

Phosphorylation of Ribosomal Protein S6 Attenuates DNA Damage and Tumor Suppression during Development of Pancreatic Cancer

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Abstract

The signaling pathways that mediate the development of pancreatic ductal adenocarcinoma (PDAC) downstream of mutant *Kras* remain incompletely understood. Here, we focus on ribosomal protein S6 (rpS6), an mTOR effector not implicated previously in cancer. Phosphorylation of rpS6 was increased in pancreatic acinar cells upon implantation of the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) or transgenic expression of mutant *Kras*. To examine the functional significance of rpS6 phosphorylation, we used knockin mice lacking all five phosphorylatable sites in rpS6 (termed rpS6^{P-/-} mice). Strikingly, the development of pancreatic cancer precursor lesions induced by either DMBA or mutant *Kras* was greatly reduced in rpS6^{P-/-} mice. The rpS6 mutants expressing oncogenic *Kras* showed increased p53 along with increased staining of γ -H2AX and 53bp1 (Trp53bp1) in areas of acinar ductal metaplasia, suggesting that rpS6 phosphorylation attenuates *Kras*-induced DNA damage and p53-mediated tumor suppression. These results reveal that rpS6 phosphorylation is important for the initiation of pancreatic cancer. *Cancer Res*; 73(6); 1–10. ©2012 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal human cancers. Despite significant recent advances in our understanding of the biology of this disease (1), the outcome remains dismal with a 5-year survival rate of 5%. It is now accepted that PDAC often develops from precursor lesions termed pancreatic intraepithelial neoplasia (PanIN; ref. 1). The cellular origins of PanIN lesions and PDAC remain controversial, with different studies in murine models implicating duct cells, centroacinar cells, acinar cells undergoing acinar-to-ductal metaplasia (ADM), and even islet cells as the cell of origin (2–6).

Oncogenic mutations in *KRAS* are found in more than 95% of PDACs and appear to drive the formation of PanIN

lesions. Loss of the *INK4A* locus is another major frequent event in the development of PDAC, with loss of *TP53*, *SMAD4*, and *BRCA2* tumor suppressor genes seen at lower frequencies (1). On the basis of these findings, reliable genetic mouse models for human PDAC have been developed. In particular, Cre-mediated activation of an oncogenic *Kras* mutant in the pancreatic epithelium induces the formation of PanIN lesions but only rarely leads to the development of carcinoma (7, 8). Additional hits such as genetic deficiency for *Ink4A* (7) or p53 (9) cause the formation of metastatic PDAC, recapitulating human pathology and lending strong support to the PanIN progression model. These mouse models have, in turn, led to significant new insights into the events that contribute to the development of PanIN lesions and PDAC. For example, inflammation was shown to trigger the progression of mouse PanIN lesions, induced by expression of mutant *Kras*, into PDAC (8). The underlying mechanism appears to be a bypass of cellular senescence (10), which is documented in acinar metaplasia and early PanIN lesions and acts as a tumor suppression mechanism (11). Mouse models have also led to a better understanding of the signaling pathways involved in PDAC. Sonic hedgehog signaling was shown to contribute to PDAC in both early and late stages (12, 13). Wnt/ β -catenin signaling is also an important mediator of the oncogenic effects of *Kras*, acting to modulate the preneoplastic process of ADM (4). Despite these and other advances, the nature of the signaling cascades operating downstream of *Kras* in the development of pancreatic cancer remains incompletely understood.

One important signaling pathway implicated in the development of PDAC is the PTEN/PI3K/AKT/mTOR pathway. AKT is amplified or hyperactivated in a large fraction of human

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PDAC (14), and very recently *PTEN*, a tumor suppressor negating phosphoinositide 3-kinase (PI3K) activity, has been identified as a major tumor suppressor in human PDAC (15). In mice, we have previously shown that *Pten* restricts the development of PanIN lesions and pancreatic cancer (16). More recently, *PTEN* loss was shown to accelerate the development of ADM, PanIN lesions, and PDAC induced by mutant *Kras* (17).

A central downstream target of Akt is mechanistic mTOR, which regulates cell growth and division in response to energy and nutrient sufficiency. mTOR exists in 2 distinct protein complexes, mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2), which target distinct sets of substrates (18). The 2 best characterized mTORC1 substrates are ribosomal protein S6 kinase (S6K) and the eukaryotic translation initiation factor 4E-binding protein (4E-BP). Phosphorylation of both S6K and 4E-BP affects translation efficiency and thereby mediates the regulatory role of mTORC1 in growth and proliferation, respectively (19). Several studies have shown that pharmacologic inhibition of mTORC1 can attenuate the growth of pancreatic cancer cell lines *in vitro* and *in vivo* (20–22), but the identity of downstream effectors remains largely unknown. S6K has been implicated in tumorigenicity (23) but not in the context of PDAC. S6K has multiple substrates, including rpS6, whose phosphorylation is tightly coupled to mTORC1 activity (24). Nonetheless, the identity of the key substrate(s) that mediate the tumorigenic function of S6K is not known.

rpS6 is 1 of only 2 ribosomal proteins known to date to be phosphorylated in a regulated manner. Its phosphorylation occurs in response to a wide variety of stimuli on 5 evolutionarily conserved serine residues, yet the physiologic function of this modification has remained enigmatic (25). Using knockin mice whose rpS6 contains alanine substitutions at all 5 phosphorylatable serine residues (rpS6^{P-/-}), we have previously shown that rpS6 phosphorylation is not essential for embryonic development or postnatal survival (26). This result contrasts with the embryonic lethality of mice lacking just one allele of the rpS6 gene (27). At the molecular level, total protein synthesis rate is not reduced in rpS6^{P-/-} mice, suggesting that phosphorylation of rpS6 impacts functions of rpS6 other than direct, essential ribosome-mediated translation. In fact, the most significant phenotype observed in these mice was a decrease in the size of pancreatic β -cells, which was associated with defective insulin secretion and glucose homeostasis (26). In addition, rpS6^{P-/-} mice have defects in the growth and energy content of their muscle (28). Notably, no role of rpS6 phosphorylation in cancer was reported before, other than descriptive studies showing increased phosphorylation in several human cancers (29, 30). Furthermore, it has been shown that Akt-induced lymphoma does not rely on rpS6 phosphorylation (31).

Notably, the key signaling components downstream of PI3K/AKT in the context of PDAC remain unexplored, as is the exact nature of the link between this pathway and *Kras* signaling. Here, we identify rpS6 as an important mediator of PanIN lesion formation induced by mutant *Kras*. Our results are consistent with a model whereby the phosphorylation of rpS6 reduces *Kras*-induced DNA damage in acinar cells and in ADM and consequently reduces p53-mediated tumor suppression.

Materials and Methods

Mice and genotyping

The following mouse strains were used: rpS6^{P-/-} (26), on a mixed ICR and C57/Bl6 backgrounds; Pdx1-Cre (32); and LSL-Kras G12D-IRES-lacZ (8). Genotyping was conducted using PCR on DNA extracted from tail biopsies using the following primers:

For rpS6 genotyping: 5'-GTCATCCAGCATGGGTGCTG-3' and 5'-GGCTGATACCTTTTGGGACAG-3'. PCR product is digested with EcoRV.

For *Kras* mice, genotyping was conducted using primers for lacZ: 5'-CAACAGTTGCGCAGCCTGAATGG-3' and 5'-AAATCGCTGATTGTGTAGTCGGT-3'.

For Pdx1-Cre genotyping: 5'-TCCCCAGGAGAGCAGTGGA-GAAC-3' and 5'-CATCAGGTTCTTGGGAACCTCAT-3'. The joint ethics committee (Institutional Animal Care and Use Committee) of the Hebrew University (Jerusalem, Israel) and Hadassah Medical Center (Jerusalem, Israel) approved the study protocol for animal welfare. The Hebrew University is an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited institute.

Implantation of 7,12-dimethylbenz(*a*)anthracene and treatment with rapamycin

Three month old mice were anesthetized with ketamine (225 mg/kg)/xylazine (5 mg/kg) intraperitoneally for induction and with isoflurane inhalation for maintenance. We conducted a median laparotomy, exposed the pancreas, and applied a purse-string suture in the tail of the pancreas between the stomach and the spleen, avoiding obstruction of the main pancreatic duct. A cotton pledget was soaked in 10 μ L of 7,12-dimethylbenz(*a*)anthracene (DMBA) dissolved in benzene (100 mg/mL) and deposited in the pocket. The purse-string suture was subsequently tied. Analgesia was provided with subcutaneous injections of buprenorphine (0.5 mg/kg), twice daily for 1 week after surgery. Rapamycin (1.5 mg/100 mL caboxymethyl cellulose) was injected at 0.3 mg/kg on the first day after DMBA implantation and then 0.15 mg/kg every other day for 1 month. This low dose of rapamycin used was selected to minimize potential nonspecific effect and was based on several articles that used a similar protocol (33–35).

Histological evaluation

To quantify the severity of PanIN lesions, we used a scoring system described previously (5). Briefly, 3 hematoxylin and eosin-stained slides from different levels were examined per pancreas. The score for each slide was calculated on the basis of the 5 highest grade PanIN lesions observed, according to: 1x (#mPanIN-1A/1B) + 2x (#mPanIN-2) + 3x (#mPanIN-3), resulting in a score from 0 to 15. Any score above 5 reflects the presence of at least one high-grade mPanIN2 or mPanIN3 lesion. The score per mouse was the average score for the 3 slides analyzed. Metaplasia was scored as the total number of metaplastic foci in a section, averaging for 3 sections examined per mouse. As no differences were seen between mice bearing wild-type or heterozygous rpS6 alleles, these genotypes were combined and referred to as wild-type in subsequent analysis. Pathologists (A. Khatib and A. Maitra) were in all cases blind to the genotype of mice scored.

Immunostaining

Tissue processing and staining were conducted as described previously. Briefly, tissue was fixed in zinc formalin for 4 hours and processed to embed in paraffin. Five-micrometer thick sections were incubated overnight with primary antibodies, in some cases after antigen retrieval in a pressure cooker, followed by secondary antibodies. Primary antibodies used were: rabbit anti-phospho-rpS6 (Cell Signaling, 1:50), mouse anti- γ H2AX (Millipore, 1:4,000), rabbit anti-p53 (Leica CM5, 1:4,000), and rabbit anti-53bp1 (Bethyl, 1:400). Secondary antibodies were from Jackson ImmunoResearch and Zytomed.

Results

Rapamycin attenuates the development of pancreatic cancer induced by chemical carcinogenesis

To study the downstream effectors of PTEN in the development of pancreatic cancer *in vivo*, we first tested the effect of rapamycin, a clinically used selective inhibitor of mTORC1. In this set of experiments, we used a chemical carcinogenesis model, based on implantation of DMBA in the pancreas of mice. Conveniently, DMBA can be implanted in wild-type mice avoiding the need for extensive breeding schemes of transgenic animals, and it initiates a temporally defined sequence of carcinogenic events starting in the adult pancreas. As shown previously (36), implantation of beads soaked with DMBA into the body of the pancreas led to the development of PanIN lesions within 3 to 4 weeks (Fig. 1A–C), which resembled histologically the lesions induced by transgenic expression of

mutant Kras (see below). At 5 to 6 months postimplantation, some of these lesions progressed to PDAC (Fig. 1; Supplementary Fig. S1). A second group of DMBA-implanted mice was treated with rapamycin starting at the day of carcinogen implantation and sacrificed at 1 month. To quantify the severity of PanIN lesions and cancer, we used a previously described scoring system, which takes into account both the number of precursor lesions and their grade in multiple sections from each pancreas (5). As shown in Fig. 1D, rapamycin-treated mice had a significantly lower score of PanIN lesions. These results suggest that mTORC1 is important for the development of PanIN lesions.

Phosphorylation of rpS6 in pancreatic cancer and its precursor lesions

To establish the effector of mTORC1 that mediates its tumorigenic activity, we set out to study the involvement of rpS6 phosphorylation in the development of PDAC. To this end, we documented the pattern of rpS6 phosphorylation in the pancreas using antibodies directed against phospho (p)-rpS6. In the wild-type pancreas, rpS6 is phosphorylated in a subset of islet cells (Supplementary Fig. S2) and in acinar cells, but not in ducts (Fig. 2A). In the pancreas of DMBA-treated mice, an overall stronger p-rpS6 signal was observed in acinar cells adjacent to the area where DMBA was implanted (Fig. 2B). Notably, PanIN lesions did not stain for p-rpS6 (Fig. 2B, arrows). These results suggest that rpS6 phosphorylation may play a role in the development of PanIN lesions.

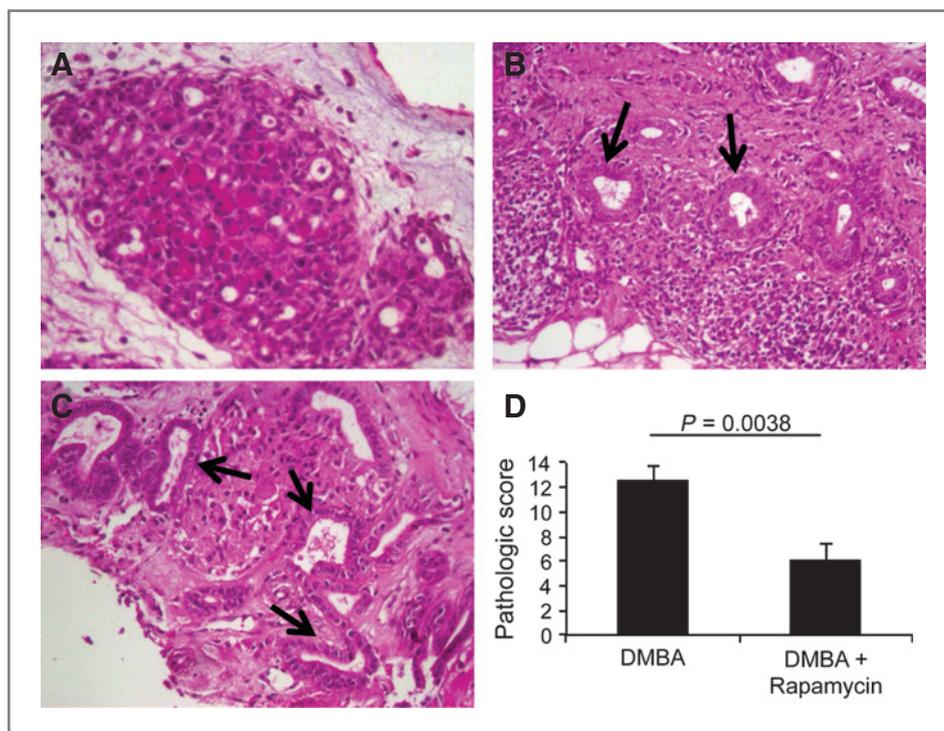


Figure 1. Rapamycin inhibits the development of DMBA-induced pancreatic cancer in wild-type mice. A, ADM foci in a wild-type mouse, 1 month after DMBA treatment. B, PanIN1/2 lesions in a wild type mouse (arrows), 1 month after DMBA treatment. C, PanIN3 lesions in a wild-type mouse (arrows), 3 months after DMBA treatment. D, a score of PanIN lesions in mice implanted with DMBA, with and without rapamycin administration starting at the time of DMBA. Mice were sacrificed 1 month after DMBA implantation. $n = 9$ mice in each group. All pictures were taken at a $\times 200$ magnification.

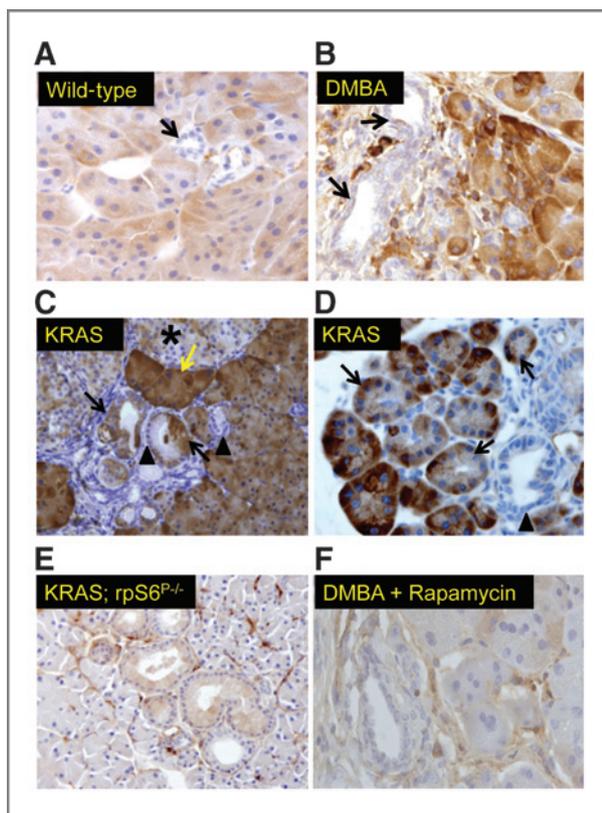


Figure 2. Pattern of rpS6 phosphorylation in the exocrine pancreas. A, wild-type pancreas. Staining of rpS6 phosphorylation (brown) is seen in a mosaic pattern in acinar tissue. Ducts do not normally stain for p-rpS6 (arrow). Original magnification, $\times 400$. B, DMBA-treated wild-type mice. Extensive p-rpS6 staining is seen in acinar tissue in areas implanted with DMBA but not in PanINs (arrows). Original magnification, $\times 400$. C and D, examples of rpS6 phosphorylation in Pdx1-Cre;LSL-Kras G12D mice. Note extensive staining in acinar cells (yellow arrow) and ADM (black arrows) but not in PanIN lesions (arrowheads). *, islet. Original magnification, $\times 200$ in C, $\times 400$ in D. E, complete lack of signal in Pdx1-Cre;LSL-Kras G12D;rpS6^{P-/-} mice, confirming specificity of antibody. Original magnification, $\times 200$. F, DMBA-treated wild-type mice, co-treated with rapamycin. No p-rpS6 staining is detected. Original magnification, $\times 400$.

To more directly examine the link between rpS6 phosphorylation and oncogenic Kras, we used Pdx1-Cre;LSL-Kras^{G12D} transgenic mice (Kras mice). These mice express a mutant Kras, driven from its endogenous promoter, throughout the pancreatic epithelium starting in embryonic development. As described before, these mice develop PanIN lesions that infrequently progress to PDAC (refs. 7, 8, 37; Supplementary Fig. S1). The pattern of p-rpS6 in Kras mice was striking: acinar cells were uniformly and strongly stained, indicating that mutant Kras directly signals to activate S6K and its phosphorylation targets in this compartment (Fig. 2C and D). Metaplastic foci were also strongly stained. In contrast, islets and ducts showed a normal staining, suggesting that excessive Kras activity is not sufficient to trigger rpS6 phosphorylation in the respective cells. Importantly, PanIN lesions were unstained for p-rpS6 (Fig. 2C and D). In some cases, mosaic p-rpS6 was observed within the same tubular structure, with strong stain-

ing in residual acinar or ADM cells and no staining in fully formed mucinous PanIN cells (Fig. 2C). Such a pattern suggests a cell autonomous nature of this signaling event. The complete lack of signal in the pancreas of rpS6^{P-/-} mice attests to the specificity of immunostaining (Fig. 2E). As expected, treatment of mice with rapamycin abolished the p-rpS6 signal (Fig. 2F).

Reduced PanIN lesion formation in rpS6^{P-/-} mice

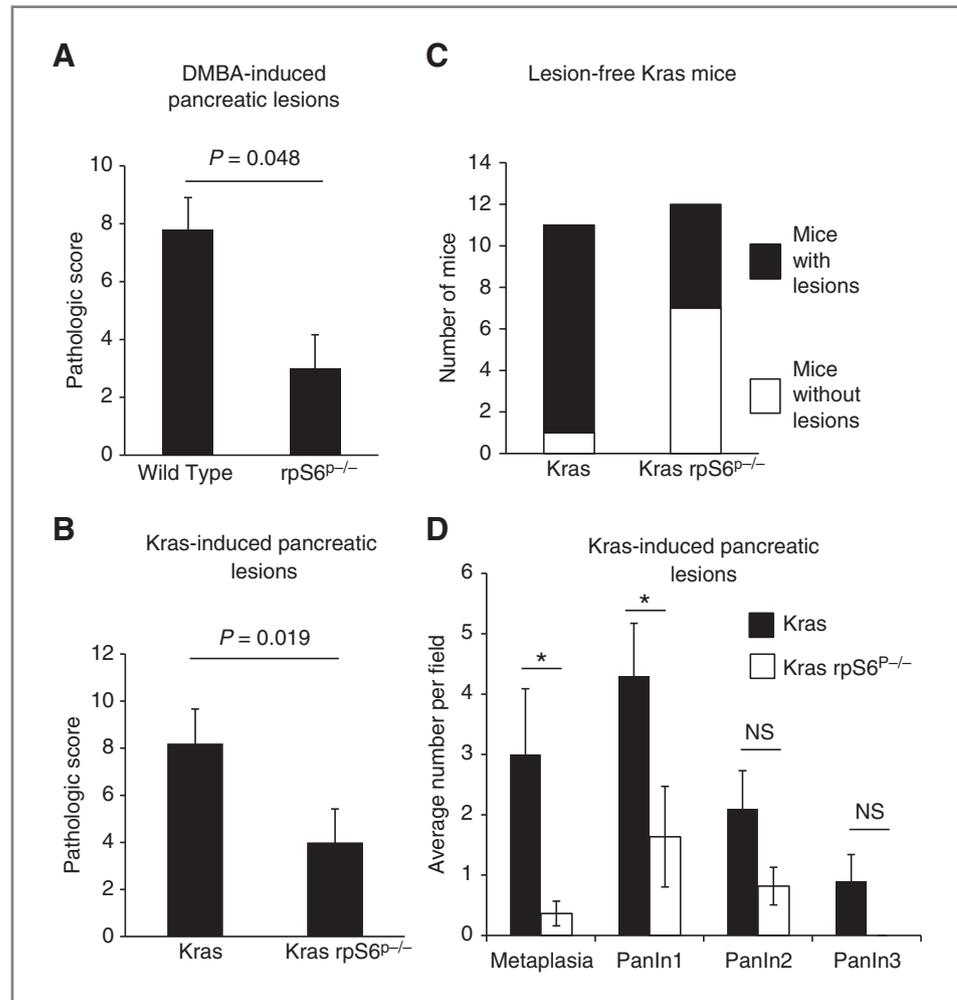
To examine the functional significance of phosphorylated rpS6 in the development of PanIN lesions, we implanted DMBA into the pancreas of rpS6^{P-/-} and control littermates, heterozygous for the phospho-mutant or wild-type. Three to 5 months later, pairs of wild-type and mutant mice were sacrificed and their PanINs were scored pathologically. rpS6^{P-/-} mice showed a highly significant attenuation of PanIN development, providing a first demonstration for the importance of rpS6 phosphorylation in cancer development (Fig. 3A). To test this notion in a genetic model for PanIN formation, we generated compound mice that expressed mutant Kras in the pancreas (Pdx1-Cre;LSL-Kras^{G12D}) in the background of wild-type, heterozygous, or homozygous phospho-rpS6 mutants. At the age of 3 months, mice were sacrificed and their PanIN lesions were pathologically scored. As shown in Fig. 3B, Kras mice lacking rpS6 phosphorylation had a significantly lower score of PanIN lesions compared with Kras mice with one or two normal rpS6 alleles. No differences were noticed between wild type and heterozygous rpS6 mutants (Supplementary Fig. S3). The reduced development of PanIN lesions was also seen when we scored the fraction of mice that were free of any PanIN lesion. Of 11 Kras mice examined, only one was free of any PanIN lesion. In contrast, of 12 Kras; rpS6^{P-/-} mice examined, 7 were free of PanIN lesions (Fig. 3C). Similar results were obtained when the overall pathologic score was broken to specific lesions. Specifically, KRAS mice lacking rpS6 phosphorylation had a trend toward lower scores in all lesion categories compared with KRAS mice containing wild-type rpS6; however, only the scores of metaplasia and PanIN1, and not more advanced lesions, have reached statistical significance (Fig. 3D). Collectively, these results show that rpS6 phosphorylation contributes to the development of PanIN lesions induced by either chemical carcinogenesis or mutant Kras expression.

As aforementioned, the origins of PanIN lesions are not known. ADM is considered a potential preneoplastic lesion, but its contribution to the development of PanIN lesions and pancreatic cancer is not clear (38). Interestingly, we observed decreased frequency of cells showing an ADM morphology in Kras mice deficient for rpS6 phosphorylation (Fig. 4A and B). These results are consistent with a model whereby the lack of rpS6 phosphorylation eliminates ADM and prevents the progression of these lesions to PanIN. However, in the absence of lineage tracing, it is impossible to claim a causal relationship between the frequency of ADM and the score of PanIN lesions.

Induction of p53 in acinar metaplasia

The inhibitory effect of rpS6 mutation on PanIN formation suggests that in the absence of rpS6 phosphorylation, a tumor suppression mechanism is activated and restrains the

Figure 3. Reduced PanIN lesion formation in rpS6 mutants. **A**, score of PanIN lesions in wild-type and in rpS6^{P-/-} mutant mice treated with DMBA. One-month-old mice were implanted with a DMBA pellet and sacrificed 2 months later. *n* = 12 wild-type mice, 11 rpS6^{P-/-} mice. **B**, score of PanIN lesions in Pdx1-Cre; LSL-Kras G12D mice expressing wild-type (*n* = 11) or mutant (*n* = 12) rpS6. Mice were sacrificed at 3 months of age. The wild-type group includes rpS6 heterozygous mice, which did not differ from true wild-type mice (Supplementary Fig. S3). **C**, fraction of lesion-free Pdx1-Cre; LSL-Kras G12D mice expressing wild-type (*n* = 11) or mutant (*n* = 12) rpS6. Mice were sacrificed at 3 months of age. **D**, average number of each type of PanIN lesion in Pdx1-Cre;LSL-Kras G12D mice expressing wild-type (*n* = 10) or mutant rpS6 (*n* = 11). Mice were sacrificed at 3 months of age. Graphs show the average number of each type of lesion per high-power field. Five fields were counted per mouse. Note that only the scores of early lesions show statistically significant differences between wild-type and mutant rpS6: metaplasia, *P* = 0.034; PanIN1, *P* = 0.048; PanIN2, *P* = 0.16; PanIN3, *P* = 0.064.



oncogenic effect of Kras activity. To address the underlying mechanism, we examined the rates of cell proliferation and apoptosis in the pancreas of Kras mice expressing wild-type or mutant rpS6. We failed to detect significant cell death in any of the genotypes [terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, data not shown], and no significant differences were observed in proliferation rates (Supplementary Fig. S4). We reasoned that the effect of rpS6 phosphorylation deficiency might be restricted to a small population of cells that plays a major role in the formation of PanIN lesions, perhaps related to the decreased abundance of ADM observed in the absence of rpS6 phosphorylation. Given the known tumor suppressor effect of p53 on Kras-driven PanIN lesion formation and PDAC development (9), we monitored the pattern of p53 expression in situ using immunostaining. Wild-type and rpS6^{P-/-} pancreata had no detectable staining for p53 (Fig. 5A). Kras mice showed strong nuclear p53 staining in rare cells, mostly in areas of ADM (Fig. 5A), consistent with a recent report on p53 expression and cellular senescence in precursor lesions of pancreatic cancer (11). Notably, PanIN lesions had minimal p53 staining, despite continued expression and activity of Kras, suggesting lack of p53-inducing stress in these structures. Kras mice deficient for

rpS6 phosphorylation had a similar pattern of p53 expression, mostly in metaplastic areas (Fig. 5A). However, the frequency of p53-positive cells in these mice was significantly higher than in Kras mice, when scoring the metaplastic areas (Fig. 5B). These results support the view that phospho-rpS6 acts to prevent Kras-induced expression of p53 in acinar cells and ADM and suggest a mechanism for the attenuation of PanIN lesion formation in Kras mice that lack phospho-rpS6.

Evidence for an increased DNA damage response in rpS6 mutants

We next asked what induces p53 expression in Kras mice that lack rpS6 phosphorylation. One major trigger of p53 activity in the context of active oncogenes is DNA damage. This pathway is a particularly attractive candidate in our case, as Kras was shown to cause oxidative stress leading to DNA damage, which serves as a powerful inducer of p53 (39). To test this idea, we stained pancreatic sections for phosphorylated H2AX (γ H2AX), a histone variant recruited to chromatin domains that flank double-strand breaks in DNA. Intestinal sections of CK1 α -knockout mice had multiple γ H2AX+ nuclei as previously shown (40) and served as a positive control (Supplementary Fig. S5). Normal pancreatic tissue showed no

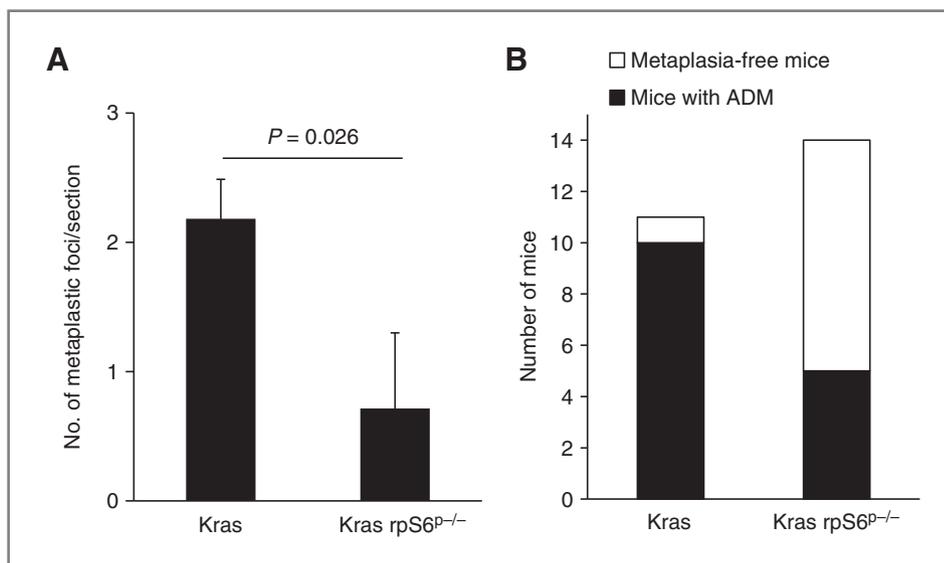


Figure 4. Reduced number of metaplastic foci in rpS6 mutants. A, number of foci showing an ADM per section in 3-month-old Pdx1-Cre;LSL-Kras G12D mice expressing wild-type ($n = 11$ mice) or mutant ($n = 12$ mice) rpS6. B, fraction of ADM-free Pdx1-Cre;LSL-Kras G12D mice expressing wild type ($n = 11$) or mutant ($n = 12$) rpS6. Mice were sacrificed at 3 months of age.

detectable staining for γ H2AX, whereas γ H2AX-positive nuclei were readily detected in Kras mice. Staining was mostly concentrated in metaplastic areas and adjacent acinar cells, reminiscent of the pattern of p53 expression. The pattern of γ H2AX staining in Kras mutants deficient for p-rpS6 was similar to that in Kras mice expressing wild-type rpS6 (Fig. 6A). However, quantification of stained nuclei revealed a 3-fold increase in the frequency of γ H2AX+ nuclei in rpS6 phosphorylation deficient mice, specifically in areas of ADM and adjacent acinar tissue (Fig. 6B). These results suggest that p53 expression in metaplastic areas of Kras mice results from DNA damage (specifically double-strand breaks), a classic trigger of p53 expression. The increased frequency of γ H2AX-positive nuclei in Kras mice lacking rpS6 phosphorylation is consistent with the increased expression of p53 in these mutants and suggests that the absence of p-rpS6 augments Kras-induced DNA damage. Finally, we have used another marker of the DNA damage response, 53bp1, known to co-localize with γ H2AX at chromatin areas flanking double-strand breaks and to be required for p53 accumulation after DNA damage (41, 42). The pattern obtained was similar to that seen with γ H2AX, namely, 53bp1 foci were more abundant in metaplastic regions of KRAS; rpS6 than in KRAS mice (Fig. 7A and B). Together, these results provide a plausible molecular mechanism for reduced PanIN lesion formation in rpS6 mutants. Phosphorylated rpS6 appears to attenuate DNA damage triggered by expression of mutant Kras and hence to reduce p53-mediated tumor suppression.

Discussion

We provide here genetic evidence for the participation of phosphorylated rpS6 in the development of pancreatic cancer. Phosphorylation of rpS6 via mTORC1 is augmented in acinar cells and metaplastic regions in the pancreas of mice implanted with DMBA or expressing mutant Kras but not in ducts and PanIN lesions. Using both rapamycin and mouse mutants deficient for rpS6 phosphorylation, we have shown that rpS6 phosphorylation is an important mediator of the

oncogenic effect of Kras. Our data suggest that in the absence of p-rpS6, mutant Kras induces DNA damage preferentially in acinar and metaplastic regions, which triggers p53 expression. In turn, p53 may attenuate PanIN lesion development. Thus, our current findings assign a surprising new function to rpS6 phosphorylation in cancer biology, preventing DNA damage induced by mutant Kras. Phosphorylated rpS6 could in principle act to reduce the formation of reactive oxygen species (ROS), a known inducer of DNA damage (43), or alternatively to reduce oncogene-induced replication stress and consequent DNA damage (39). Future studies will determine exactly how rpS6 interfaces with DNA metabolism. Regardless, our results suggest that inhibition of rpS6 phosphorylation can attenuate cancer development only in the presence of an intact p53 pathway (see below). We acknowledge however that our findings do not prove that DNA damage and p53 induction are the key mediators of cancer inhibition in rpS6 mutants. Experiments with KRAS;rpS6^{P-/-} mice that lack p53 can provide a causal link: our model predicts that p53 deficiency will cancel the antitumorigenic effects of rpS6 phosphorylation deficiency. Experiments to test this idea are underway.

It will also be important to study why rpS6 phosphorylation is augmented specifically in acinar cells and metaplastic regions of DMBA-treated or mutant KRAS-expressing mice and is absent from more advanced lesions. This might be related to the fact the DNA damage and p53 activation are seen preferentially in metaplastic regions and not in PanIN lesions (see below).

While the importance of mTORC1 in cancer is well-appreciated, it is usually believed that the key downstream effector of this pathway in cancer is 4E-BP. S6K is also known as an important player in the development of cancer (ref. 23; although not PDAC), but relatively little attention has been given to the role of rpS6 phosphorylation in tumorigenicity. Our work provides a first direct functional link between rpS6 phosphorylation and cancer. The role of p-rpS6 in development of pancreatic cancer is particularly

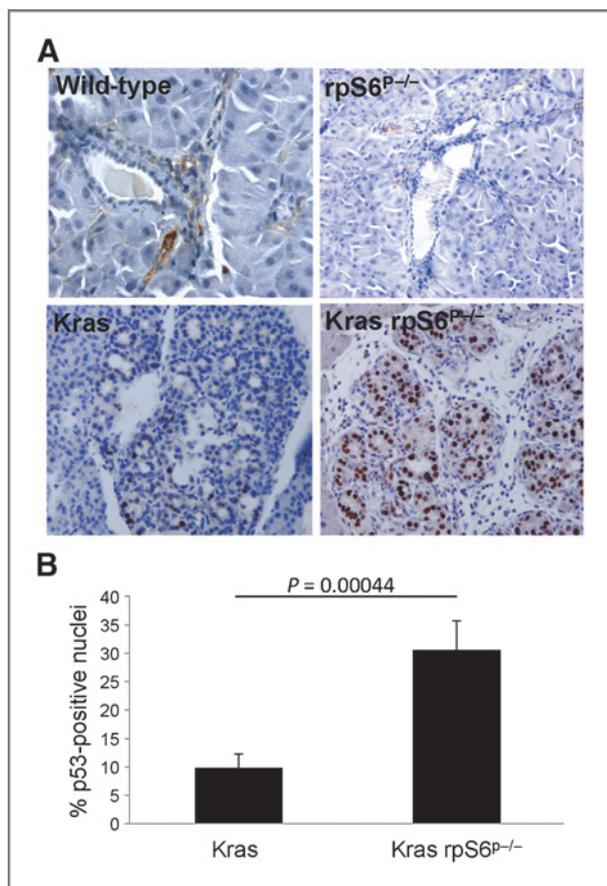


Figure 5. Lack of p-rpS6 increases expression of p53 in acinar metaplasia. **A**, representative images of p53 staining (brown). Top, wild-type and rpS6^{P-/-} mice do not stain for p53 in acinar cells or ducts. Bottom, Pdx1-Cre;LSL-Kras G12D mice containing an intact (left) or mutant (right) rpS6 gene stain for p53 in areas of metaplasia but to a different extent. Original magnification, $\times 400$. **B**, quantification of p53 expression in metaplastic regions of 3-month-old Pdx1-Cre;LSL-Kras G12D mice as a function of rpS6 genotype. Five wild-type rpS6 and 6 mutant rpS6 mice were analyzed. For each mouse, 15 random fields were analyzed and a minimum of 200 cells from foci of metaplasia were analyzed. The graph depicts the percentage of p53+ nuclei observed per metaplastic focus. Note that in this figure (as well as in Fig. 6), comparisons between Kras mice containing wild-type or mutant rpS6 were done on similar histologic structures, namely metaplastic regions.

interesting in light of the mild phenotype of rpS6^{P-/-} mice. Conceptually, it is reminiscent of synthetic lethal interactions, which expose a requirement for a certain gene only in the context of oncogene expression and of the phenomenon of "non-oncogene addiction" (44). In principle, gene products required for cancer development but having little effect on normal cells represent ideal targets for cancer treatment, with minimal expected toxicity. While pharmacologic inhibitors of rpS6 phosphorylation do not exist at this time, inhibitors of mTORC1 such as rapamycin are in clinical use, including clinical trials in human PDAC. Unfortunately, the results of these trials have been disappointing so far (45, 46). Our data may suggest at least a partial explanation for this outcome. The rapamycin trials have been conducted on

patients with advanced PDAC. In such cases, it is highly likely that p53 or the p53 pathway has already been inactivated, and therefore, inactivation of rpS6 phosphorylation has lost its tumor-suppressive effect. Our data suggest that inhibition of rpS6 phosphorylation (and potentially mTORC1) in pancreatic cancer is more likely to have an effect in the context of intact p53, that is in very early stages of disease. Hence, chronic use of mTOR inhibitors and/or the development of a more specific drug to target rpS6 phosphorylation for the prevention of disease in individuals at risk for PDAC are interesting possibilities. Whether inhibition of rpS6 phosphorylation can lead to regression or PanIN lesions is an important question to be addressed in future experiments. Interestingly, a recent article using a different model of pancreatic cancer suggested that acute inhibition of mTOR signaling can cause proliferation arrest and senescence in existing KRAS-driven PanIN lesions (47), but downstream effectors were not studied.

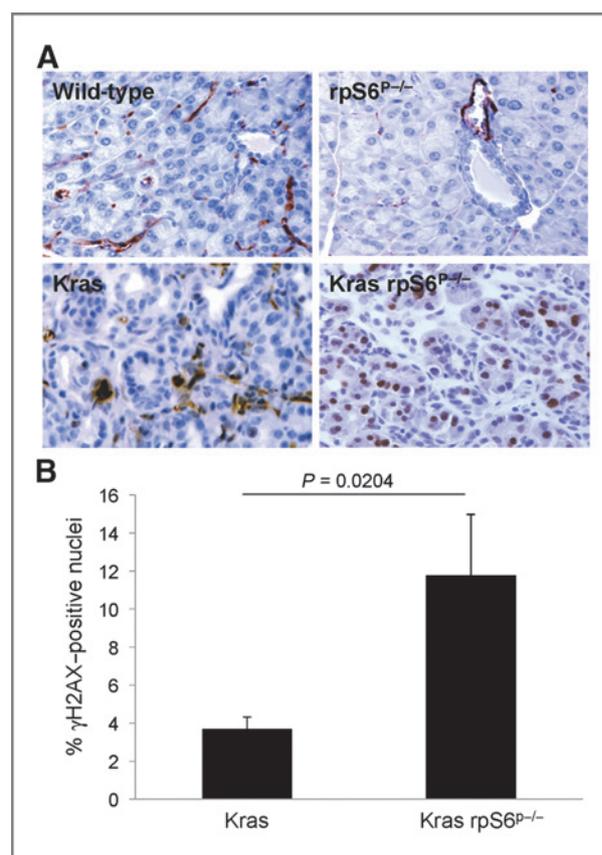


Figure 6. Increased activation of γ H2AX in rpS6 mutants. **A**, representative images of γ H2AX staining (brown). Top, wild-type (left) and rpS6^{P-/-} (right) mice do not show specific nuclear staining for γ H2AX in acinar cells or ducts. Bottom, Pdx1-Cre;LSL-Kras G12D mice containing an intact (left) or mutant (right) rpS6 gene stain for γ H2AX in areas of metaplasia but to a different extent. Original magnification, $\times 400$. **B**, quantification of γ H2AX staining in metaplastic areas of Pdx1-Cre;LSL-Kras G12D mice as a function of rpS6 genotype. For each mouse, more than 250 cells from foci of metaplasia were analyzed. $n = 6$ mice for each genotype. The graph depicts the percentage of γ H2AX nuclei observed per metaplastic focus.

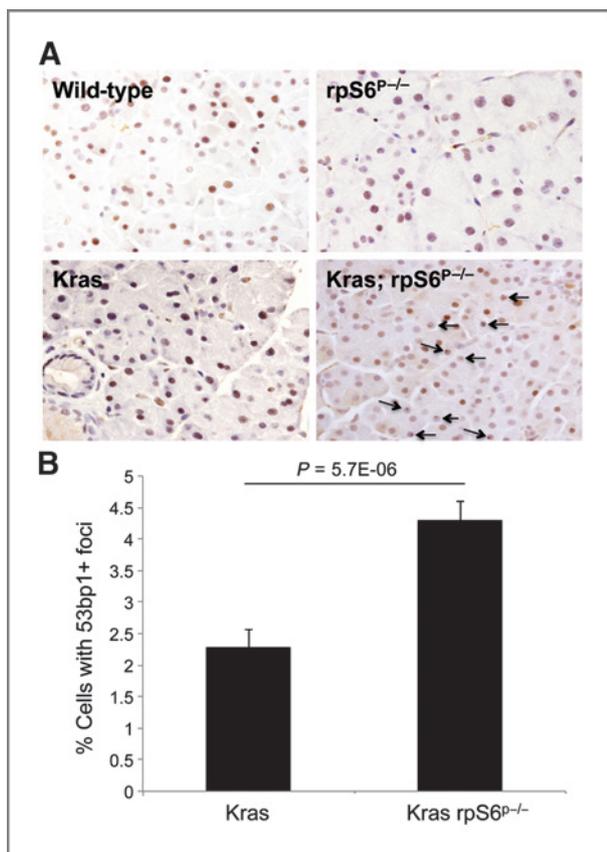


Figure 7. More 53bp1+ nuclear foci in metaplastic areas of $rpS6^{P-/-}$ mice. **A**, representative images of 53bp1 staining (brown). Positive staining is defined as the localization of 53bp1 at discrete nuclear foci (arrows), whereas diffuse nuclear staining reflects inactive 53bp1. Top, wild-type (left) and $rpS6^{P-/-}$ (right) mice exhibit diffuse nuclear staining for 53BP1 in acinar cells. Bottom, Pdx1-Cre;LSL-Kras G12D mice containing an intact (left) or mutant (right) $rpS6$ gene exhibit discrete nuclear foci of 53bp1 but more abundantly in $rpS6$ mutants. Original magnification, $\times 400$. **B**, quantification of 53bp1 staining in metaplastic areas of Pdx1-Cre;LSL-Kras G12D mice as a function of $rpS6$ genotype. For each mouse, more than 60 foci of metaplasia were analyzed. $n = 5$ mice for each genotype. The graph depicts the average percentage of nuclei containing discrete nuclear 53bp1 foci observed per metaplastic focus.

Obviously, mTORC1 inhibition (e.g., by rapamycin) has broader effects than just inhibiting $rpS6$ phosphorylation. Interestingly, 4E-BP1, an mTORC1 target implicated in pancreatic cancer, is resistant to rapamycin relative to S6K (48–50), consistent with our conclusion on the importance of the S6K- $rpS6$ pathway in pancreatic cancer. Administration of rapamycin to $rpS6^{P-/-}$ mice implanted with DMBA or expressing mutant KRAS could in principle reveal if rapamycin adds to the antitumorigenic effect of genetic $rpS6$ deficiency. In preliminary experiments, we failed to detect a significant additive beneficial effect of rapamycin combined with $rpS6$ deficiency (not shown), however large numbers of animals will be needed to reach statistical significance on this issue.

The cellular origins of PanIN lesions remain controversial. Absence of definitive lineage tracing in our study cannot resolve this debate. However, it is interesting to note that the

tumor suppression events, which we observed (γ H2AX, 53BP1 foci, and p53 expression), occur preferentially in areas of ADM, consistent with a recent report that p53 and senescence are present mostly in such regions (11). Moreover, the frequency of cells with ADM morphology is lower in the absence of phosphorylated $rpS6$ (Fig. 4). It is thus tempting to speculate that ADM is a key step in the formation of PanIN and a central point of intervention by the p53 tumor suppression pathway.

Another key question is why Kras triggers (and phospho- $rpS6$ deficiency further augments) p53 activation preferentially in ADM areas. One possibility is that in more advanced stages (PanIN lesions) the p53 pathway is already inactivated by mutations. We believe that this is unlikely for several reasons. First, γ H2AX (a likely trigger for p53 activation) is induced in acinar cells and ADM and much less so in PanIN lesions. Second, loss of p53 is known to dramatically accelerate the formation of PDAC; the fact that Kras mice rarely develop PDAC suggests that they retain largely intact p53. Finally, loss of p53 is usually manifested as stabilization of the mutant protein. PanIN lesions have no evidence for this, suggesting lack of trigger for activation. We thus propose that the metabolic wiring of metaplastic cells is more prone to Kras induction of p53 compared with either normal acinar cells or PanIN lesions. Further studies are needed to clarify this important point.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Khalailieh, A. Dreazen, O. Meyuhas, Y. Dor, G. Zamir

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Khalailieh, A. Dreazen, R. Apel, A. Swisa, O. Meyuhas, G. Zamir

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Khalailieh, A. Dreazen, A. Khatib, R. Apel, A. Maitra, O. Meyuhas, Y. Dor

Writing, review, and/or revision of the manuscript: A. Dreazen, A. Maitra, O. Meyuhas, Y. Dor, G. Zamir

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Dreazen, N. Kiddess-Bassir

Study supervision: Y. Dor, G. Zamir

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Phosphorylation of Ribosomal Protein S6 Attenuates DNA Damage and Tumor Suppression during Development of Pancreatic Cancer

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