T187A mice, and compared it to mRNA levels in *K-ras** normal lung tissue. We found a strong downregulation of p27 mRNA levels in *K-ras** (76-92%) and T187A/*K-ras** lung cancer tissue (65-93%) compared to normal lung (Fig. 2B). Identical results were obtained when p27 mRNA expression in lung cancer tissue micro-dissected from the ENU treated mice was compared to surrounding wild-type tissue (see below; data not shown), indicating that independent of the inducing agent p27 downregulation occurs by modulating the p27 transcript levels in these tumors.

Regulation of the p27 transcript in human breast cancers

Our study of *K-ras** induced tumorigenesis pointed towards a role of p27 mRNA regulation as a mechanism involved in the control of p27 expression in cancer cells. To ascertain whether p27 mRNA regulation occurs in human cancers, the p27 protein and mRNA levels were assayed in breast cancers, a tumor entity in which p27 protein expression levels predict the clinical outcome of the affected patients (24). In order to determine whether low p27 protein levels correlate with decreased p27 mRNA, the quantity of p27 transcript was analyzed in tumors with known p27 protein amounts.

Twenty-seven breast tumors were assayed for p27 protein expression. From this analysis, 13 tumors were identified that clearly exhibited either high nuclear or low nuclear p27 protein staining (Fig. 2C). The other 14 specimens had intermediate levels of p27 protein or cytoplasmic staining. Cytoplasmic mislocalization of p27 in response to oncogenic signals contributes to functional inactivation of the p27 protein in breast cancer tissues(25, 26). In such tumors overall p27 levels are of questionable significance in predicting patients survival.

We therefore focused our analysis on breast cancer samples with predominant nuclear p27 staining. The relative amount of p27 transcript in the 13 tumors with nuclear staining was determined by quantitative RT-PCR (Fig. 2D). While the transcript levels vary among the samples, all of the tumors in the lowest quartile of p27 mRNA amounts were also low for p27 protein, suggesting the decreases in transcript levels in these specimens caused the decrease in p27 protein. Additionally, a previous study analyzing only p27 mRNA levels in breast cancers indicated that overall survival was significantly decreased for patients in the lowest quartile of p27 mRNA compared to patients with higher p27 transcript levels²⁵. Overall, this pilot analysis indicated that in more than half (62%) of human breast cancers downregulation of the p27 protein may be caused by decreased abundance of the p27mRNA. This was consistent with our mouse studies, which showed that inactivation of the major pathway for p27 proteolysis did not affect its downregulation during lung tumorigenesis.

Stabilization of p27 interferes with colon cancer development

To extend our analyses of the p27T187A mutation to a broader spectrum of tumors, we injected 15 day old mice intraperitoneally with the carcinogen N-ethyl-N-nitrosourea (ENU). We chose this agent as its carcinogenic effect had previously been shown to be increased by loss of p27 (3). These mice predominantly develop colon tumors, and ultimately succumb to this disease by one year of age. The effect of the T187A mutation on colon tumorigenesis is considered in detail, below. However, these mice also developed liver carcinomas, albeit at a relatively lower frequency. Nevertheless, we found that the p27 protein was downregulated in these liver carcinomas equally in the wild-type and p27T87A mice (not shown), which was consistent with what we had observed during lung tumorigenesis.

As in our initial study in the *K-ras** strain, our primary question was whether p27 stabilization would lead to an improved overall survival of the mutant mouse as compared to wild-type controls. Therefore we observed the entire colony of ENU treated animals for a

total of 58 weeks after injection of the carcinogen and only killed moribund mice when clear signs of fatal disease were obvious. Figure 3A shows that wild-type and p27T187A animals died with comparable frequencies between 18 and 58 weeks; thus, expression of p27T187A did not lead to an improved overall survival. Necropsies were performed on all mice followed by a complete histological analysis of all internal organs. As shown in Figure 3B most animals showed multiple tumors at the time of necropsy with no obvious differences in the spectrum of tumors that arose in wild-type p27 and mutant p27T187A mice.

Interestingly, we did however observe a significant difference in the frequency of intestinal carcinomas with wild-type mice showing double the number of carcinomas of the intestine at the time of death ($p < 0.05$) compared to p27T187A (Fig. 3B). The development of intestinal carcinomas has been studied extensively in humans and in transgenic mouse models, and it is generally agreed that adenomatous lesions represent an early, non-malignant stage that can progress through a series of molecular steps to invasive carcinomas (27). Several previous studies which examined primary human colon cancer tissue as well as experimental mouse models of intestinal tumorigenesis (28) had shown a correlation between a more malignant phenotype and reduced p27 expression (29, 30). The observation that p27T187A mice developed fewer intestinal carcinomas after treatment with ENU suggested that a T187 dependent degradation mechanism might indeed represent a key step in the progression of this tumor.

Stabilization of p27 affects tumor development in the colon by a mechanism independent of controlling cell proliferation

As shown in Figure 4C untreated p27T187A mice had reduced numbers of PCNA positive cells in the epithelial lining of the bowel compared to wild-type controls. This reduction in the number of proliferating cells in the intestinal epithelium of p27T187A mice correlated with increased expression of p27. Figure 4A (panels E, F) shows representative

sections through p27 wild-type and p27T187A intestinal epithelia after staining with a p27 specific antibody. Figure 4B shows a quantification of the number of cells which stained positive for p27 in normal, adenomatous and carcinoma tissues derived from the different mouse strains. Interestingly, this baseline difference in cell proliferation did not result in a decreased incidence of ENU-induced intestinal adenomas in p27T187A mice (31%) compared to wild-type (21%) mice probably due to the fact that the intestinal tumors arising in the p27T187A strain had a tendency to accumulate at the adenoma stage rather than progressing to carcinoma. We then determined the expression levels of p27 in adenomas and carcinomas from wild-type and p27T187A mice. p27 staining was clearly detectable in the tissue surrounding adenomatous lesions in wild-type mice (Fig. 4A, panel C) while the adenomas expressed p27 at much reduced levels. p27T187A mice expressed significantly more p27 protein in both adenomas and surrounding normal tissue (Fig. 4A, panel D). The increased levels of p27 in adenomas from p27T187A mice however did not lead to a reduced proliferative activity as p27 wild-type and p27T187A mice showed similar levels of PCNA staining (Fig. 4C) and no significant differences in the number of apoptotic cells (data not shown). This observation is in agreement with previous studies which showed correlations between the histological differentiation and expression levels of p27, but failed to detect a correlation between p27 expression levels and proliferative indices in human tissue samples representing various stages of colon carcinogenesis (29, 30).

We concluded that the SCF^{skp2} pathway for p27 downregulation is essential for the normal degradation of p27 in intestinal epithelia and facilitates the progression from intestinal adenoma to carcinoma. Moreover, in contrast to what we observed in lung tumors, we found only a 20% reduction in the levels of p27 mRNA in intestinal tumors arising in the p27T187A strain compared to surrounding wild-type tissue (data not shown).

Discussion

By comparing the mechanisms of p27 downregulation in lung and intestinal cancers, we discovered that these pathways are more complex than previously anticipated. While phosphorylation induced degradation of the p27 protein is part of the progression of intestinal adenomas to carcinomas, inhibition of this pathway did not prevent p27 downregulation in lung tumors. We and others have previously shown that degradation of p27 by the Skp2-dependent SCF complex controls the size, cell number and ploidy of lung epithelial cells (31, 32). The results of this study however point to an entirely different mechanism of p27 regulation in lung epithelial cells under conditions of tumorigenesis, namely decreased p27 mRNA abundance. Our results underscore the fact that p27 levels can be regulated by several independent mechanisms, and suggests that lung cancer cells may switch between different modes of regulation during the course of tumor development.

Previously, we and others demonstrated that the RAF-MEK-ERK and PI3-kinase-PDK1-AKT signal transduction pathways downstream of Ras control p27 mRNA levels (17, 33). We tested whether these signalling pathways were activated in lung tumors from *K-ras** or *K-ras**p27T187A/ p27T187A mice using phospho-epitope specific antibodies. AKT activation and phosphorylation of the downstream FoxO1-3 transcription factors was not observed (data not shown). However, we detected activation of ERK1/2 in most of the lung tumors from both mouse strains (data not shown), and the tumors expressing the lowest levels of p27mRNA had strong phosphorylation of ERK1/2. Additionally, in human lung tumors we observed a correlation between loss of p27 protein and phosphorylation of ERK1/2 (data not shown). In vascular smooth muscle cells ERK decreases p27 transcript levels posttranscriptionally through the 3'UTR (17), and c-myc, one of the transcription factors functioning downstream of ERK, represses p27 transcription (34). Therefore, ERK may regulate p27 transcript abundance in lung tumors by decreasing gene expression and/or RNA stability.

In addition to analyzing p27 mRNA regulation in lung tumors, we examined human breast cancers with nuclear p27 staining where the protein levels are known to be predictive of outcome. Reduced survival for breast cancer patients with the lowest quartile of p27 transcript has been reported, but the p27 protein levels were not assessed. In our pilot study, all of the tumors in the lowest quartile for p27 mRNA levels had low p27 protein, suggesting that p27 mRNA was regulated in a subset of human breast cancers. In our analysis low p27 transcript level was a good predictor of low p27 protein abundance, but the reciprocal was not true. No activation of ERK1/2 was detected in these specimens; however, other transcription factors known to regulate p27 gene expression or different mechanisms affecting p27 mRNA abundance may function in breast tumors, and the mode of p27 regulation may depend on the tissue types. Despite increasing evidence that p27 mRNA levels can be regulated by diverse transcriptional and post-transcriptional mechanisms, our *in vivo* studies represent the first demonstration that p27 mRNA mis-regulation is important during tumorigenesis.

Our observation regarding the role of p27 turnover in intestinal tumor progression is also relevant to the choice of clinical settings in which inhibition of p27 degradation may have therapeutic benefit. Previous studies using purified extracts from primary colon cancer tissue suggested that increased degradation of p27 by the SCF^{skp2} dependent mechanism correlated with increasing aggressiveness of the respective tumor. Our previous analysis of the p27T187A mouse strain had not shown major differences in p27 levels in most tissues compared to wildtype mice(13). However, in this study we directly show that the expression of p27T187A decreases turnover of p27 in intestinal epithelia, thereby pointing to a specific role of the T187/SCF^{skp2} pathway in the regulation of intestinal cell proliferation. The high degree of tissue specificity is also reflected by the observation that while ENU treatment induced a wide variety of different epithelial cancers only intestinal tumorigenesis was significantly affected in the p27T187A mouse as compared to wild-type. Moreover our

observation that the progression of the adenomatous polyp to invasive intestinal cancers is impaired when p27 is stabilized points to a stage specific role of p27 turnover in the development of colon cancers. In agreement with previous studies in human colon cancer samples we did not find a significant difference in the proliferative activity of tumors which express high levels of p27 (T187A) and low levels of p27 (wild-type). Our results therefore suggest that p27 turnover increases the tendency of intestinal tumors to progress to a more malignant state but that this function is not directly connected to a change in cell proliferation rates. Studies in mice which express a cyclin/cdk non-binding form of p27 recently provided evidence for a role of p27 in regulating the stability of a tissue specific transcription factor which controls cell differentiation (35). Whether similar mechanism exist in intestinal epithelial cells and control tumor progression dependent on p27 expression levels is currently unknown.

Thus, interference with the Skp2-mediated p27 turnover might have a role in disorders associated with high risk of developing malignant intestinal carcinomas, like ulcerative colitis or the adenomatous polyposis syndrome. In light of the tissue specific differences we observed in p27 mis-regulation, it will be equally important to address whether pharmacological approaches aimed at preventing p27 downregulation should target proteolytic, transcriptional, or posttranscriptional mechanisms.

Materials and Methods

Mice and treatment

The animals studied for ENU mutagenesis were C57B6/J 129/Sv hybrid, littermates produced from heterozygous crosses. The animals for the K-ras study were all 129 littermates produced from heterozygous crosses. The p27T187A and p27^{Kip1} knockout mutations were recombined on to the *K-ras*^{LA2} chromosome. All mice were genotyped by PCR as described (36) and were weaned at 3-4 weeks. The p27 wild-type and p27T187A mice were intraperitoneally injected with ENU (0.5mmol per g mouse) at the age of 15+/-2 days (3). Mice were killed at the first sign of morbidity which included: excessive weight loss, rectal bleeding, abdominal swelling, hunched posture and rapid breathing, or anemia. Complete necropsies of all internal organs were performed for ENU injected mice, and focused necropsies of lungs from *K-ras**, p27^{-/-}/*K-ras**, and p27T187A/*K-ras** mice. For histological and immunohistochemical analysis of intestinal tumors longitudinal cross sections (5 µm thick) through intestinal rolls spanning the entire intestine were used. All haematoxylin-and-eosin stained sections were assessed by a mouse pathologist (K.K.). To distinguish adenoma from intestinal carcinoma the following morphological criteria were used: malignant cellular morphology, i.e. nuclear hyperchromatism and pleomorphism, increased number of mitotic figures especially atypical mitosis, irregularly shaped glands and invasion through the lamina muscularis mucosae by atypical glands. Statistical analysis was done on *K-ras** (n=65), p27^{-/-}/*K-ras** (n=13), p27T187A/*K-ras** (n=38) and p27T187A (n=19), p27 wild-type (n=14) after ENU treatment. The R program algorithm (<http://www.R-project.org>) was used to determine statistical significance between mortality rates for *K-ras**, p27^{-/-}/*K-ras**, p27T187A/*K-ras** mice. Differences in the occurrence of intestinal adenomas and carcinomas were tested using Fisher's Exact Test and Pearson Chi Square Test. Kaplan-Meier survival curves were used to display the time to tumor mortality. All experiments were done according to the rules and regulations of the animal health authorities of the state of Lower Saxony, Germany and the

Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

Immunostaining

Lung tissue from wild-type, p27 ^{-/-}, *K-ras**, p27T187A/*K-ras** mice were fixed in 10% formalin, processed as paraffin sections, and deparaffinized. p27 and Cks1 immunostaining were performed according to the methods provided in the ImmunoCruz Staining Systems Kit (Santa Cruz Biotechnology, CA). Briefly, rehydrated tissue sections were steamed for 30 min in 0.1% citrate acid and blocked in serum block for 20 min at room temperature. Samples were subsequently incubated with anti-p27 antibody (C19, 1:100 dilution, Santa Cruz Biotechnology, CA) or anti-Cks1 antibody (C-term, 1:100 dilution, Zymed Laboratories Inc, CA) for 1 hr and biotinylated secondary antibody for 30 min, and visualized by the chromogen 3,3'-diaminobenzidine (DAB). Intestinal tissue was stained using the Histostain-Plus-Kit (Zymed Laboratories), p27 monoclonal antibody (Transduction Laboratories, 1:50 dilution) and PCNA antibody (DAKO) following the manufacturers instructions. Samples were counterstained with hemalaun solution. The percentage of p27 and PCNA positive cells was determined by counting the number of positive cells in 20 low-power (10x) magnification fields chosen at random and expressing the number of positive cells as a percentage of all cells counted. At least 3 sections and 3 visual fields of each collected sample were quantified.

RNA isolation and RT-PCR

cDNA synthesis and semi-quantitative RT-PCR in intestinal cancer tissue were done as described previously (Kossatz et al, 2004). For semi-quantitative RT-PCR 1 μ l cDNA was amplified using the following cycle profile: denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 45 s using Platinum[®] Taq Polymerase (Invitrogen), for 30 cycles. For quantitative real-time PCR, RNA was extracted from pieces of frozen lung tumors (*K-ras** and p27T187A/*K-ras**) and normal lungs (*K-ras** and p27T187A) or sections of

frozen human breast tumors following the Trizol protocol (Invitrogen). cDNAs were generated by reverse transcribing 1 µg of total RNA using oligo dT and the Taqman reverse transcription kit (Applied Biosystems). The cDNAs were diluted 1:10 and 5 µl added to each reaction containing Taqman master mix at 1X concentration and the p27 Mm00438167_g1 and Hs or Hprt1 Mm00446968_m1 and Hs99999909_m1 Assay on Demand primers and probe (Applied Biosystems). The results were verified using a second p27 primer and probe set and a GAPDH control primers and probe (Applied Biosystems). The sequences for the p27 primers are 5'AGGAGAGCC AGGATGTCAGC3' and 5'CAGAGTTTGCCTGAGACCCAA3' and probe sequence 5'AGCCGCCAGGCGGTGCCT3'. Each 50 µl reaction was done in triplicate. Taqman real-time PCR reactions were performed using an ABI PRISM 7900HT sequence detector and analyzed by the SDS2.2 software. Sequences for the Assay on Demand probes were as follows:

Mouse p27: AGGAAGCGACCTGCTGCAGAAGATT

Mouse HPRT: AGGTTGCAAGCTTGCTGGTGAAAAG

Human p27: ACCTGCAACCGACGATTCTTCTACT

Human HPRT1: AGATGGTCAAGGTCGCAAGCTTGCT

Lasermicrodissection of stained histological tissue sections and subsequent RNA isolation was performed essentially as described (37).

Acknowledgement:

We thank Tyler Jacks for providing *K-ras^{LA2}* transgenic mice and Holly Sundberg Malek, Lenhard Rudolph, and Matthew Fero for valuable discussion and comments on the manuscript. Work in N.P.M. lab is funded by the Deutsche Krebshilfe (Max-Eder-Program) and by the Deutsche Forschungsgemeinschaft. C.M.G.-E. is funded by a NCI postdoctoral fellowship.

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Figure legends:

Figure 1. Expression of p27T187A does not prevent p27 downregulation in lung cancer

A: Survival curve of homozygous p27KO, p27T187A and wild-type mice bred to *K-ras** expressing mice. Mice were sacrificed when signs of morbidity were obvious. p27^{-/-}/*K-ras** mice die significantly faster than *K-ras** and p27T187A/*K-ras** mice which die with comparable kinetics. **B:** Immunohistochemical staining of lung cancer and surrounding normal tissue of the indicated strains with an antibody specific for p27^{kip1}. No expression of p27 or p27T187A is detectable in the tumor tissue while normal surrounding tissues stain positive with a p27 specific antibody.

Figure 2. Lung cancer tissues express reduced levels of p27 mRNA

A: Staining of representative tissue sections of wild-type p27 lung tissue and tumor tissue from *K-ras** and p27T187A/*K-ras** mice with an antibody specific for the cks1 protein shows reduced expression of the cks1 protein in tumor tissue. **B:** p27 gene expression in *K-ras** normal lung, *K-ras** tumors, and p27T187A/*K-ras** tumors was determined by real-time PCR using probe and primers at the border of exons 1 and 2. The amount of RNA in each sample was normalized to HPRT1 gene expression. The fold change observed is relative to p27 transcript levels in the *K-ras** normal lung sample.

C: p27 protein staining in representative tumor sections demonstrates high nuclear staining (A) and low nuclear staining (B). In panel B the tumor tissue has low p27 staining, but infiltrating lymphocytes are positive for p27 protein. **D:** p27 mRNA levels in the high and low p27 protein tumors was assayed by quantitative real-time PCR. The quantity of RNA in each sample was normalized to HPRT1 gene expression.

Figure 3. Expression of p27T187A interferes with intestinal tumor progression

A: Survival curve of wild-type and p27T187A mice injected with ENU at 12 d of age. No significant differences in survival were observed between wild-type and p27T187A mice. Mice were killed when clear signs of morbidity were present. **B:** Incidence of different tumor types which were identified at necropsy and after histological examination of all organs. A significant difference was observed in the numbers of intestinal adenomas and carcinomas which arose in the p27T187A strain ($p < 0.05$) compared to p27 wild-type controls.

Figure 4. The p27T187A protein is stabilized in intestinal tumors

A: Characteristic sections through normal tissue (E, F), adenomas (C, D) and carcinoma tissue (A, B) from wildtype and p27T187A mice after staining with a p27 specific antibody showing strong p27 expression in p27T187A derived intestinal epithelium and wildtype non-cancerous tissue but not in wildtype tumor tissue. **B:** Percentage of cells which stained positive with a p27 antibody in normal intestinal epithelium, adenomas and carcinomas from wildtype and p27T187A mice. **C:** Percentage of PCNA positive cells in normal intestinal epithelium, adenomas and carcinomas from wildtype and p27T187A mice shows a significant (**, $p < 0.05$) difference in proliferative activity.

Fig.1 Timmerbeul et. al

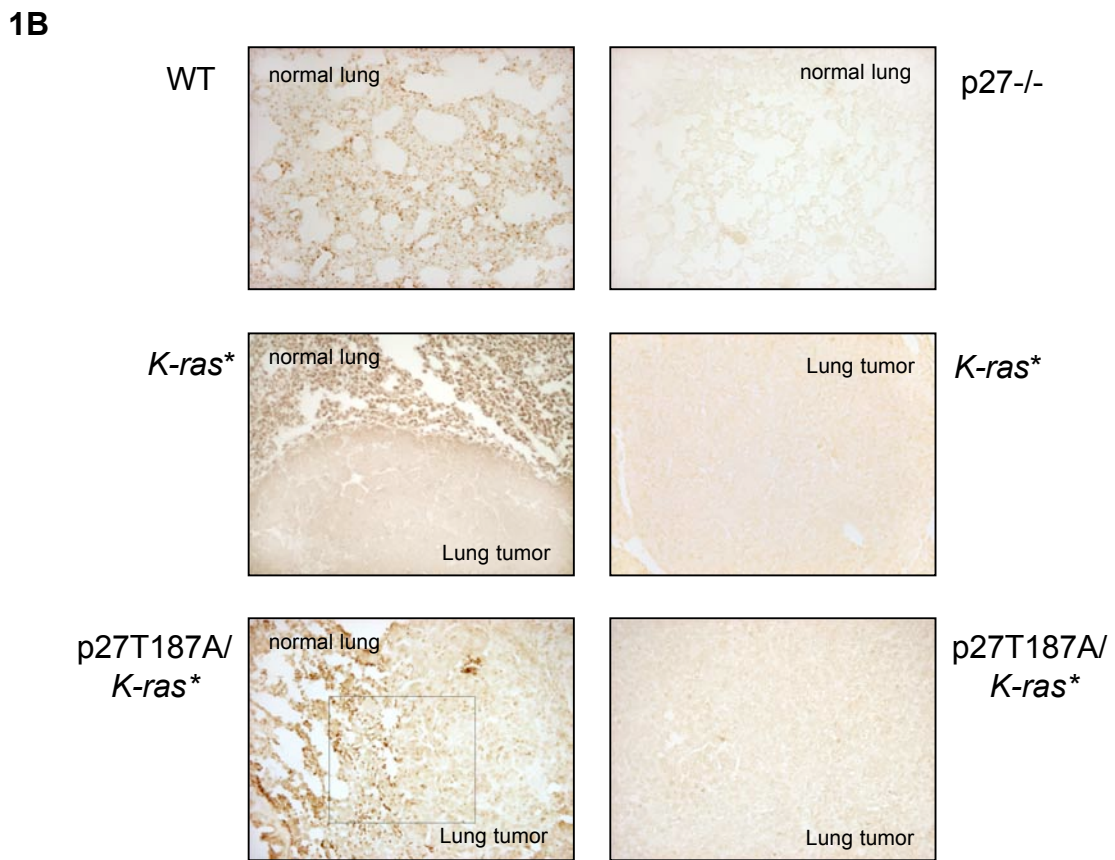
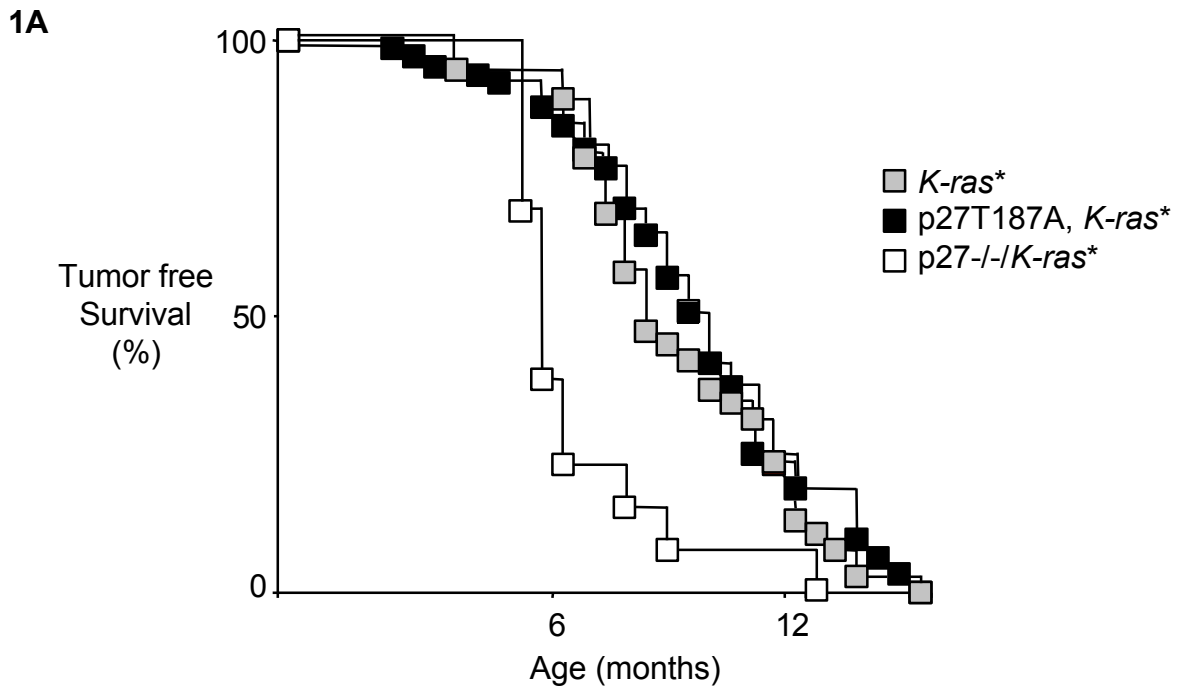


Fig.2 Timmerbeul et. al

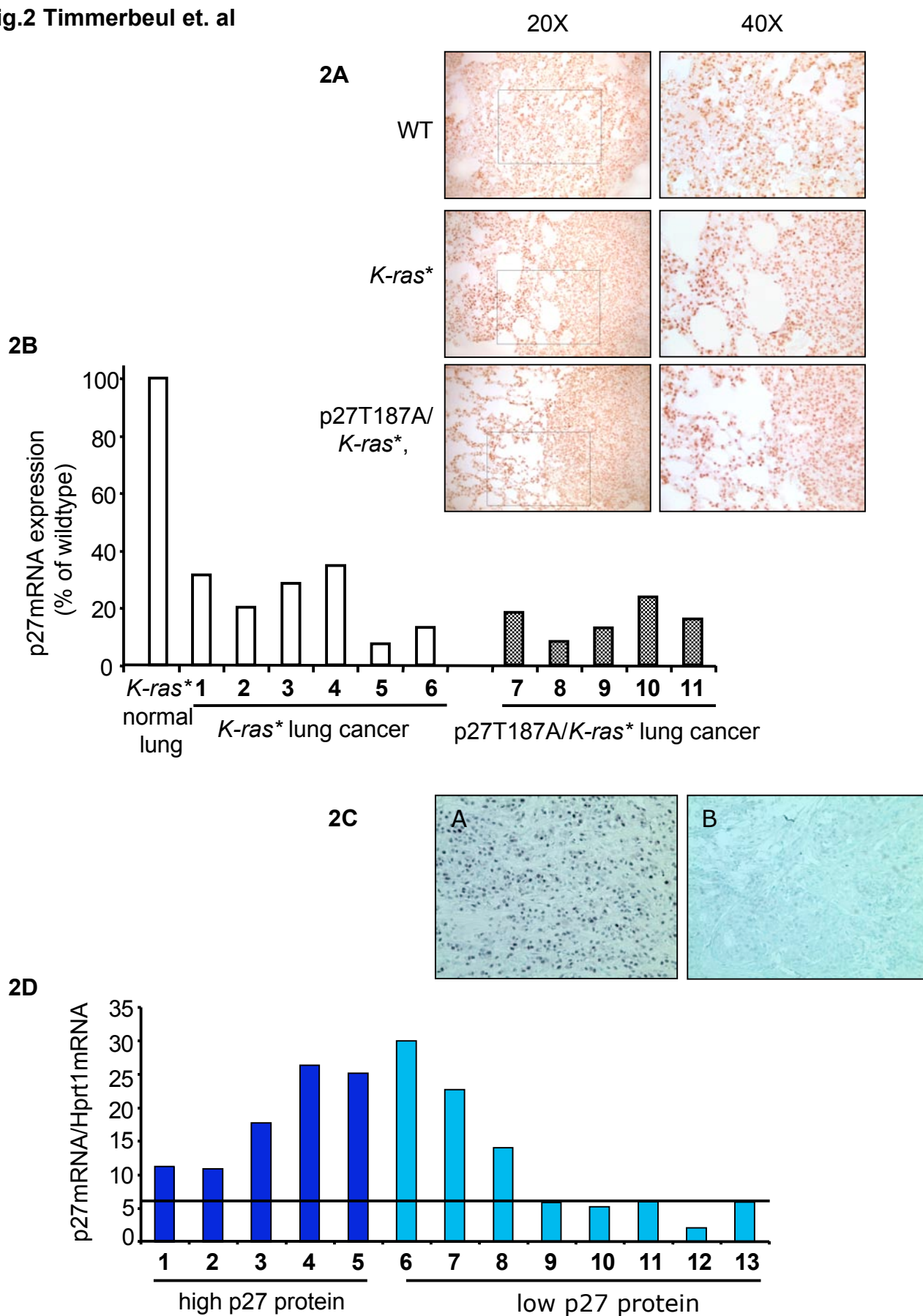
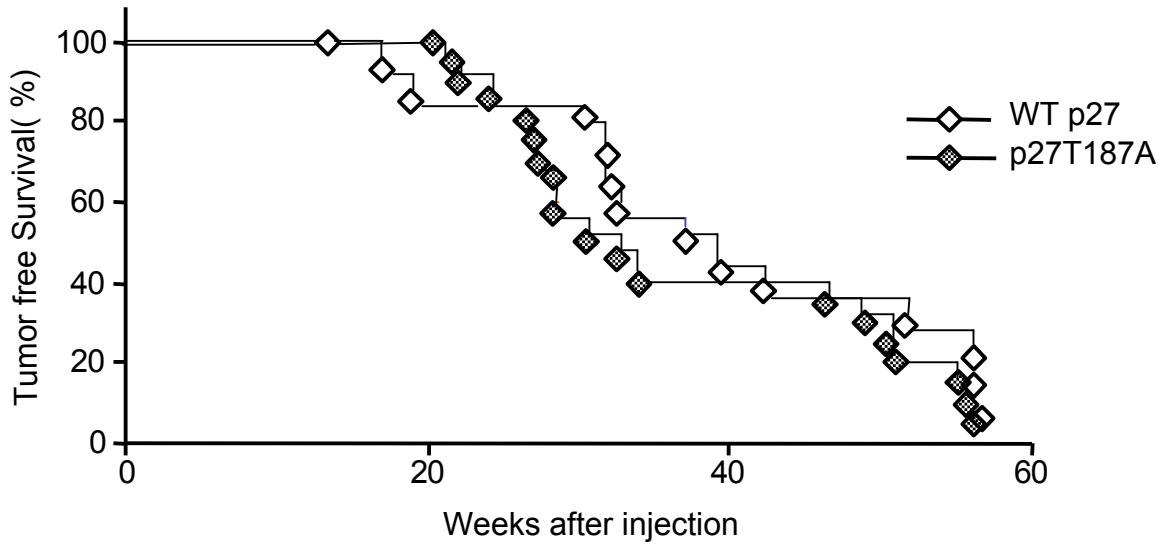


Fig.3 Timmerbeul et. al

3A



3B

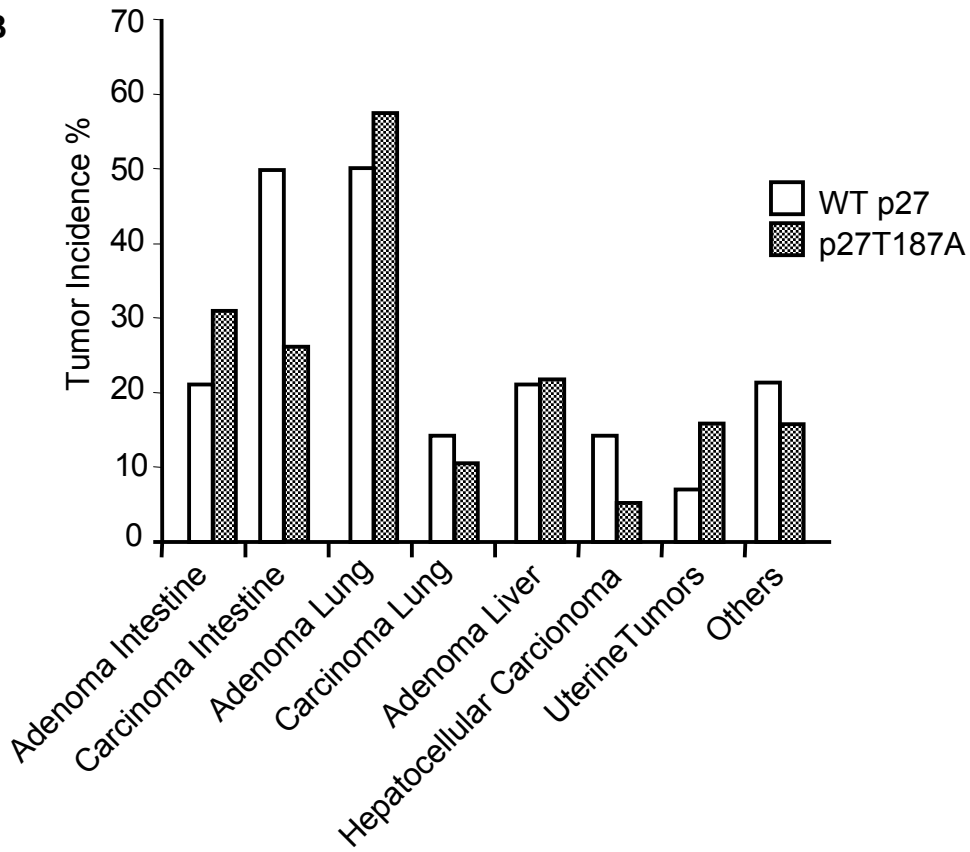


Fig.4 Timmerbeul et. al

