Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer

Jennifer P. Morton[a,b], Paul Timpson[a], Saadia A. Karim[a], Rachel A. Ridgway[a], Dimitris Athineos[a,b], Brendan Doyle[a], Nigel B. Jamieson[b], Karin A. Oien[b], Andrew M. Lowy[c], Valerie G. Brunton[d], Margaret C. Frame[d], T. R. Jeffry Evans[a,b], and Owen J. Sansom[a,1]

[a]Beatson Institute for Cancer Research, Glasgow G61 1BD, United Kingdom; [b]Centre for Oncology and Applied Pharmacology, Division of Cancer Sciences and Molecular Pathology, University of Glasgow, Glasgow G61 1BD, United Kingdom; [c]Division of Surgical Oncology, Moores UCSD Cancer Center, La Jolla, CA 92093; and [d]Edinburgh Cancer Research Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh EH4 2XR, United Kingdom

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TP53 **mutation occurs in 50–75% of human pancreatic ductal adenocarcinomas (PDAC) following an initiating activating mutation in the *KRAS* gene. These p53 mutations frequently result in expression of a stable protein, $p53^{R175H}$, rather than complete loss of protein expression. In this study we elucidate the functions of mutant p53 ($Trp53^{R172H}$), compared to knockout p53 ($Trp53^{fl}$), in a mouse model of PDAC. First we find that although $Kras^{G12D}$ is one of the major oncogenic drivers of PDAC, most $Kras^{G12D}$-expressing pancreatic cells are selectively lost from the tissue, and those that remain form premalignant lesions. Loss, or mutation, of *Trp53* allows retention of the $Kras^{G12D}$-expressing cells and drives rapid progression of these premalignant lesions to PDAC. This progression is consistent with failed growth arrest and/or senescence of premalignant lesions, since a mutant of p53, $p53^{R172P}$, which can still induce p21 and cell cycle arrest, is resistant to PDAC formation. Second, we find that despite similar kinetics of primary tumor formation, mutant $p53^{R172H}$, as compared with genetic loss of p53, specifically promotes metastasis. Moreover, only mutant $p53^{R172H}$-expressing tumor cells exhibit invasive activity in an in vitro assay. Importantly, in human PDAC, p53 accumulation significantly correlates with lymph node metastasis. In summary, by using 'knock-in' mutations of *Trp53* we have identified two critical acquired functions of a stably expressed mutant form of p53 that drive PDAC; first, an escape from $Kras^{G12D}$-induced senescence/growth arrest and second, the promotion of metastasis.**

Kras | metastasis | p53 | pancreatic cancer | senescence

Pancreatic ductal adenocarcinoma (PDAC) is the fifth leading cause of cancer deaths in Europe and the United States, with an estimated 5-year overall survival of less than 5% (1, 2). Poor prognosis results from the aggressive nature of the disease, with as many as 90% of patients at the time of diagnosis harboring unresectable cancer that is extremely resistant to chemotherapy. PDAC arises from precursor lesions called pancreatic intraepithelial neoplasms (PanINs), which are characterized by the sequential accumulation of alterations in the *KRAS* oncogene and loss of the *CDKN2A*, *TP53,* and/or *SMAD4* tumor suppressors in many cases (3). Although we know the frequencies of such mutations in PDAC, their specific functions during the development of pancreatic cancer remain unclear. Here we have used a genetically engineered mouse model of pancreatic cancer (4) to aid in understanding of the respective roles of gain-of-function *Kras* and *Trp53* mutations.

KRAS is mutated in almost all human PDACs (5), and this is one of the earliest genetic events driving development of human PanINs. Studies in murine models have further shown that activating *KRAS* mutation represents an initiating step in PDAC (6–9). The *TP53* tumor suppressor gene is also frequently mutated in human pancreatic cancer (50–75%), predominantly through missense mutations (10). These often result in accumulation of mutant p53 protein, with potentially gain-of-function or dominant-negative properties. The fact that *TP53* is mutated, rather than deleted, in the majority of cancers suggests that mutant p53 provides some tumor cell growth advantage. Murine models support this, as mice expressing the accumulating p53 mutants $p53^{R172H}$ or $p53^{R270H}$ have increased incidence of osteosarcomas and epithelial carcinomas, some of which spread to distant organs (11, 12). In contrast, mice that harbor a *Trp53* null allele rarely develop metastases.

Despite its role as an oncoprotein, $Kras^{G12D}$ induces a senescence program in normal human and mouse cells in culture, and this can be prevented by inactivation of either p53 or $p16^{INK4A}$ (13). Recent studies using mouse models and human tissue samples have suggested that induction of senescence may restrain progression of premalignant lesions in vivo (14–17). However as yet, few studies have addressed this following activation of *Kras* in vivo. $Kras^{G12V}$ expression in the intestine, for example, does not alter intestinal homeostasis (18), whereas $Kras^{G12V}$-induced senescence has been reported in a mouse model of lung cancer (15). The authors of another study in which $Kras^{G12D}$ was expressed within the lung failed to detect senescence (19). Thus, it remains controversial whether, and in which contexts, oncogenic Kras induces senescence in vivo.

Targeting endogenous expression of $Kras^{G12D}$ and $p53^{R172H}$ to the mouse pancreas (using Cre-Lox technology), via the *Pdx1* pancreatic progenitor cell gene promoter, results in the formation of preinvasive PanIN lesions that develop into invasive and metastatic pancreatic cancer reminiscent of the human disease, with malignant disease burden apparent in animals by 10 weeks of age (4). As this model recapitulates the human disease in many features, it provides an excellent system to address the precise functions of individual genes and mutations in vivo. Here we show, through our ability to image recombined GFP-expressing cells in vivo, that expression of $Kras^{G12D}$ within the pancreas causes selective outgrowth of the majority of "recombined" cells, with the few cells that are retained forming senescent PanIN lesions that rarely progress to PDAC. Inactivation of both copies of *Trp53* overcomes both the selective loss and outgrowth of $Kras^{G12D}$-expressing cells and induction of growth arrest/senescence, showing a genetic interaction between the two oncogenic events. This also demonstrates a direct role for p53 in overcoming Kras-induced growth arrest/senescence in the mouse pancreas. Moreover, we show that, despite inducing similar escape from growth arrest/senescence and tumor formation, only mice expressing mutant $p53^{R172H}$, in contrast to p53 deficiency, develop pancreatic cancer metastasis. This identifies a novel role for mutant p53 that is distinct from p53 knockout.

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[1]To whom correspondence should be addressed. E-mail: o.sansom@beatson.gla.ac.uk.

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Results and Discussion

In Vivo Imaging Allows Visualization of Pancreatic Tumor Development. To better assess pancreatic cancer development and progression in vivo, we adapted a murine model of PDAC to express GFP. We crossed (*Lox-STOP-Lox*) *LSL-Kras*$^{G12D/+}$ mice (20) with *LSL-Trp53*$^{R172H/+}$ mice (12), *Pdx1-Cre* mice (21), and *Z/EGFP* mice (22). In the *Z/EGFP* reporter mouse, the *Z/EGFP* transgene results in the expression of β-galactosidase by most tissues via a *β-geo* insert, which is flanked by *lox-p* sites (22). Thus, in *Pdx1-Cre-Z/EGFP* mice (referred to hereafter as *Pdx1-Cre-GFP*), the presence of Cre-recombinase results in the excision of the *β-geo* gene, and so constitutive expression of GFP in Pdx1-expressing cells. We interbred progeny from these crosses to generate cohorts of *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$ (KPC) mice, *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) mice, *Pdx1-Cre-GFP, LSL-Trp53*$^{R172H/+}$ (PC) mice, and *Pdx1-Cre-GFP* (C) mice. This enabled us to image the pancreata of GFP-positive mice, using the Olympus OV100 in vivo imaging system. This imaging was noninvasive and permitted repeated visualization of tumor progression in situ, for monitoring tumor development and progression over time in individual mice.

The presence of GFP expressed in Pdx1-expressing cells within the pancreas did not influence pancreatic development or tumor phenotypes in the mice (Fig. S1 *A–C*). As described previously, animals expressing pancreas-specific endogenous Kras^{G12D} and p53^{R172H} developed PanIN lesions at approximately 6 weeks of age; as expected, these lesions developed into invasive PDAC, with a latency ranging from 2 to 10 months (Fig. S1*D*) (4). Pancreatic ducts appeared normal with regular cuboidal epithelium (Fig. 1*A*), whereas PanIN lesions exhibited characteristic histological changes including conversion of the duct epithelial cells to a columnar phenotype with mucin accumulation (Fig. 1*B*). This was confirmed by Alcian blue staining (Fig. 1*D*). Formation of papillary architecture and luminal budding was observed, with loss of polarity and appearance of atypical nuclei. The resulting tumors exhibited prominent ductal differentiation with a marked desmoplastic reaction (Fig. 1*C*) and frequent metastasis to the liver (see Fig. 3).

We observed that, from early after birth, *Pdx1-Cre-GFP* (C) and *Pdx1-Cre-GFP, LSL-Trp53*$^{R172H/+}$ (PC) mice expressed GFP uniformly within the pancreas, as detected by in vivo imaging. However, whereas GFP was visible in *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$ (KPC), and *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) animals after birth, this was gradually lost over time (period between 2 and 6 weeks shown in Fig. 1 *E–H,* KPC mouse shown in Fig. S1*E*). Imaging of pancreata from 3-week-old mice expressing Kras^{G12D} revealed areas that did not visibly express GFP when compared with *Kras* wild-type mice (Fig. 1*F*), and the fluorescence signal was completely lost by 6 weeks of age (Fig. 1*H*). Thus, GFP imaging allowed us to examine the fate of cells expressing both GFP and the gain-of-function Kras^{G12D} protein, and we found that these cells were lost from the pancreas during the first weeks after birth. This indicated that there was selective loss of Kras^{G12D}-expressing cells within the pancreas. To test for loss of recombined cells, we isolated DNA from the pancreata of 6-week-old mice with or without conditional Kras^{G12D} expression and/or conditional p53 mutation, and from tumors arising in these mice. We performed PCR to detect the recombined p53 allele, and found high levels in pancreata where Kras^{G12D} was not expressed (Fig. S1*H*). In Kras^{G12D}-expressing pancreata, however, levels of recombined p53 were substantially lower, providing further evidence for a selective loss, or competitive outgrowth, of recombined cells in which Kras^{G12D} was expressed. We found that a subpopulation of Kras^{G12D}-expressing cells did persist to a sufficient extent to enable tumors to form. Although whole-body in vivo imaging of GFP fluorescence was not sensitive enough to detect this subpopulation of cells at the stage of PanIN formation, we did observe

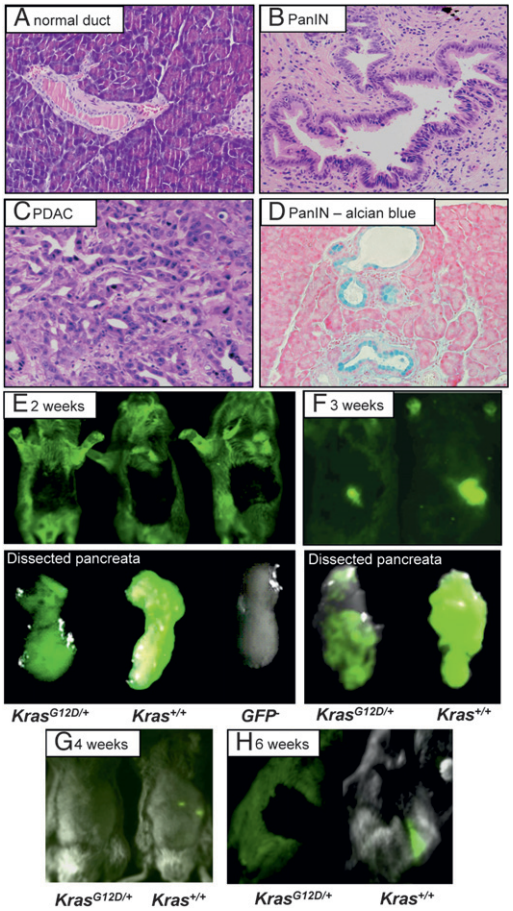

Fig. 1. Noninvasive in vivo imaging in a murine model of pancreatic ductal adenocarcinoma (PDAC). (*A–C*) H&E staining of (*A*) a normal pancreatic duct in a *Pdx1-Cre-GFP, LSL-Trp53*^{R172H} (PC) mouse, (*B*) a PanIN lesion in a *Pdx1-Cre-GFP-LSL-Kras*^{G12D}*, LSL-Trp53*^{R172H} (KPC) mouse, and (*C*) an invasive adenocarcinoma from a KPC mouse. (*D*) Alcian blue staining of a PanIN lesion from a KPC mouse. (*E* and *F*) In vivo imaging, using the Olympus OV100 in vivo imaging system, of GFP fluorescence within the pancreata of (*E*) 2-week-old and (*F*) 3-week-old *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) mice and PC mice, as indicated. (*Top*) Whole-body imaging; (*Bottom*) ×8 magnification of excised pancreata. (*G* and *H*) In vivo imaging, using the Olympus OV100 in vivo imaging system, of GFP fluorescence within the pancreata of (*G*) 4-week-old and (*H*) 6-week-old KC mice and PC mice, as indicated.

tumor formation and growth by in vivo imaging (Fig. S1 *E–G*). Moreover, we demonstrated high levels of GFP expression within PanINs and tumors, compared with significantly lower levels in normal pancreata, by immunohistochemistry (IHC) staining for GFP protein (Fig. S1 *I–K*). Consistent with this, higher levels of recombined p53 were observed by PCR in tumors compared with the low levels we observed in normal pancreata from the same mice (Fig. S1*H*). Together, these results show that there is selection against expression of activated Kras in the pancreas during the growth and development of the adult pancreas (for model see Fig. S2).

The expression of nestin marks a restricted exocrine progenitor cell compartment within the pancreas (23). Targeting of *Kras*^{G12D} to this compartment was sufficient for the formation of PanINs, with the same frequency as observed in *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) mice (24), where expression was widespread throughout the pancreas. As the frequency of Cre-mediated recombination should be much higher using the *Pdx1*-driven Cre recombinase (21); and, given that the majority of recombined Kras^{G12D}-expressing cells were selectively lost from the pancreas, we hypothesized that the mutant Kras^{G12D}-expressing cells that persisted were derived from the nestin lineage. IHC staining

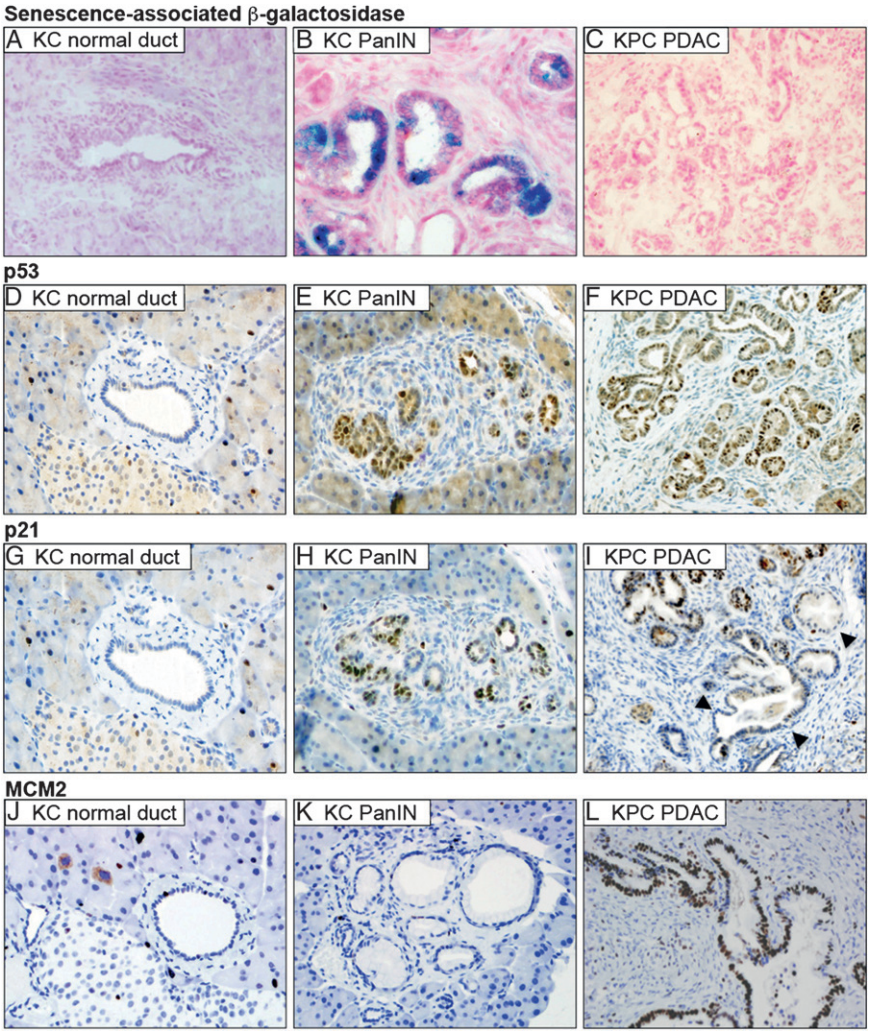

Fig. 2. Senescence program is activated in $Kras^{G12D}$-expressing cells in the normal pancreas but not in pancreatic tumors. (*A–C*) β-Galactosidase staining at pH 6, (*D–F*) p53 immunohistochemical staining, (*G–I*) p21 immunohistochemical staining, and (*J–L*) MCM2 immunohistochemical staining, in sections of frozen (β-galactosidase) or formalin-fixed paraffin-embedded pancreatic tissue. (*A, D, G,* and *J*) Normal pancreatic ducts in pancreata harvested from 6-week-old *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$ (KC) mice, (*B, E, H,* and *K*) PanIN lesions in pancreata harvested from 6-week-old KC mice, and (*C, F, I,* and *L*) pancreatic ductal adenocarcinoma harvested from *Pdx1-Cre-GFP-LSL-*$Kras^{G12D}$*, LSL-*$Trp53^{R172H}$ (KPC) mice. Arrowheads indicate areas where p21 up-regulation has been lost.

confirmed that, in contrast with normal pancreatic ducts, all PanINs and carcinomas that arose did indeed express nestin (Fig. S3 *D–F*); and although it is not possible to rule out up-regulation of nestin in $Kras^{G12D}$-expressing cells, these data support the hypothesis that the nestin-positive progenitor lineage was not selectively lost upon expression of $Kras^{G12D}$. We hypothesize that it is activation of oncogenic *Kras*^{G12D} in the nestin-positive progenitor cell population that ultimately gives rise to PDAC, with the non–nestin-expressing pancreatic cells selectively lost or outcompeted during growth of the pancreas after oncogenic *Kras* mutation.

Loss of p53 Allows Retention of $Kras^{G12D}$-Expressing Cells in the Pancreas. To assess the mechanism by which $Kras^{G12D}$-expressing cells were selectively lost, we generated mice that carried two copies of mutant p53: *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$*, LSL-*$Trp53^{R172H/R172H}$ (KPPC) mice. Unlike *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$ (KC) and *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$*, LSL-*$Trp53^{R172H/+}$ (KPC) mice, in which we had observed loss of GFP with time, GFP was now retained throughout development, and this correlated with rapid tumorigenesis (Fig. S3*A*). Higher levels of the recombined p53 allele were observed by PCR analysis of DNA from *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$*, LSL-*$Trp53^{R172H/R172H}$ (KPPC) pancreata, compared with *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$*, LSL-*$Trp53^{R172H/+}$ (KPC) pancreata, indicative of the retention of recombined cells (Fig. S3*B*). Given that the entire pancreas was now composed of recombined $Kras^{G12D}$-expressing cells, we hypothesized that cells outside the nestin lineage could now be retained and could form tumors, and this was indeed the case (Fig. S3 *C, G,* and *H*).

Senescence/Long-Term Growth Arrest of $Kras^{G12D}$-Expressing Cells Limits Tumor Progression in the Pancreas. We next investigated the mechanism by which wild-type p53 was blocking tumorigenesis in the pancreas. By 2 months of age, *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$ (KC) mice had developed multiple premalignant PanIN lesions that rarely progressed to carcinomas. However, *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$*, LSL-*$Trp53^{R172H/+}$ (KPC) mice rapidly developed PDAC with a median onset of 130 days (Fig. S1*D*).

As it has been suggested that cellular senescence is a key tumor suppressor pathway downstream of Ras signaling, we investigated whether p53 loss was suppressing Ras-induced senescence. Cellular senescence is associated with senescence-associated β-galactosidase, and induction of cell cycle regulators such as $p16^{Ink4a}$, p53, and $p21^{CIP1}$. Thus, we first performed β-galactosidase staining on cryosections of pancreata from 6-week-old mice expressing conditional $Kras^{G12D}$ and tumors with conditional $Kras^{G12D}$ and mutant p53 expression (Fig. 2 *A–C* and Fig. S4*A*). β-Galactosidase staining was evident in the premalignant PanINs (but not in normal ducts) arising in *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$ (KC), and *Pdx1-Cre-GFP, LSL-*$Kras^{G12D/+}$*, LSL-*$Trp53^{R172H/+}$ (KPC) mice, indicative of growth arrest/senescence. In contrast, β-galactosidase staining was not detected in pancreata from *Pdx1-Cre-GFP* (C) or *Pdx1-Cre-GFP, LSL-*$Trp53^{R172H/+}$ (PC) mice. Critically, tumors that developed in

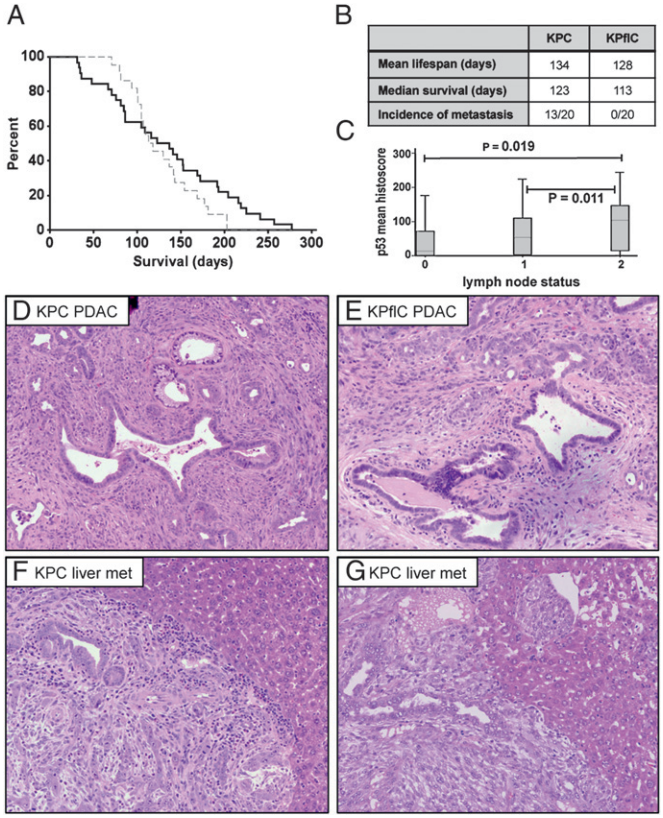

Fig. 3. p53 Drives metastasis of pancreatic ductal adenocarcinoma. (*A*) Kaplan-Meier survival curve shows no significant difference in survival between *Pdx1-Cre-GFP, LSL-Kras*^{G12D}, *LSL-Trp53*$^{R172H/+}$ (KPC) mice (solid line), and *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{loxP/+}$ (KP^{fl}C) mice (dashed line). $P = 0.479$. (*B*) Table showing that mean lifespan and median survival are not significantly different in KPC mice compared with KP^{fl}C mice, whereas incidence of metastasis in KPC mice is significantly increased compared with KP^{fl}C mice, in which metastasis is not observed at all. (*C*) p53 Histoscore in relation to lymph node status in cases of human PDAC (0, lymph node negative; 1, metastatic disease present in <50% of lymph nodes sampled; 2, metastatic disease present in >50% of lymph nodes sampled; mean number of nodes reviewed per resection, 21). (*D* and *E*) H&E-stained sections from (*D*) a KPC tumor and (*E*) an age-matched KP^{fl}C tumor show there is no difference in tumor stage or grade between the two genotypes. (*F* and *G*) H&E-stained sections of liver metastases arising in KPC mice.

Pdx1-Cre-GFP, LSL-Kras$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice did not exhibit detectable β-galactosidase staining (Fig. 2*C*). Given that previous studies have shown that the remaining wild-type allele of p53 is lost within PDACs that arise in the pancreatic cancer model, this implies that p53 loss allows cells to escape from Kras^{G12D}-induced growth arrest/senescence. Tumors that developed within *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice exhibited GFP expression as judged by in vivo imaging and IHC, suggesting that they have likely progressed from the nestin-positive GFP-expressing PanINs (Fig. S1 *E–G* and *I–K*).

We also performed IHC analysis for additional markers of senescence/long-term growth arrest. We first carried out IHC for p53 and its target p21^{CIP1}, which is known to play a role in senescence/growth arrest (25, 26), in PanINs from *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) mice and *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice. In the PanINs, we observed high nuclear levels of both p53 and p21, compared with normal ducts (Fig. 2 *D, E, G,* and *H*, and Fig. S4 *B* and *C*). Within *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice, it has previously been shown that tumor formation occurs after loss of the remaining wild-type p53 allele, and that this correlates with the accumulation of mutant p53^{R172H} protein. Hence, this complicates analysis of p53 activation in the premalignant versus the malignant tumors. However, in our case, staining of PanINs from *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice showed an accumulation of nuclear p53 and p21^{CIP1}, arguing that p53 is functional, whereas PDACs and some more advanced lesions from these mice maintained high levels of p53 but crucially had lost p21 (consistent with mutant p53 protein that is unable to transcriptionally activate p21) (Fig. 2 *F* and *I*). We also performed IHC for another marker of senescence Igfbpb7. Like p21, early PanIN lesions exhibited high levels of this marker, again consistent with senescence, whereas levels were reduced in carcinomas (Fig. S4 *D–G*). However, a more stringent test for a long-term growth arrest or senescence is the presence of very low levels of proliferation within PanINs. To test this, we looked at two markers of proliferation: the nuclear proliferation marker Ki67 (Fig. S4 *H–K*) and the replication-licensing protein MCM2. We found that PanIN lesions exhibited only a few positive-staining cells (MCM2 staining shown in Fig. 2*K*). High levels of proliferation and MCM2 staining were observed in PDACs (Fig. 2*L*). Thus, these data provide clear evidence that long-term growth arrest/senescence occurs in Kras^{G12D}-expressing cells in the mouse pancreas, and that this must be overcome in order for gross tumor development to occur.

p53 Suppression of p21 Is Required for PDAC Development. There is considerable evidence that p21 gene activation is a key target of p53 in eliciting a growth arrest/senescence response (27). However, it has been unclear whether this occurs in vivo. p21-Deficient mice are viable and have only a weak predisposition to cancer, and there is very little evidence that p21 can act as a bona fide tumor suppressor in vivo (28). To test whether p21-mediated growth arrest/senescence downstream of p53 activation is important for the formation of PDAC, we intercrossed mice carrying the p53^{R172P} mutation to *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) mice and investigated whether they formed PDAC with similar kinetics to *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice. This mutation of p53 promotes the transactivation of p21 and downstream growth arrest but not some other p53 target genes (29, 30). To assess whether mice developed PDAC, we aged *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *Trp53*$^{R172P/+}$ (KP^{p21}C) mice to 100 days and compared PDAC formation to that observed in *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice. We found that 0/9 *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172P/+}$ (KP^{p21}C) mice had PDAC, compared with 7/9 *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice ($P = 0.01$ χ^2). Importantly, these mice developed similar numbers of PanINs to *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) mice but they did not progress to PDAC (Fig. S5 *A* and *B*). The PanINs at this stage were still minimally proliferative and exhibited senescence-associated β-galactosidase. Indeed, even in mice aged up to 250 days, pancreata had numerous PanINs but no PDAC (Fig. S5*C*). These data are compatible with those of previous studies showing that tumor onset is delayed in p53^{R172P}-expressing mice compared with p53 knockout mice (31–33).

One of the difficulties in assigning a long-term cell cycle arrest as senescence in vivo is that the definition of senescence ascribed in vitro may not completely apply. A premalignant lesion would have expanded from a single mutant clone, and it is therefore unlikely that a complete irreversible growth arrest really occurs in vivo. Indeed, given recent work showing that senescent cells can be cleared by the immune system (34, 35), it may be that the majority of senescent cells in the body are cleared and thus, would not be detected. Obviously some proliferation must occur to allow the formation of premalignant lesions, but once formed, these display very low proliferation rates and express markers of the senescence that is associated with reduced proliferation and tumor progression. These findings explain some of the contradictions in the literature where so-called hyperplastic benign lesions are defined as senescent.

Mutant p53 Drives Metastasis of Pancreatic Ductal Adenocarcinoma. One vital question that we could address was whether accumulating mutation of p53 was having a greater impact on tumorigenesis than p53 deficiency. One reason that we examined this arises from the observations that *TP53* mutations in human PDAC can occur downstream of *CDKN2A* (*INK4A*) mutations, which are frequently an early event following *KRAS* activation (36). Mutation of *INK4A* causes an escape from long-term growth arrest/senescence similar to loss of function p53 mutation (Fig. S6 showing the results when we crossed the *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$ (KC) mice to *LSL-Cdkn2*$^{+/-}$ (KIC) mice). This implies that mutant p53^{R172H} must confer additional, or distinct, tumorigenic properties on pancreatic cancer cells in addition to loss of the growth arrest/senescence response. To test this, we intercrossed mice carrying *Trp53*^{loxP} to *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, LSL-Trp53$^{R172H/+}$ (KPC) mice to generate cohorts of *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{loxP/+}$ (KP^{fl}C), and sibling *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice. We found no significant difference in PanIN development, tumor latency, or tumor stage between these two cohorts (Fig. 3 *A, D,* and *E*). In vivo imaging and β-galactosidase staining indicated that, once again, most Kras^{G12D}-expressing cells were not retained in the pancreas and that those that were retained formed growth-arrested/senescent premalignant lesions (Fig. S7). However, there was a striking difference in the incidence of metastasis (Fig. 3 *B, F,* and *G*). The incidence of metastatic spread to the liver was 13/20 (65%) in *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice, compared with 0/20 (0%) in *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{loxP/+}$ (KP^{fl}C) animals ($P < 0.001$). These results demonstrate a prometastatic function for the accumulating mutant p53^{R172H} that is distinct from, and in addition to, the tumor-promoting effects of p53 loss. Previous studies have shown that IHC analysis of nuclear p53 accumulation corresponds well with p53 mutation status in human tumors (37). To examine whether our findings in the genetically engineered mouse model were relevant to human PDAC, we investigated whether p53 accumulation, by IHC staining of a human tissue microarray of 114 resected human PDAC, correlated with any clinicopathological findings in PDAC. Although no significant associations were found with tumor progression parameters, we found a significant correlation between p53 accumulation and lymph node metastasis. In particular, lymph node negative resections were associated with a significantly lower tumor accumulation of p53, as measured by IHC, compared with lymph node–positive resections (median histoscore 12.3 vs. 64.7; $P = 0.019$). Furthermore, resection specimens with less than 50% of lymph nodes involved in metastatic spread were associated with lower tumor p53 accumulation, compared with resections with greater than 50% of lymph nodes involved (median histoscore 56.5 vs. 103.8; $P = 0.011$) (Fig. 3C). Importantly, it has been previously shown that patients with a high degree of lymph node involvement have an increased incidence of liver metastasis (38). Taken together, these data suggest that this prometastatic function of mutant p53 is conserved between mice and humans.

We next investigated which part of the metastatic process mutant p53 was affecting, and examined whether this phenotype was intrinsic to the tumor cells. To do this, we established tumor cell lines from the *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{loxP/+}$ (KP^{fl}C) and *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice and investigated the capacity of these cells to invade in an in vitro invasion assay into collagen gels. Although tumor cell lines from *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice were proficient at invasion in this assay, cell lines from *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{loxP/+}$ (KP^{fl}C) mice did not invade to any great extent (Fig. 4 *A* and *B*). To show that this was a dominant effect of mutant p53, we expressed mutant p53^{R175H}, the human equivalent of murine p53^{R172H}, in the *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{loxP/+}$ (KP^{fl}C) tumor cell line and found that this cell line was now capable of invasion in vitro (Fig. 4 *C* and *D*). Taken together, our data show that, in a genetic model of human pancreatic cancer in vivo, mutant p53 drives metastasis, most likely by conferring invasive properties on the tumor cells that express it. Our findings differ somewhat from a previous study in which p53 deletion within the pancreas in combination with viral PyMT delivery to elastase-expressing cells led to the formation of metastatic pancreatic cancer (39). In this study, metastases were detected only in animals that developed acinar carcinomas, and these metastatic tumors did not exhibit any ductal characteristics (39). It is therefore likely that the ability of mutant p53 to promote metastasis is influenced by primary tumor type. These data are consistent with a broader literature in which mutant p53 has been suggested to play an important role in metastasis beyond that of knockout p53; however, these studies have generally yielded quite subtle increases in metastasis (11, 12, 40). Our data conclusively show that mutant p53 drives the metastatic process in vivo, particularly as there is no difference in tumor latency between mice bearing mutant p53 compared with p53 deletion.

Mice carrying endogenous p53 missense mutations have been studied as a model of Li Fraumeni syndrome (11, 12). These mice develop a distinct spectrum of tumors compared with those arising in p53 heterozygous null mice, with an increased incidence of osteosarcomas and carcinomas (11, 12). These results indicate that the p53^{R172H} mutant is not simply a loss-of-function allele; rather, its tumorigenicity is enhanced through a gain-of-function or dominant negative mechanism. These data also suggest that, depending on tumor type and collaborating oncogenic or tumor suppressive events, mutant p53 may confer additional properties on tumor cells, thus influencing proliferation, differentiation, and metastasis.

In summary, we have characterized the functions of p53 mutation in pancreatic cancer, namely, an escape from growth arrest/

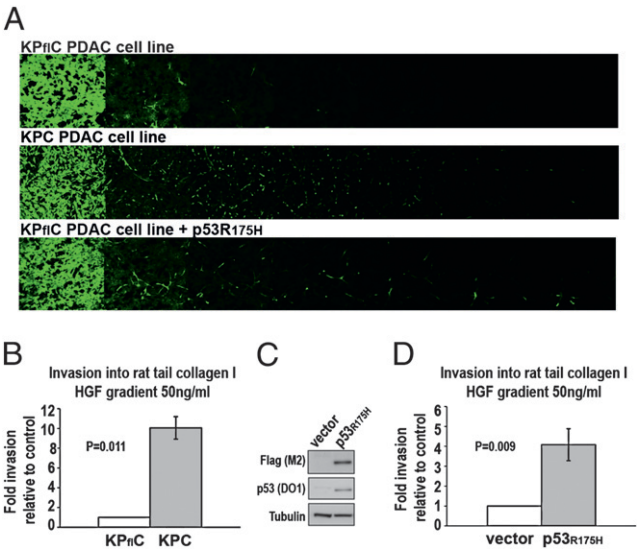

Fig. 4. Mutant p53 promotes invasion of PDAC cells in vitro. Inverted invasion assays were performed on murine PDAC tumor cell lines with or without mutant p53. (*A*) Tumor cell lines bearing mutant p53^{R172H}, from *Pdx1-Cre-GFP, LSL-Kras*^{G12D}, *LSL-Trp53*$^{R172H/+}$ (KPC) tumors (*Middle*), invade further than tumor cells grown from *Pdx1-Cre-GFP, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{loxP/+}$ (KP^{fl}C) tumors (*Top*). Introduction of exogenous expression of mutant p53^{R175H} into these KP^{fl}C tumor cells, however, promotes invasion (*Bottom*). (*B*) Bar graph showing increased invasive capacity of KPC tumor cells compared with KP^{fl}C tumor cells. Representative images of at least three independent experiments are shown. Columns indicate mean; bars indicate SE. *$P \leq 0.01$. (*C*) Western immunoblotting shows expression of flag-tagged p53^{R175H} in stably transfected KP^{fl}C cells. β-Tubulin represents a loading control. (*D*) Bar graph showing increased invasive capacity of KP^{fl}C tumor cells following exogenous expression of mutant p53^{R175H}. Representative images of at least three independent experiments are shown. Columns indicate mean; bars indicate SE. *$P < 0.01$ by unpaired Student's *t* test.

senescence and a promotion of metastasis. Our data provide definitive evidence, in a matched genetic system, that mutant p53 has extra functions over and above loss of p53, in a tumor model that closely recapitulates the human disease.

Materials and Methods

Genetically Modified Mice and Animal Care. The *Pdx1-Cre*, *ZIEGFP*, *LSL-Kras^{G12D}*, *LSL-Trp53^{R172H}*, *LSL-Cdnk2a^{loxP}*, *LSL-Trp53^{loxP}*, and *LSL-Trp53^{R172P}* mouse strains have been previously described (8, 12, 20, 22, 29, 41, 42). Animals were kept in conventional animal facilities and monitored daily. Experiments were carried out in compliance with U.K. Home Office guidelines. Mice were genotyped by PCR analysis. Tumor and metastatic burden was assessed by gross pathology and histology.

Tumor Harvest and Histology. Animals were killed as per institutional guidelines. Organs/tumors were removed and either fixed in 10% buffered formalin overnight at room temperature or snap frozen in liquid nitrogen. Fixed tissues were paraffin embedded and 5-μm sections placed on sialynated/poly-L-lysine slides for IHC analysis.

Fluorescence Imaging in Live Mice. The Olympus OV100 Whole Mouse Imaging System (Olympus), containing an MT-20 light source (Olympus Biosystems) and DP70 CCD camera (Olympus), was used for imaging in live mice. Anesthesia was induced and maintained with a mixture of isoflurane and oxygen.

PCR. Primers and details of PCR are given in *SI Materials and Methods.*

Immunohistochemistry. IHC analysis was performed as described previously. Further information is given in *SI Materials and Methods.*

Senescence-Associated β-Galactosidase Staining. We stained cryosections of mouse pancreas or tumor for senescence-associated β-galactosidase activity according to the protocol of the manufacturer (Cell Signaling Technology) and counterstained with nuclear fast red.

Tissue Microarray analysis. The human pancreatico-biliary tissue microarray was created within the West of Scotland Pancreatic Unit, University Department of Surgery, Glasgow Royal Infirmary. Further information is provided in *SI Materials and Methods*.

Inverted Invasion Assay. Inverted invasion assays were adapted from (43). Further details are given in *SI Materials and Methods.*

Transfection and Immunoblotting. Cells were transfected using polyfect (Qiagen) according to the manufacturer's instructions. Cells were washed with PBS and then lysed in cell extraction buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 μg/mL aprotinin, 125 mM PMSF, 100 μM Na_3VO_4, and 0.5 mM NaF). Lysates were resolved by 10% Bis-Tris gel electrophoresis (Invitrogen). Proteins were transferred to PVDF membrane, blocked, and probed with antibodies against Flag (M2, Sigma), p53 (D01, Pharmingen), and β-tubulin (Sigma).

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