Trp53^{R172H} and *Kras*^{G12D} cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice

Sunil R. Hingorani,[1,2,*] Lifu Wang,[2] Asha S. Multani,[4] Chelsea Combs,[2] Therese B. Deramaudt,[1,3] Ralph H. Hruban,[5] Anil K. Rustgi,[1,3] Sandy Chang,[4] and David A. Tuveson[1,2,*]

[1]Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104
[2]Department of Cancer Biology, Abramson Family Cancer Research Institute, Abramson Cancer Center at the University of Pennsylvania, Philadelphia, Pennsylvania 19104
[3]Gastroenterology Division, University of Pennsylvania, Philadelphia, Pennsylvania 19104
[4]Department of Molecular Genetics, M.D. Anderson Cancer Center, Houston, Texas 77030
[5]Departments of Pathology and Oncology, Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287
*Correspondence: srhingo@mail.med.upenn.edu (S.R.H.); tuvesond@mail.med.upenn.edu (D.A.T.)

Summary

To define the genetic requirements for pancreatic ductal adenocarcinoma (PDA), we have targeted concomitant endogenous expression of *Trp53*^{R172H} and *Kras*^{G12D} to the mouse pancreas, revealing the cooperative development of invasive and widely metastatic carcinoma that recapitulates the human disease. The primary carcinomas and metastases demonstrate a high degree of genomic instability manifested by nonreciprocal translocations without obvious telomere erosion—hallmarks of human carcinomas not typically observed in mice. No mutations were discovered in other cardinal tumor suppressor gene pathways, which, together with previous results, suggests that there are distinct genetic pathways to PDA with different biological behaviors. These findings have clear implications for understanding mechanisms of disease pathogenesis, and for the development of detection and targeted treatment strategies.

Introduction

Ductal adenocarcinoma of the pancreas (PDA) is an almost uniformly lethal disease, largely because it eludes diagnosis until very advanced stages. Indeed, greater than 80% of patients with PDA have locally unresectable or frankly metastatic disease at the time of presentation (Warshaw and Fernandez-del Castillo, 1992; Yeo et al., 2002b). PDA is also unusually resistant to all forms of cytotoxic chemotherapies and ionizing radiation, and as a result, as many people die of PDA each year as are newly diagnosed with it. Now the fourth leading cause of cancer-related mortality among men and women in the United States, approximately 31,000 new cases and deaths were expected from PDA in 2004 (Jemal et al., 2004).

The current standard of care for advanced PDA is infusional gemcitabine, a deoxycytidine analog and inhibitor of nucleic acid synthesis, which prolongs survival by only a few weeks and provides symptomatic improvement in a minority of patients (Burris et al., 1997). For those rare patients able to undergo complete surgical resection of their primary tumor, five-year survival can be as high as 20%–40%; however, even these highly selected patients eventually succumb to both locally recurrent and metastatic disease (Allison et al., 1998; Yeo et al., 2002a). Thus, in addition to methods to detect preinvasive disease, therapies that can kill invasive and metastatic pancreatic cancer cells are needed.

Considerable insight into potential mechanisms of disease pathogenesis has been gleaned from static analyses of resected pancreatic tumor specimens giving rise to histologic (Brat et al., 1998; Hruban et al., 2001a; Klimstra and Longnecker, 1994) and molecular (Hruban et al., 2000; Hruban et al., 2001b) frameworks for disease progression. These studies of sporadic pancreatic cancers have suggested a model of disease evolution through a preinvasive state, termed pancreatic intraepithelial neoplasia (PanIN), involving progressive cellular and architectural atypia accompanied by increasingly frequent mutations in a key oncogene and select tumor suppressor

SIGNIFICANCE

Cancer is a genetic disease driven by the stochastic acquisition of mutations and shaped by natural selection. Genomic instability, a hallmark of human epithelial cancers, propagates these mutations, allowing cells to overcome critical barriers to unregulated growth, and may therefore herald a defining event in malignant transformation. How and when during the course of tumor progression significant genomic instability arises, and whether a cancer can be cured or even contained after that point, represent pivotal and largely unanswered questions. We describe here a murine model of pancreatic ductal adenocarcinoma, characterized by the development of widespread and complex chromosomal instability, which may prove useful in investigating these issues.

genes (TSG). Specifically, activating point mutations in the *KRAS2* proto-oncogene are encountered in greater than 90% of pancreatic carcinomas, along with inactivation of the *CDKN2/INK4A* locus in a similar number. Point mutations of the *TP53* tumor suppressor have been described in approximately 75% of pancreatic cancers, while 55% harbor deletions or mutations in *SMAD4* (also known as *DPC4* and *MADH4*). *BRCA2* is mutated in less than 10% of sporadic pancreatic cancers and perhaps as many as 19% of familial cases (Hahn et al., 2003; Murphy et al., 2002). Mutations in DNA mismatch repair genes, such as *MSH2* and *MLH1*, similarly occur in fewer than 10% of cases. Interestingly, such mutations are found in rare tumors with a distinct syncytial growth pattern, and are not associated with *KRAS2* or *TP53* mutations (Goggins et al., 1998; Yamamoto et al., 2001). Ductal adenocarcinomas of the pancreas also manifest an unusual degree of numerical and structural chromosomal instability (reviewed in Hansel et al., 2003).

The genetic epidemiology described above has richly informed attempts to model the disease in animals (reviewed in Leach, 2004). However, such molecular compendia are necessarily speculative and cannot distinguish causal from coincident events, nor which combinations of events might be required to establish disease. Corroborative support for the involvement of certain tumor suppressor genes in disease pathogenesis is provided by a number of heritable syndromes that substantially increase the lifetime risk of pancreatic cancer, as seen, for example, in FAMMM syndrome patients possessing germline *CDKN2/INK4A* mutations and in Li-Fraumeni patients with germline *TP53* mutations. Definitive evidence can be provided by engineering these events systematically into the mammalian genome and observing the resultant phenotype. By this means, we now know that endogenous expression of oncogenic $Kras^{G12D}$ serves to intiate PanIN, which can spontaneously progress to fully invasive and frankly metastatic disease (Hingorani et al., 2003). The molecular details of disease progression remain largely unknown, although some conclusions have recently been established. When placed in the context of concomitant biallelic *Ink4a/Arf* deletion, mice expressing endogenous $Kras^{G12D}$ developed an aggressive, locally invasive, and poorly differentiated disease accompanied, on occasion, by microscopic metastases (Aguirre et al., 2003). Interestingly, these tumors did not manifest mutations in any of the other TSG pathways noted above. This raises the question of whether other TSG mutations alter the biology of the disease. For example, additional mutations may be required to develop a significant metastatic burden. Alternatively, the abrogation of each TSG pathway individually may constitute a distinct genetic route to PDA with a potentially unique phenotype.

To directly address questions concerning the requirements for tumor progression, we have targeted endogenous expression of $Trp53^{R172H}$, an ortholog of one of the most common *TP53* mutations in human PDA (Olivier et al., 2002), to progenitor cells of the mouse pancreas. We find that physiologic expression of $Trp53^{R172H}$, in the context of concomitant endogenous $Kras^{G12D}$ expression, promulgates the development of invasive and widely metastatic pancreatic ductal adenocarcinoma that recapitulates the principal clinical, histopathological, and genomic features of the cognate human condition.

Results

LSL-$Kras^{G12D/+}$*;LSL-*$Trp53^{R172H/+}$*;Pdx-1-Cre* mice develop metastatic PDA

We have previously described the targeting of endogenous $Kras^{G12D}$ to the mouse pancreas (Hingorani et al., 2003). Using similar methods, we have generated a conditionally expressed point mutant allele of the Li-Fraumeni human ortholog, $TP53^{R175H}$ (Figure 1 and Olive et al., 2004). Activation of both the $Kras^{G12D}$ and the $Trp53^{R172H}$ alleles occurs in tissue progenitor cells of the developing mouse pancreas through interbreeding with *Pdx-1-Cre* transgenic animals. The presence of each rearranged, activated allele can be detected in the pancreata, but not tails, of compound mutant animals by specific PCR (Figure 1). Thus, tissues not expressing *Cre* recombinase remain functionally heterozygous for these loci. We note that activation of the respective point mutant alleles occurs in the context of expression of their wild-type counterparts, as is presumably encountered in the spontaneous acquisition of such mutations in the human disease.

LSL-$Kras^{G12D/+}$*;LSL-*$Trp53^{R172H/+}$*;Pdx-1-Cre* mice have a dramatically shortened median survival of approximately 5 months as compared with their control littermates (wild-type, *Pdx-1-Cre* and *LSL-*$Trp53^{R172H/+}$*;Pdx-1-Cre* animals). In the triple mutant cohort (n = 28), 100% mortality occurred by 12 months (Figure 1B); only one animal lacked invasive PDA at necropsy (Table 1). As expected from previous analyses of $Trp53^{+/-}$ animals (Donehower et al., 1992; Jacks et al., 1994), thymic lymphomas (n = 2) or teratocarcinomas (n = 1) were also rarely found at necropsy. Triple mutant animals also succumb much earlier than *LSL-*$Kras^{G12D/+}$*;Pdx-1-Cre* animals, which spontaneously develop PDA with a proscribed latency after manifesting preinvasive disease. The majority of *LSL-*$Kras^{G12D/+}$*;LSL-*$Trp53^{R172H/+}$*;Pdx-1-Cre* animals develop cachexia and abdominal distension, highly reminiscent of clinical findings seen in the human disease (Figure 2A). From the time of development of abdominal distension, reflecting the accumulation of hemorrhagic ascites, imminent demise is rapid and predictable, typically occurring within 48–72 hr. Upwards of 7 milliliters of ascitic fluid could be recovered from these animals. At necropsy, a large, firm, fibrotic head-of-the-pancreas tumor was almost invariably seen (Figures 2B and 2E), frequently accompanied by signs of biliary and small bowel obstruction (Figures 2B and 2C), reflecting compression and invasion of adjacent structures, respectively, classic features of human pancreatic cancer. The remainder of the pancreas was typically multinodular (Figure 2C). Metastatic foci could be readily appreciated studding the surface of the liver (Figures 2D and 2E), lungs (Figures 2D and 2F), diaphragm, and adrenals (not shown) in many animals; in addition, although not systematically evaluated in all cases, metastatic spread to peripancreatic, mesenteric, and retroperitoneal lymph nodes was noted in several of the mice (Table 1). The specific distribution of metastatic disease also recalls that of human PDA, in which metastases are frequently encountered to the liver (80%), lung (50%–60%), adrenals (20%), and peritoneum (20%–30%) (reviewed in Lillemoe et al., 2000).

In very young *LSL-*$Kras^{G12D/+}$*;LSL-*$Trp53^{R172H/+}$*;Pdx-1-Cre* mice (e.g., 4–6 weeks), the vast majority of the pancreatic parenchyma was histologically normal, characterized by abundant acinar tissue and scattered islets. Early stage PanIN

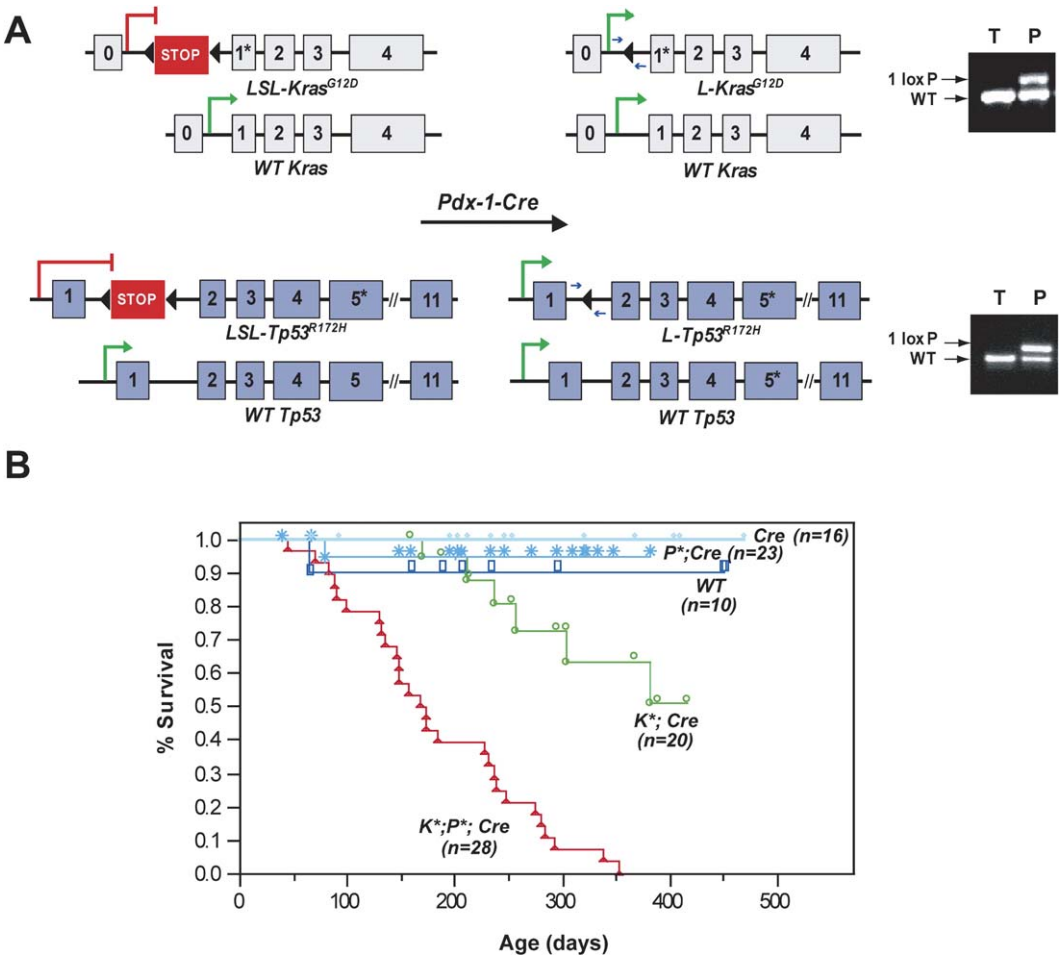

Figure 1. Targeting endogenous *Kras^{G12D}* and *Trp53^{R172H}* expression to the mouse pancreas

A: Endogenous alleles of *Kras^{G12D}* and *Trp53^{R172H}* are conditionally activated in the pancreata of *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* (triple mutant) mice. Specific PCR analysis of genomic DNA from the pancreata, but not the tails, of triple mutant mice reveals the expected "1LoxP" recombination product for each targeted allele.

B: Survival of *LSL-Kras$^{G12D/+}$;LSL- Trp53$^{R172H/+}$;Pdx-1-Cre* mice is significantly decreased. Kaplan-Meier curves reveal a median survival in triple mutant mice of approximately 5 months, significantly less than wild-type (WT), *LSL-Trp53$^{R172H/+}$;Pdx-1-Cre*, and *LSL-Kras$^{G12D/+}$;Pdx-1-Cre* mice ($p < 0.001$, log-rank test, for each pairwise combination).

lesions in these young animals, with demonstrated rearrangement of the mutant alleles, were observed infrequently (data not shown). A similar stage and degree of disease burden occurs in animals expressing only endogenous *Kras^{G12D}* (Hingorani et al., 2003). Thus, invasive tumors did not appear to develop in utero or even in the immediate postnatal period, a latency that suggests the requirement for additional genetic events for disease progression.

A significant disease burden did become apparent in animals by 10 weeks of age at the earliest. Importantly, the full spectrum of preinvasive lesions was apparent in these mice (Figure 2G and data not shown). Histologic analyses of primary pancreatic carcinomas from triple mutant animals revealed a predominant moderately well-differentiated to well-differentiated morphology organized in a glandular architecture (Figure 2H) in the majority of mice (n = 22/25; Table 1), as is observed in the human disease. The carcinomas expressed CK-19 (Figure 2O) and frequently contained mucin, as demonstrated by Alcian blue staining (Figure 2N). Metastases to the liver (Figures 2I and 2J) and lungs (Figures 2K and 2L) were morphologically similar to the pancreatic primaries.

Some tumors also contained minor, poorly differentiated, or undifferentiated components with anaplastic (n = 7/25; Supplemental Figure S1A) or sarcomatoid features (n = 2/25; Supplemental Figures S1B and S1C). Noninvasive cystic papillary neoplasms (n = 3/25; Supplemental Figure S1D) were occasionally noted, as were areas of focal adenosquamous morphology (n = 3/25, Supplemental Figures S1E and S1F), an uncommon form of human pancreatic ductal cancer comprising 3%–4% of reported cases (Kardon et al., 2001). Finally, several animals developed esophageal papillomas (Supplemental Figure S1G) and hyperplasias, or papillomatosis, of the biliary tree (Supplemental Figures S1H and S1I), likely reflecting expression of *Pdx-1* in the developing foregut endoderm.

Table 1. Clinical spectrum of disease in *LSL-Kras$^{G12D/+}$;LSL- Trp53R$^{172H/+}$;Pdx-1-Cre* mice

			Histology							
ID	Age (days)	PDA	1°	2°	Liver	Lung	Diaphragm	Adrenal	Ascites	Other
1	150	Y	G		Y^M	Y^M	Y^m	Y^m	Y	HCC
2	89	N	NA		N	N	N	N	N	TL
3	132	Y	G		Y^M	N	N	Y^m	N	NSCLC
4	137	Y	G		Y^m	Y^M	N	N	Y	NP^m
5	85	*	*		*	*	*	*	*	*
6	92	Y	G	U	Y^M	Y^M	N	N	Y	
7	170	Y	G		Y^M	Y^M	Y^m	Y^m	Y	M^m
8	174	Y	G		N	ND	N	N	N	
9	147	Y	ND		Y^M	Y^M	N	N	N	
10	240	Y	G		Y^M	Y^M	Y^M	N	Y	BP
11	230	Y	S	G	N	N	Y^m	N	N	pLN, NSCLC
12	186	Y	G	AS	Y^M	Y^M	N	N	N	HCC, BP
13	175	Y	G		Y^M	N	Y^M	N	Y	
14	47	Y	CPN		Y^M	N	Y^M	N	Y	Teratocarcinoma
15	340	Y	G		N	N	N	N	N	BP
16	281	Y	G		N	N	N	N	N	
17	295	Y	G	U	Y^M	Y^M	Y^M	Y^m	Y	R^M
18	149	Y	G	AS	Y^M	N	Y^M	N	N	
19	71	Y	G	U, CPN	N	Y^M	N	N	Y	
20	101	Y	G	U, CPN	N	N	N	N	Y	
21	158	Y	G	U	N	N	N	N	N	TL, BP
22	276	Y	G		N	N	Y^M	N	Y	pLN
23	239	Y	G	U	Y^M	N	N	N	N	R^m, pLN, SN
24	133	Y	G		N	N	N	N	Y	
25	355	Y	G	AS	Y^M	Y^M	Y^M	Y^M	Y	R^m, BP
26	286	Y	G	U	Y^M	N	N	N	Y	M^m, R^m
27	232	Y	G		Y^M	Y^M	N	Y^M	Y	pLN, M^m, R^M, mLN, LN^m
28	249	Y	S	G	Y^m	N	N	N	Y	mLN
Totals		26/27			17/27	11/25	10/27	6/27	16/27	
%		96.3			63.0	44	37	22.2	59.3	

^{M}, macrometastasis; ^{m}, micrometastasis; A, adrenal capsule; TL, thymic lymphoma; NP, nervous plexus; M, mesentery; R, renal capsule; pLN, peripancreatic lymph node; mLN, mediastinal LN; ND, not determined; *, tissue not evaluable secondary to necrosis; 1°, predominant pancreatic histology noted (present in >50% of evaluated tissue); 2°, secondary pancreatic histologies noted; G, glandular; U, undifferentiated; S, sarcomatoid; CPN, cystic papillary neoplasm; AS, adenosquamous; HCC, hepatocellular carcinoma; BP, biliary papillomatosis; NSCLC, non-small cell lung cancer; SN, spindle cell neoplasm.

Molecular heterogeneity of tumor progression

To characterize events of potential importance in tumor progression, we began by assessing the expression patterns of proteins in critical signaling pathways. Pancreatic tumorigenesis has been frequently associated with overexpression of members of the *Erbb* family of receptor tyrosine kinases and their associated ligands (Hansel et al., 2003). In particular, increased expression of *Errb1*/*Egfr* and *Errb2*/*Her2* has been noted, and these receptors have even been suggested as potential therapeutic targets for the disease. We surveyed a large number of preinvasive, invasive, and metastatic lesions for expression of these receptors, and also for increased activity of the downstream MAPK effector pathway (Supplemental Figure S2 and Supplemental Table S1). Overall, we observed a surprising degree of heterogeneity in the expression patterns of key signaling components in invasive and metastatic lesions, with less variability across the spectrum of PanIN lesions (Supplemental Figure S2 and not shown). More precisely, we observed discrete types of heterogeneity that spanned stages of progression and states of differentiation, and which even manifested stochastically across individual lesions of similar differentiation within the same tissue. For example, intense focal *Errb1*/*Egfr* expression was seen in some cells of PanINs, but not others, against the backdrop of more diffusely positive PanIN cells (Supplemental Figure S2E). Only very weakly positive expression of *Erbb1*/*Egfr* was observed in primary invasive carcinomas, regardless of differentiation state; finally, metastases uniformly lacked such expression. Thus, *Erbb1*/*Egfr* expression manifested principally progression-stage-dependent heterogeneity. In comparison, *Erbb2*/*Her2* was invariably and intensely expressed in PanIN lesions (Supplemental Figure S2F). Moreover, high levels of expression were maintained, for the most part, in the invasive carcinomas (Supplemental Figure S2J) and the metastases (Supplemental Figures S2N and S2R). Nevertheless, there were discrete regions within both primary tumors (Supplemental Figure S2J) and among distinct metastatic lesions in a given organ that displayed significant heterogeneity. Thus, in some cases, regions of well-differentiated PDA strongly expressed *Erbb2*/*Her2*, while more poorly differentiated areas were less robust, a form of differentiation-state-dependent heterogeneity. However, there were also glandular liver metastases that showed intense levels of expression, while others were completely lacking (Figures 3I–3L), a stochastic form of heterogeneity. Thus, the pattern of *Erbb2*/*Her2* in invasive and metastatic disease was complex and not predictable even within a given animal, consistent with the heterogeneous nature of *Erbb2*/*Her2* expression in human pancreatic cancer (Day et al., 1996). Interestingly, activation of the MAPK pathway, as assessed by phosphorylated Erk (pErk) levels, correlated highly with *Erbb2*/*Her2* expression (Supple-

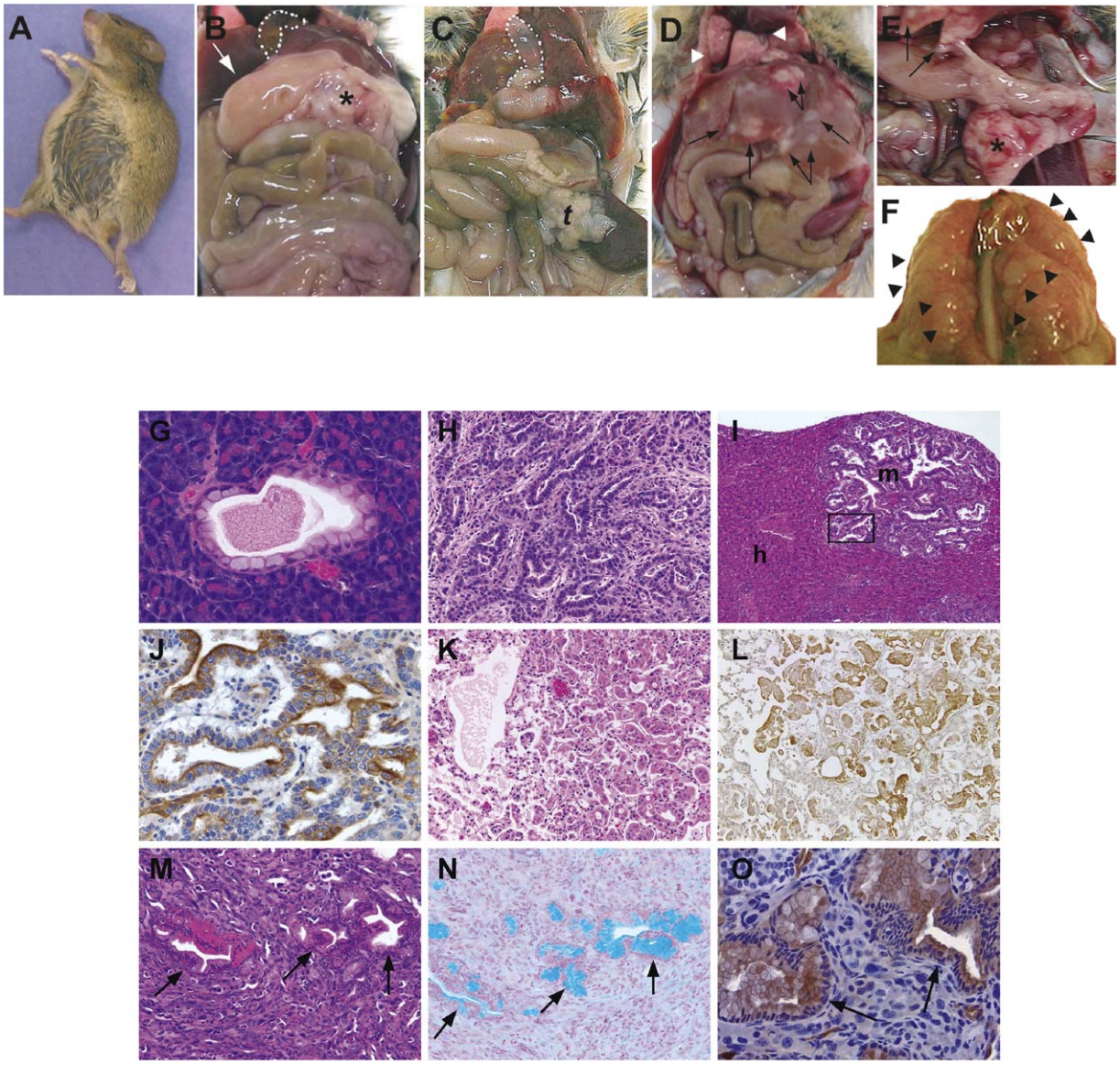

Figure 2. *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* mice succumb to invasive and widely metastatic pancreatic ductal adenocarcinoma (PDA)

A–F: Gross pathological photographs of metastatic PDA in a representative series of triple mutant mice. **A:** Abdominal distension due to the accumulation of malignant ascites was commonly noted. **B:** Primary PDA in the head-of-the-pancreas (asterisk) resulting in the distended loop of the small bowel due to direct invasion and compression of the proximal duodenum (arrow); also note biliary obstruction and gallbladder distension (outlined). **C:** Gallbladder distension due to a pancreatic tumor in the head (unlabeled); diffuse nodularity was noted in the tail (t) of the pancreas. **D and E:** Specimens from same mouse demonstrating multiple gross hepatic (black arrows) and diaphragmatic metastases (white arrowheads) and a large head-of-the-pancreas primary tumor (asterisk). **F:** Multiple pleural metastases (arrowheads).

G–O: Histological features in *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* mice. **G:** PanIN-1A lesion in a 4-month-old triple mutant mouse (400×). **H–J:** Well-differentiated glandular PDA (**H**) (200×) and well-differentiated liver metastasis (**I**) (100×) in a 4.5-month-old animal. The liver metastasis highly expressed the ductal marker, CK-19 (**J**) (400×). **K and L:** 8-month-old mouse with well-differentiated lung metastasis (**K**) (200×) demonstrating CK-19 expression (**L**) (200×). **M–O:** Ductal adenocarcinoma from 3-month-old mouse with both well-differentiated (arrows) and surrounding poorly differentiated areas (**M**) (200×). The abundant mucin content of well-differentiated areas is revealed by Alcian blue staining (**N**) (arrows, 200×), while CK-19 expression confirms their ductal phenotype (**O**) (arrows, 400×).

mental Figures S2G, S2K, S2O, and S2S), but much less highly with *Erbb1/Egfr* (with a few exceptions—see, for example, Supplemental Figures S2J and S2K). To the extent that activation of the MAPK pathway is important for pathogenesis, therefore, *Erbb2/Her2* may represent a better therapeutic target.

Aberrant expression of *Sonic hedgehog (Shh)*, a member of the Hedgehog ligand family, has recently been described in both preinvasive and invasive human PDA and suggested as a novel therapeutic target (Berman et al., 2003; Thayer et al., 2003). Expression of *Shh* is normally inhibited during development to allow proper pancreatogenesis from endodermal structures that are otherwise biased toward intestinal and he-

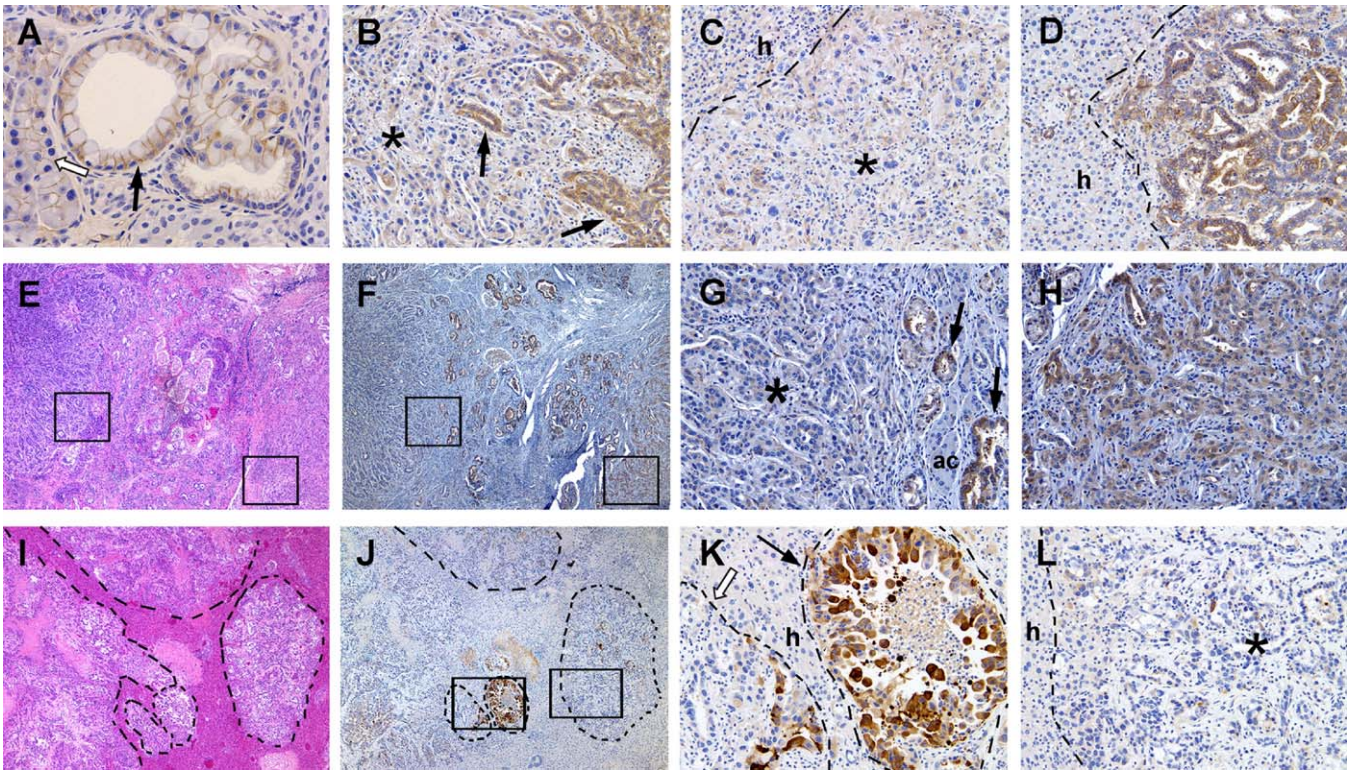

Figure 3. Molecular heterogeneity of signaling pathways

A–D: Heterogeneity in E cadherin expression as a function of degree of differentiation. **A:** Membranous staining of E cadherin in normal acinar cells (white arrow) and PanIN-1A (black arrow, 400×). **B:** 4.5-month-old mouse with intense E cadherin staining in the well-differentiated areas of a primary PDA (arrows), but not in the poorly differentiated areas (asterisk, 200×). **C and D:** Liver metastases from the same mouse as in **B**, demonstrating lack (**C**, asterisk) or presence (outlined in **D**) of E cadherin staining, depending on the degree of differentiation. Normal hepatic parenchyma (h) is also denoted (200×).

E–H: Stochastic heterogeneity demonstrated by Her2 expression in primary PDA from a 4.5-month-old mouse. H&E (**E**) (40×) and immunohistochemical detection of Her2 (**F**) (40×) demonstrate a large, invasive PDA with high levels of Her2 expression in one area (right box, magnified in **H**, 200×) and essentially no expression in another (left box, magnified in **G**, 200×). The latter area shows significant Her2 expression in PanINs (**G**, arrows), but lack of expression in adjacent normal acinar tissue (ac) and the moderately differentiated PDA (asterisk).

I–L: Stochastic heterogeneity of Her2 expression in liver metastases. **I:** H&E (40×) and Her2 immunohistochemistry (**J**) in serial section demonstrates multiple liver metastases (outlined) but only one area of Her2-positive staining (**J**, 40×). Higher magnification of the Her2-positive (arrow, **K**, 200×) and Her2-negative (asterisk, **L**, 200×) liver metastases.

patic fates. We found *Shh* to be aberrantly and highly expressed in both preinvasive (Supplemental Figure S2H) and invasive murine PDA (Supplemental Figure S2L), and at least moderately expressed in metastases (Supplemental Figures S2P and S2T), representing a consistent pattern across disease progression.

Finally, expression of E cadherin, a member of the integrin family of adhesion molecules, also displayed impressive and unexpected variability. Loss of E cadherin expression has been implicated in the epithelial-to-mesenchymal transition (EMT) thought to precede the development of an invasive and metastatic phenotype, perhaps by altering critical interactions in a structured epithelium (Cavallaro and Christofori, 2004). In addition, E cadherin may suppress cellular transformation by sequestering β-catenin and preventing its translocation to the nucleus (Gottardi et al., 2001). As expected, E cadherin expression was demonstrable in normal acini and preinvasive ductal lesions (Figure 3A). In pancreatic ductal tumors, expression was robust in the more differentiated regions and much less so in the more poorly differentiated regions, consistent with a role in disease progression (Figure 3B). This same pattern of expression persisted in metastases, however, where once again robust levels were observed in well-differentiated metastases (Figure 3D), but not in poorly differentiated lesions (Figure 3C) in the same organ. If we consider each metastasis to represent an independent and clonal event, then this example encompasses both stochastic and differentiation-state heterogeneity.

Requirements for disease progression

We wondered whether combinations of additional mutations in critical tumor suppressor gene pathways might explain the striking degree of molecular heterogeneity in the primary tumors and metastases of *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* mice. To assess this possibility directly, we investigated primary cell lines established from ductal cells at various stages of disease progression using methods we described recently (Schreiber et al., 2004). Portions of each resected specimen from wild-type, *LSL-Kras$^{G12D/+}$;Pdx-1-Cre*, and *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* animals were preserved in parallel for histologic analysis. The recovered cells were passaged a total of four to five times on discrete matrices to favor the growth of ductal elements over other cellular components

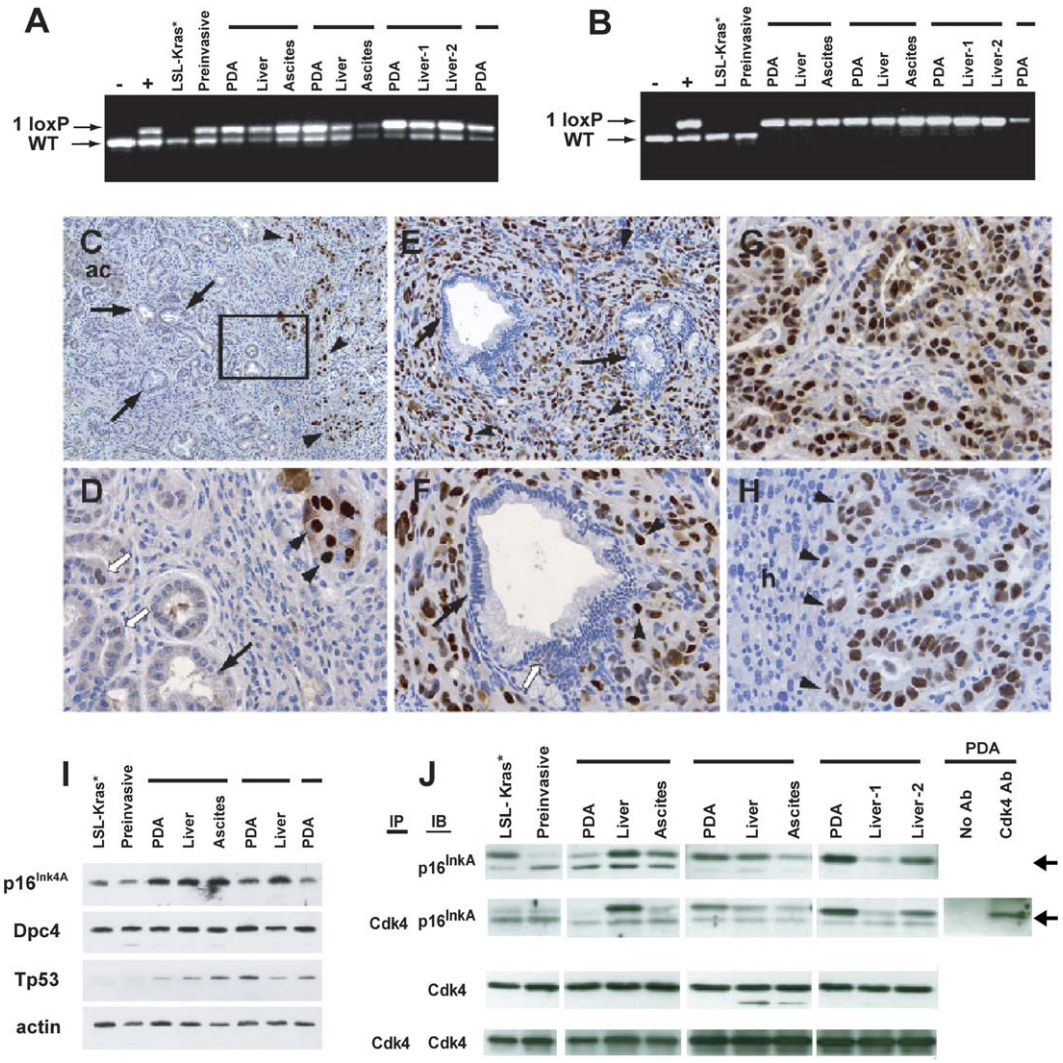

Figure 4. Uniform loss of wild-type *Trp53* and lack of mutations in additional tumor suppressor genes in primary PDAs and metastases

A: Activated mutant (1 LoxP, upper band) and wild-type (lower band) *Kras* alleles are present in early passage cell lines prepared from matched sets of primary carcinomas and metastases. *LSL-Kras* ductal cells were prepared from *LSL-Kras*$^{G12D/+}$ animal. Preinvasive cell line was prepared from *LSL-Kras*$^{G12D/+}$; *Pdx-1-Cre* animal. Control DNA was prepared from MEFs that had (+) or had not (−) recombined the conditional allele.

B: Wild-type *Trp53* allele is consistently lost (lower band) in all primary tumors and metastases.

C–H: Immunohistochemical assessment of Trp53 levels in tumors from triple mutant mice. **C and D:** p53 expression in PanIN lesions is absent (arrow) or weakly positive (open arrow), whereas intense expression of Trp53 (arrowheads) is noted in the adjacent invasive component of PDA (**C**, 100×) (**D**, 400×). **E and F:** Absent (arrow) or weak (open arrow) expression of Trp53 in PanINs, but intense expression (arrowheads) in invasive PDA cells (**E**, 200×) (**F**, 400×). **G and H:** Intense expression (arrowheads) of Trp53 in a primary PDA (**G**, 400×) and liver metastasis (**H**, 400×) from the same animal.

I: Expression of critical tumor suppressors in a representative panel of control, preinvasive, invasive, and metastatic cell lines. Note that Trp53 levels are highest in cell lines that have lost the wild-type allele.

J: Functional interaction of p16^{Ink4a} and Cdk4 in control, preinvasive, invasive, and metastatic cell lines. Top panel: p16^{Ink4a} levels in the lysates of cell lines (arrow denotes the migration of p16^{Ink4a}). Second panel: Cdk4 immunoprecipitation followed by p16 immunoblotting demonstrates coimmunoprecipitation of the two proteins. Third panel: Cdk4 levels in lysates. Bottom panel: Cdk4 levels in the Cdk4 immunoprecipitates. Bars over adjacent lanes indicate samples prepared from the same animal.

of the pancreas and stroma. These procedures result in essentially uniform populations of ductal or tumor cells as assessed by light microscopy (Supplemental Figures S3A–3E) and biochemical assays (Schreiber et al., 2004). We note that ductal cells from *LSL-Kras*$^{G12D/+}$*;Pdx-1-Cre* mice contain a mixture of cells at different PanIN stages; it is also formally possible that a rare "invasive" cell may be copurified. However, by a number of criteria detailed below, *LSL-Kras*$^{G12D/+}$*;Pdx-1-Cre* ductal cells clearly demonstrated properties reflecting an earlier stage of progression than their invasive counterparts. Thus, for simplicity, we refer to these cells interchangeably as *LSL-Kras*$^{G12D/+}$*; Pdx-1-Cre* or preinvasive ductal cells.

We first established the genetic status of the *Kras* and *Trp53* loci in a panel of primary control, preinvasive, and tumor cell lines. Rearrangement, or activation, of the *LSL-Kras*^{G12D} allele was seen in preinvasive and tumor cell lines, as expected, to-

gether with the presence of the wild-type *Kras* allele (Figure 4A). Resected pancreatic tissue from a 6-week-old *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* mouse demonstrated activation of the *Trp53^{R172H}* allele along with retention of the wild-type *Trp53* gene (not shown). Each one of the primary pancreatic tumor cell lines, as well as all of the metastatic cell lines, similarly revealed the presence of the activated point mutant *Trp53* allele; however, in every instance, the wild-type allele was lost (Figure 4B). This combination of point mutation and loss of heterozygosity (LOH) of *Trp53* is also the principal way in which mutations in this TSG manifest themselves in human pancreatic cancer (Rozenblum et al., 1997; Scarpa et al., 1993). Thus, it would appear that LOH is a requisite step in tumor progression in the setting of *Trp53^{R172H}*. Indeed, the immunohistochemical assessment of Trp53 expression in vivo provides further evidence of LOH as a key step in progression. In normal cells, steady-state levels of the Trp53 tumor suppressor lie below the detection limits of conventional immunoblot and immunohistochemical methods. The specific point mutations associated with tumorigenesis, however, confer increased stability on the protein and enable detection. In animals with germline transmission of the *Trp53^{R172H}* allele, for example, normal tissues retain the wild-type allele, and Trp53 is not detected immunohistochemically; however, in the tumors that developed in these animals with demonstrated LOH, Trp53 expression was readily detectable (Olive et al., 2004). Thus, the absence of detectable Trp53 expression in PanIN lesions adjacent to invasive carcinomas (Figures 4C–4F) suggests retention of the wild-type allele and, therefore, a largely intact rate of protein degradation. Occasional PanINs do show moderate, or intermediate, levels of detectable Trp53, potentially suggesting early loss of the wild-type allele or activation of additional mechanisms of p53 stabilization (Figure 4F). Intense expression of Trp53 is seen only in the invasive cancers and metastases, consistent with LOH at the wild-type locus (Figures 4C–4H).

As a first step toward evaluating the integrity of other key tumor suppressor pathways, we performed immunoblot analyses of whole-cell lysates on a representative sampling of control, preinvasive, and tumor cell lines. As expected, Trp53 expression was not discernible in either wild-type or preinvasive *LSL-Kras$^{G12D/+}$;Pdx-1-Cre* ductal cells; demonstrable levels were seen in the primary and metastatic tumor cell lines from *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* animals, consistent with expression of the activated point mutant allele (Figure 4I). Smad4 expression, an integral component of the TGFβ pathway, was uniformly present. Somewhat to our surprise, we found that all of the cancer cell lines also retained expression of the p16/Ink4a tumor suppressor (Figure 4I) and the p19/Arf tumor suppressor (not shown). Expression of other proteins of potential importance in pancreatic cancer, including Rb, Akt, and Myc, was similarly intact and not significantly different among the various cell lines analyzed (Supplemental Figure S4).

The *Cdkn2/Ink4a* pathway can be inactivated by homozygous deletion of the locus, by monoallelic deletion coupled with an inactivating point mutation in the remaining allele, or, less frequently, by promoter methylation resulting in epigenetic silencing (Caldas et al., 1994; Schutte et al., 1997). In addition, specific point mutation of its cellular target, Cdk4, can inhibit the interaction between these two proteins. Thus, the functional integrity of the *Cdkn2/Ink4a* axis can be demonstrated by the ability of the tumor suppressor to bind Cdk4, which correlates with inhibition of its kinase activity. Protein lysates from primary ductal cells, preinvasive cells and tumor cells contained comparable amounts of Cdk4 and variable, though always detectable, levels of Cdkn2/Ink4a (Figure 4J). Importantly, the binding between Cdkn2/Ink4a and Cdk4 was always retained, as manifested by the ability to coimmunoprecipitate the two proteins.

The integrity of the TGFβ-Smad4 tumor suppressor pathway in these cell lines, from surface receptor binding of ligand to activation and nuclear translocation of intracellular Smad2/3/4 complexes, was assessed by following the intracellular localization of Smad4 before and after treatment with TGFβ. In preinvasive, primary carcinoma, and metastatic cells, diffusely distributed Smad4 was efficiently translocated to the nucleus in response to TGFβ, demonstrating that the pathway was intact (Supplemental Figure S5).

As a final method of interrogating the fidelity of these critical pathways, we directly sequenced the open reading frames for *Cdkn2/Ink4a*, *Cdk4,* and *Smad4*. Several steps were taken to ensure faithful rendering of the sequences and to minimize the introduction of artifacts. First, cDNA was reverse-transcribed from total RNA preparations of the various cell lines and then amplified by PCR. Single bands of the correct molecular weight were recovered in all circumstances. The isolated cDNAs were then subcloned and eight independent clones for each cell line were sequenced bidirectionally. A mutation was considered significant only if: (1) it was detected in both strands of a given clone (to obviate potential sequencing artifacts) and (2) the same mutation appeared in more than one of eight clones (i.e., $\geq$25%), to avoid potential errors introduced by PCR. Very few mutations of any kind were discovered by this method and, by the exclusion criteria noted, no reproducible mutations were found in *Cdkn2/Ink4a*, *Cdk4,* or *Smad4*. As a further test of the methodology, we isolated and sequenced 5 subclones each for both *Kras2* and *Trp53* from five tumor and two control cell lines. In all cases, at least two or three of the sequenced clones from each tumor cell line revealed the presence of the *Kras^{G12D}* point mutant allele, while the remaining clones contained the corresponding wild-type alleles. In contrast, every clone from each of the five tumor cell lines showed only the presence of the *Trp53^{R172H}* mutation and no wild-type *Trp53* sequence, consistent with LOH. Wild-type cells contained only wild-type alleles for both genes in all clones, while in preinvasive ductal cells from *LSL-Kras$^{G12D/+}$;Pdx-1-Cre* animals, we detected both mutant and wild-type *Kras* alleles, but only wild-type *Trp53*, as expected. Thus, LOH in the setting of *Trp53^{R172H}* was an invariant event in pancreatic tumor progression and, by several different methodologies, no mutations in the other principal tumor suppressor gene pathways were apparent.

Genomic instability

A pathognomonic feature of human PDA is its high degree of genomic, principally chromosomal, instability. Chromosomal instability (CIN), manifested by simple aneuploidy or by complex structural abnormalities, would provide a ready explanation for the molecular heterogeneity observed in the primary carcinomas and metastases noted above. Wholesale gains or losses of chromosomes are associated with centrosomal amplification and subsequent chromosomal missegregation, known sequelae of *Trp53* deficiency (Tarapore and Fukasawa, 2002). We therefore first examined isolated tumor cell lines for

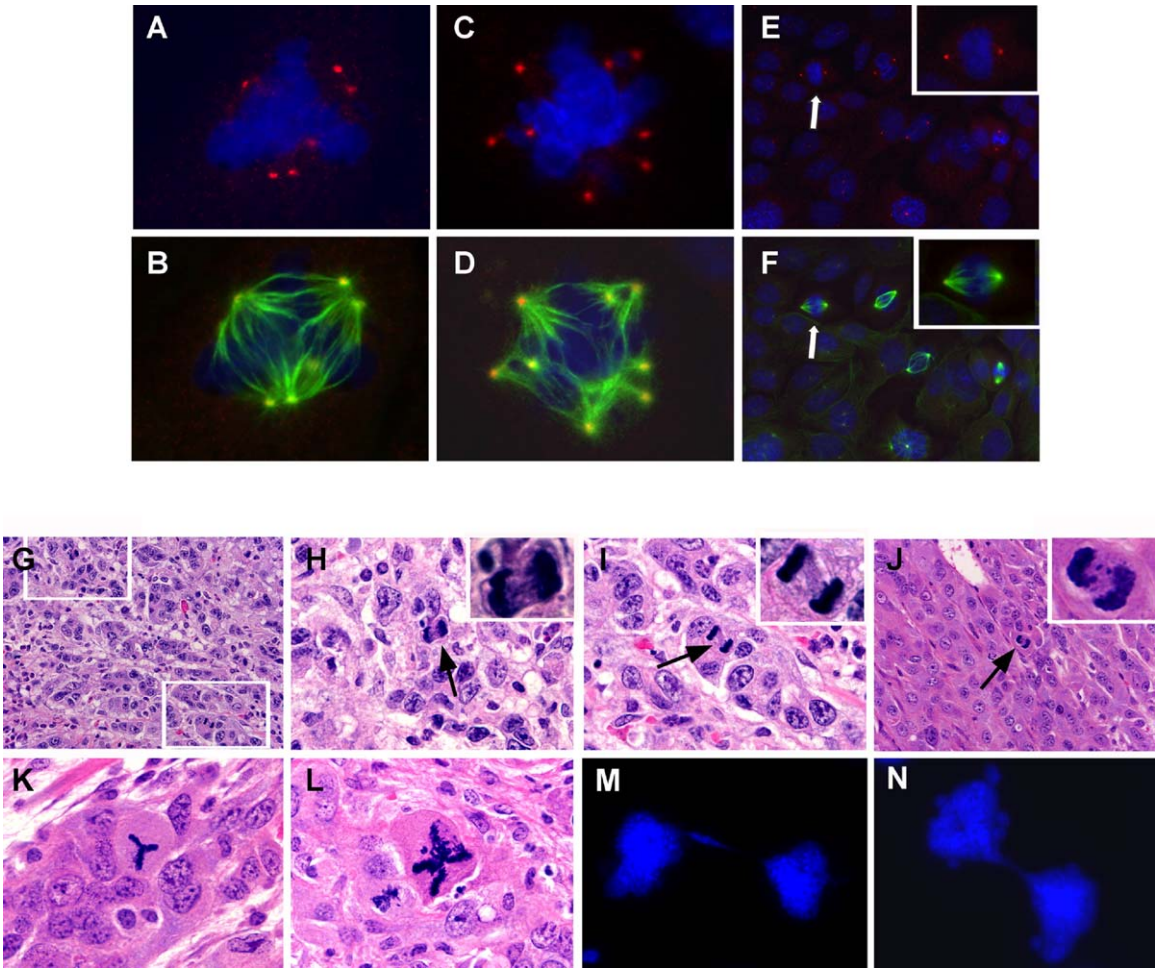

Figure 5. Chromosomal instability in *Pdx-1-Cre;LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$* mice

A–F: Centrosome amplification in pancreatic carcinoma cell lines. Pancreatic cell lines prepared from primary PDA (**A** and **B**, 60×) and liver metastases (**C** and **D**, 60×) of triple mutant mice frequently contain greater than two centrosomes as demonstrated by γ-tubulin staining (**A** and **C** red dots), whereas pancreatic cell lines prepared from *Pdx-1-Cre;LSL-Kras$^{G12D/+}$* mice have a normal number of centrosomes (**E** and **F** 40×). α-tubulin staining (green, **B**, **D**, and **F**) reveals the abnormal mitotic spindles in carcinoma cell lines in comparison to the normal spindles of *LSL- Kras$^{G12D/+}$;Pdx-1-Cre;*cells. Cells were also stained with DAPI (blue).

G–I: Anaphase bridges are common in triple mutant animals. **G:** Abundant anaphase figures in murine PDA (boxes, 40×) demonstrate classic bridges (arrows) (**H** and **I**, 400×).

J: Abnormal anaphase that does not meet strict criteria for designation as anaphase bridge (400×).

K and L: Abnormal mitotic figures in tumor sections (400×).

M and N: Nuclear bridges in primary cell lines prepared from two separate PDAs (400×).

increased numbers of centrosomes. Centrosome amplification, sometimes involving as many as 30 identifiable centrosomes per cell, was uniformly observed in primary carcinoma and metastatic cell lines (Figures 5A and 5B). On average, at least one or two cells with increased centrosomes were observed per high-power field. *LSL-Kras$^{G12D/+}$;Pdx-1-Cre* cells, however, only rarely contained more than one or two centrosomes (Figure 5C).

The existence of CIN in vivo can be inferred from the presence of anaphase bridges (AB), which presumably occur when di- or multicentric chromosomes attempt to enter anaphase and are trapped between opposing forces from the two spindle poles. The inevitable resulting break in the chromatin sets the stage for another end-to-end fusion event. Such repeated breakage-fusion-bridge cycles are thought to represent the primary driving force behind the large-scale CIN associated with human epithelial carcinogenesis (Lengauer et al., 1997; Lengauer et al., 1998). We found a large number of AB (Figures 5G–5I) and highly abnormal mitotic figures (Figures 5K and 5L) in our survey of primary pancreatic carcinomas, and even occasionally noted more than one AB in a single low-power field (Figure 5G). In a total of 171 anaphase figures evaluated (5–30 per tumor) from 13 primary tumors, 67 (2–12 per tumor) met strict criteria for anaphase bridges, revealing an overall anaphase bridge index of 39% (range 20%–40%). This value is comparable to that seen in human PDA (17%) (Montgomery et al., 2003) and implies a very high degree of chromosomal instability.

Finally, direct assessments of chromosomal structure as a function of disease evolution were performed. Immunofluores-

Table 2. Cytogenetic analyses of primary control, preinvasive, invasive, and metastatic ductal cell lines

Cell line ID[a]	Description	n	% diploid	Chromosomal aberrations	Chromatid breaks	Chromosome fragments	Chromosome fusions	p:p fusions	q:q fusions (dicentrics)	Markers[b]	Clonal abnormalities[c]
A	normal PDC	36	55.6	8.3	2.8	5.6	2.8	0	2.8	0	ND
B	Preinvasive	38	65.8	18.4	2.6	13	2.6	2.6	0	0	2–4 copies all chromosomes
C	PDA	33	3	97	24	100	24.2	12	15	3	5–6 copies chrom 6, 3–5 copies Y
D	PDA	30	0	100	13.3	96.7	96.7	0	0	96.7	t(1;11;10;1), t(4;1), chrom 4 & 15 fragments
E	PDA	35	37.1	25.7	8.6	17.1	8.6	5.7	2.9	0	ND
F	PDA	31	0	96.8	12.9	90.3	32.2	29	0	9.7	ND
G	PDA	31	6.4	93.5	9.7	90.3	9.7	6.4	0	3.2	ND
H	PDA	31	3.2	96.8	22.6	96.8	32.2	16.1	9.7	0	ND
I-1	PDA	30	0	100	0	93	90	20	0	86.7	t(15;15) x 2; t(12;X), t(8;2); 6–12 copies of chromosomes 2,6,7,12,15
I-2	Liver metastasis	30	0	100	7	100	53.3	13	3	33.3	2-5 copies all chromosomes
J-1	PDA	33	30.3	30.3	3	21.2	9.1	3.03	3.03	3.03	ND
J-2	Liver metastasis	33	0	100	39.4	90.9	42.4	18.2	12.1	18.2	6–10 copies all chromosomes
J-3	Ascites	33	30.3	60.6	24.2	48.5	33.3	18.2	9.1	12.1	3–6 copies chromosome 6
K-1	PDA	33	30.3	69.7	6.1	63.6	24.2	9.1	0	18.2	t(7;17), 6 copies chromosome 2; 5–13 copies of chromosome 13
K-2	Liver metastasis	36	36.1	36.1	2.8	16.7	25	13.9	13.9	5.6	3–12 copies all chromosomes
K-3	Liver metastasis	35	42.9	31.4	8.6	31.4	20	17.1	2.9	2.9	ND

[a] Cell lines with a shared letter identification were isolated from the same animal.
[b] Percentage of metaphases that contain a marker chromosome(s), the result of a nonreciprocal translocation.
[c] Clonal abnormalities assessed by SKY refer to gain of chromosomes in at least 2 metaphases or loss of specific chromosomes in at least 3 metaphases (out of 10 examined).

cence of nuclei in isolated tumor cell lines revealed the presence of nuclear bridges (Figures 5M and 5N), the presumptive correlate of anaphase bridges seen by light microscopy. The banding patterns of metaphase chromosomes from a large series of preinvasive and carcinoma cell lines were subsequently analyzed. Each of the malignant cell lines contained significant chromosomal aberrations and fusion events and, importantly, the vast majority (n = 12/14) contained nonreciprocal translocations (NRT), a hallmark of CIN (Table 2). In addition, these cells were markedly aneuploid. In comparison, the majority of *LSL-Kras$^{G12D/+}$* and *LSL-Kras$^{G12D/+}$;Pdx-1-Cre* ductal cells were diploid with relatively few chromosomal aberrations.

To determine if particular translocations predominated in the carcinomas and metastases, we performed spectral karyotyping on a number of primary cell lines from pancreatic carcinomas and associated metastases (Table 2 and Figure 6). Clonal NRTs were discovered in 3 of the 8 carcinoma cell lines examined, and chromosome 6, which notably contains *Kras*, was overrepresented in 6 of these 8 lines. Finally, the spectral karyotypes of a number of metastases revealed completely distinct genomic abnormalities when compared with their parent primary pancreatic cancers, consistent with dissemination of metastatic cells early in disease evolution.

The laboratory mouse is relatively resistant to the development of CIN and, in particular, structural abnormalities such as inversions, deletions, and nonreciprocal translocations. Murine chromosomes are distinguished by inordinately long telomeres as compared with their human counterparts. Indeed, this difference has been thought to preclude the development of sufficient telomere attrition to drive genomic instability, and has therefore been offered as an explanation for the propensity toward hematologic and mesenchymal malignancies in mice at the expense of the more karyotypically complex epithelial tumors common in humans (Rangarajan and Weinberg, 2003). The high degree of CIN observed in *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* mice therefore prompted us to examine whether telomere erosion had somehow contributed. We found that the chromosomes from these animals contained intense telomeric signals by PNA-FISH, suggesting the presence of significant telomeric sequence (Figures 5O–5Q). Moreover, the chromosomal fusion points also contained significant telomeric signals, demonstrating that these aberrant events can occur in the setting of preserved telomeric length.

Discussion

Understanding the genetic requirements for the initiation and progression of pancreatic ductal adenocarcinoma is a necessary first step toward the development of specific detection

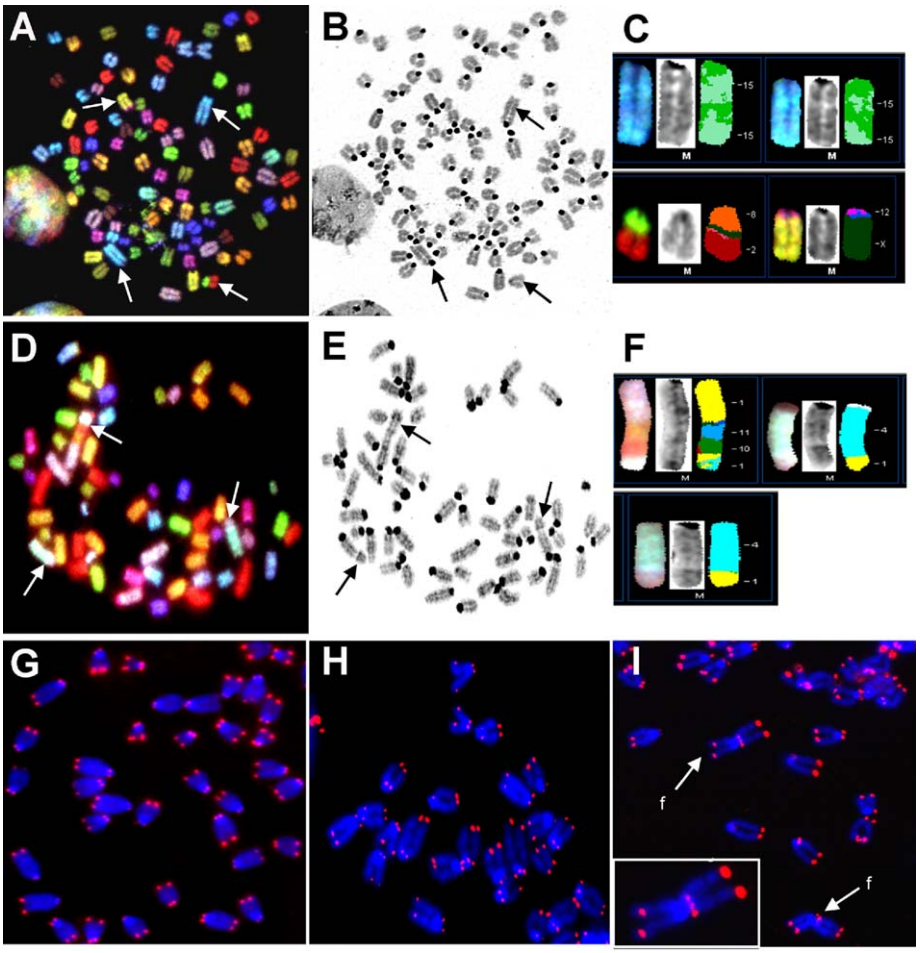

Figure 6. Nonreciprocal translocations and preserved telomeres in pancreatic cancer cell lines from *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* mice

A–C: SKY analysis (**A** and **C**) of PDA cell line I-1 demonstrates four clonal nonreciprocal translocations (NRTs), including two different NRTs of chromosome 15 (t[15;15] "long" and t[15;15] "short") as well as t(X;12) and t(8;2). Geimsa stain of the metaphase spread (**B**) demonstrates the marker chromosomes (arrows).

D–F: Spectral karyotype analysis (SKY) (**D** and **F**) of ductal carcinoma cell line demonstrates two recurrent nonreciprocal translocations, t(1;11;10;1) and t(4;11) (arrows) (cell line D in Table 2). The corresponding Giemsa image (**E**) for **D** is also shown, demonstrating the marker chromosomes (arrows).

G–I: Preserved telomeric sequence at chromosomal ends and fusion points in carcinoma cells. **G** and **H:** Normal metaphase spreads and PNA-FISH of telomeres typical of *LSL-Kras$^{G12D/+}$* primary pancreatic ductal cells (**G**) and *LSL-Kras$^{G12D/+}$;Pdx-1-Cre* preinvasive ductal cells (**H**). **I:** Preserved telomeres and strong telomeric signal at the sites of p-p arm fusions (f) in an invasive carcinoma cell line of a triple mutant mouse.

and therapeutic modalities. The assembled compendium of molecular changes that appear to chronicle human pancreatic tumorigenesis has provided important clues to mechanisms of disease pathogenesis. As part of a systematic effort to elucidate the requisite elements for disease progression and their biologic consequences, we have genetically engineered specific mutations suggested by these data into the endogenous loci of the mouse genome. We have shown here that targeted physiologic expression of point mutant *Trp53* promotes the development of invasive and widely metastatic PDA from preinvasive disease initiated by *Kras^{G12D}*. The resultant disease faithfully recapitulates the clinical syndrome, histopathology, and metastatic profile of the cognate human condition. Disease progression from preinvasive to invasive disease involved loss of the wild-type *Trp53* allele, a nearly invariant feature of human pancreatic cancers that harbor point mutations in this gene. The primary and metastatic lesions seen in these animals manifested a high degree of chromosomal instability, another genetic hallmark of the human disease. Moreover, the development of nonreciprocal translocations and other chromosomal aberrations occurred in the presence of preserved telomere sequence, suggesting that *Trp53^{R172H}* and *Kras^{G12D}* expression promotes such events independently of telomere erosion. Finally, no mutations or functional defects in either the p16^{Ink4a} or TGFβ-Smad4 tumor suppressor pathways were discovered in primary carcinoma or metastatic cells, suggesting that concomitant endogenous *Trp53^{R172H}* and *Kras^{G12D}* expression defines a unique genetic pathway to invasive and metastatic pancreatic ductal adenocarcinoma.

Distinct genetic pathways to pancreatic ductal adenocarcinoma

We have previously shown that endogenous expression of *Kras^{G12D}* is sufficient to initiate pancreatic tumorigenesis (Hingorani et al., 2003). Concomitant expression of *Kras^{G12D}* with *Trp53^{R172H}* or with biallelic deficiency of *Ink4a/Arf* leads to profoundly distinct phenotypes, including latency to tumor progression, survival, presence of macrometastatic disease, and histological morphology. *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* animals have a median survival of approximately 5 months, manifest widely metastatic pancreatic ductal adenocarcinoma that recapitulates the human spectrum, and show predominantly glandular histology, consistent with their designation as adenocarcinomas. By comparison, *LSL-Kras$^{G12D/+}$;Ink4a/Arf$^{(flox/flox)}$;Pdx-1-Cre* animals (Aguirre et al., 2003) have a greatly curtailed median survival of approximately 8 weeks, develop primarily locally invasive disease, and show a prominent spindle cell or sarcomatoid histology, a rare phenotype in human pancreatic cancer (albeit evaluated in a limited number of specimens in direct comparisons [R.H.H. et al., unpublished

data]). In either experimental system, additional mutations in the respective remaining critical tumor suppressor pathways were not observed, suggesting that mutation of either *Trp53* or the combined *Ink4a/Arf* loci is sufficient to induce invasive cancer in the setting of endogenous $Kras^{G12D}$ expression.

LOH at the wild-type *Trp53* locus was an invariant event in the primary and metastatic tumors seen in *LSL-*$Kras^{G12D/+}$*;LSL-*$Trp53^{R172H/+}$*;Pdx-1-Cre* animals, as was the development of widespread chromosomal instability. Thus, a model for tumor progression involving initiation by $Kras^{G12D}$ and progression through acquisition of $Trp53^{R172H}$ point mutation, followed by induction of chromosomal instability and LOH, is suggested. The development of mutations in the *Trp53* pathway may greatly favor the elaboration of metastatic disease, while concomitant *Ink4a/Arf* deletion instead promotes aggressive, locally invasive tumors and infrequent microscopic metastases to the liver. It may be that the rapidity of tumor growth and lethality in the latter setting precludes sufficient time for overt metastases to develop, and that expressing $Kras^{G12D}$ in mice heterozygous for one or both of the *Ink4a/Arf* tumor suppressor loci would sufficiently slow disease progression to allow for the elaboration of macroscopic metastases. We note, however, that of the three *LSL-*$Kras^{G12D/+}$*;LSL-*$Trp53^{R172H/+}$*;Pdx-1-Cre* animals that succumbed within a comparable time frame to *LSL-*$Kras^{G12D/+}$*;Ink4a/*$Arf^{(flox/flox)}$*;Pdx-1-Cre* mice (i.e., less than 11 weeks), all developed grossly evident metastases. Thus, there may indeed be distinct biological sequelae to these two genetic routes of tumor progression.

It is worthwhile to note that the available information on genetic events in human pancreatic cancer has been gleaned almost entirely from resectable carcinomas in patients without obvious metastatic disease (Hahn et al., 1995; Schutte et al., 1997). Thus, the nearly universal finding of *CDKN2A/INK4A* inactivation, for example, in human pancreatic cancer may be skewed by the study of a population presenting only with locally invasive disease, and may not accurately reflect the mutational spectrum occurring in the majority of patients who instead present with metastatic disease. Moreover, many of these studies have relied on xenografts propagated in nude mice. Thus, the available information may not entirely reflect the natural genetic progression of pancreatic cancer.

There are some data from resected human pancreatic ductal adenocarcinomas that nevertheless suggest the existence of distinct genetic trajectories to invasive disease. In a study of signature genetic mutations in 42 human pancreatic ductal adenocarcinomas, all the tumors harbored activating *KRAS* mutations, consistent with their role as initiating events (Rozenblum et al., 1997). Only one-third of the carcinomas, however, contained mutations in all three of the tumor suppressor genes (*CDKN2A/INK4A, TP53,* and *SMAD4*) most frequently implicated in human pancreatic cancer. An additional one-third of the cancers contained mutations in two of the three TSG, while 15% of tumors harbored mutations in only one TSG. Interestingly, in only two of the tumors were mutations restricted to *KRAS* and *CDKN2A/INK4A*, while four were found to have mutations in *KRAS* and *TP53* only. Thus, among tumors harboring only one TSG mutation in the context of oncogenic *KRAS*, pairing with a *TP53* mutation was at least as common as, if not more common than, paired inactivation of *CDKN2A/INK4A*. Finally, in three of the tumors fully evaluated for mutations at all three TSG loci in addition to *KRAS*, only an activating mutation in the latter was found. Thus, for these tumors, as yet undiscovered genetic events must exist that underlie disease progression. No particular associations between biological behavior, metastatic potential or survival, and mutational composition could be delineated, although the sample sizes of each of the respective combinations may have been insufficient to reveal such relationships. We have previously described the spontaneous development of invasive and metastatic pancreatic cancers in two of a cohort of 29 mice engineered to express endogenous $Kras^{G12D}$ alone (Hingorani et al., 2003); the majority of these animals have now succumbed to pancreatic ductal adenocarcinoma, with a median survival of approximately 15 months (S.R.H. and D.A.T., unpublished observations). It will be of interest to determine whether there are preferred genetic routes to PDA in vivo and if particular genetic profiles correspond to definable biological behaviors.

Point mutant *TP53* and gain of function

The TP53 tumor suppressor functions as a homotetrameric complex that transactivates key target genes in response to a variety of cellular insults, resulting in cell cycle arrest or apoptosis (Kastan et al., 1991; Kern et al., 1991; Prives, 1998). The importance of *TP53* in oncogenesis was initially discerned from the discovery of point mutant forms of the gene. Indeed, because selectively mutated and highly expressed forms of the protein were frequently associated with malignancy, *TP53* was originally thought to be a proto-oncogene that could cooperate with other oncogenes, notably mutant *RAS*, to induce transformation (Eliyahu et al., 1984; Parada et al., 1984). Subsequent findings led to the suggestion of TP53 as a conventional tumor suppressor (Finlay et al., 1989; Hinds et al., 1989), and point mutant forms are now thought to act as dominant negative components in heterotetramers of wild-type and mutant proteins. The $TP53^{R175H}$ mutation ($Trp53^{R172H}$ in mice) results in a partially denatured structure that impairs DNA binding (Cho et al., 1994).

It remains formally possible that point mutant forms of the protein actually exhibit a gain of function. According to this supposition, point mutant Trp53 combined with LOH would not be functionally equivalent to the homozygous null state. One postulated mechanism for gain of function of point mutant Trp53 involves inhibition, through direct binding, of the p53 family members Trp63 and Trp73 (Di Como et al., 1999; Gaiddon et al., 2001). It is interesting to note in this context that contrary to virtually all other TSG loci, mutations in *TP53* associated with human malignancies involve not homozygous loss of the wild-type genes, but rather point mutation of one allele followed by LOH at the remaining locus. Heritable cancer predispositions, such as the Li-Fraumeni syndrome (LFS) involving "hot spot" mutations in *TP53*, further substantiate these functional differences (Birch et al., 2001; Kleihues et al., 1997). Not all such hot spot mutations are phenotypically equivalent, nor are they necessarily equivalent to the homozygous null state, as different inherited alleles generate somewhat different tumor spectra (Olivier et al., 2003). Important differences between point mutant and homozygous null *Trp53* are also manifest in mice. $Trp53^{-/-}$ mice develop lymphomas and sarcomas almost exclusively, and succumb between 4–6 months of age (Donehower et al., 1992; Jacks et al., 1994). In comparison, carcinomas frequently develop in mice with germline expression of point mutant forms of *Trp53*, recapitulating the phenotype seen

in LFS (Olive et al., 2004). Finally, our findings of complex structural CIN in the setting of point mutant *Trp53*, in addition to the more simple numerical instability seen in a $Trp53^{-/-}$ background, suggest a potential molecular basis for the observed phenotypic gain of function (although formally establishing this conclusion awaits direct comparison of animals expressing $Kras^{G12D}$ in the context of point mutant or homozygous null *Trp53*). Thus, we can tentatively propose the following mechanism by which *Kras* and "oncogenic" *Trp53* cooperate to induce tumorigenesis, as initially suggested by some of the earliest studies of these genes. *Kras* initiates tumorigenesis by expanding a pool of progenitor cells (Hingorani et al., 2003) which in the context of point mutant $Trp53^{R172H}$ first acquire LOH at the wild-type *Trp53* locus. Indeed, the presence of the point mutant allele of *Trp53* may itself potentiate the chromosomal instability required to develop LOH. LOH of the wild-type locus then allows for the unfettered elaboration of chromosomal instability, driving tumor progression through combinations of widespread translocations, amplifications, and deletions as shaped ultimately by in vivo selection pressures. In this scenario, *Trp53* plays a "caretaker" role in addition to its usual "gatekeeper" function in cellular and genetic homeostasis.

Epithelial tumorigenesis in mice and men

The role of genomic instability in tumor progression remains a subject of intense inquiry and some controversy, with evidence and opinion divided between essential (Lengauer et al., 1997; Lengauer et al., 1998) and accessory, or even coincident, roles (Sieber et al., 2003). On balance, the consensus opinion posits that widespread CIN generates the genetic repertoire necessary to subvert critical restraints on unregulated growth. Telomere attrition is thought to drive this genomic instability, leading to a state of global chromosomal aberrations, or "crisis," which serves as the final selection barrier through which an emerging malignant cell must pass (Harley et al., 1994). Seen in this light, the lack of epithelial carcinomas in genetically engineered mice arguably results from their inordinately long telomeres, which in *Mus musculus* average 40–60 kilobases (kb), in contrast to the more modest 10 kb of human chromosomes (Rangarajan and Weinberg, 2003). Indeed, $mTert^{-/-}$ animals bred for several generations to achieve critically short telomeres do develop carcinomas, the occurrence of which is hastened in a $Trp53^{+/-}$ background (Artandi et al., 2000). We have shown here that locally invasive and widely metastatic pancreatic ductal adenocarcinoma can be modeled in the experimental mouse without the direct manipulation of genes involved in telomere length or structure. Further, these carcinomas possess complex karyotypes, manifested by chromosomal breaks, fragments, and fusions. Intriguingly, significant telomeric sequence was observed at sites of chromosomal fusions, potentially implicating other mechanisms such as those involved in uncapping telomeres or otherwise disrupting telomere integrity (de Lange, 2002; Smogorzewska et al., 2002).

In summary, we have shown here that the initiation of pancreatic tumorigenesis by endogenous $Kras^{G12D}$ expression in the context of $Trp53^{R172H}$ greatly hastens the development of locally invasive and widely metastatic pancreatic ductal adenocarcinoma that faithfully recapitulates all of the extant features of the human disease. In this model, neither telomere attrition nor mutation of other major tumor suppressor gene pathways is required. LOH at the wild-type *Trp53* locus is uniformly seen, along with widespread chromosomal instability, suggesting a requirement for other, as yet undiscovered, genetic events. Thus, it appears that at least for the *Trp53* and combined *Ink4a/Arf* TSG pathways, abrogation of either in the context of oncogenic *Kras* is sufficient to induce invasive PDA, albeit with distinct biological phenotypes. The same may be true for the remaining principal tumor suppressor pathway implicated in pancreatic cancer, namely *TGFβ-Smad4*. Whether various combinations of mutations confer additional survival advantages or are merely incidental events remains to be determined. Finally, it will be of interest to perform similar molecular and genetic analyses on the preinvasive lesions and spontaneous tumors that arise in the context of $Kras^{G12D}$ expression alone (Hingorani et al., 2003) to determine which pathways are evolutionarily favored for tumor progression, and at what point significant CIN manifests. The answers to these questions will likely influence the ability to detect and eradicate this formidable disease.

Experimental procedures

Mouse strains

Conditional $LSL\text{-}Trp53^{R172H/+}$ (Olive et al., 2004), $LSL\text{-}Kras^{G12D/+}$, and *Pdx-1-Cre* (Hingorani et al., 2003) strains were interbred to obtain $LSL\text{-}Kras^{G12D/+}$; $LSL\text{-}Trp53^{R172H/+}$;*Pdx-1-Cre* triple mutant animals on a mixed 129/SvJae/C57Bl/6 background. All studies were conducted in compliance with the University of Pennyslvania IACUC guidelines.

Histological, cytological, molecular, and biochemical analyses

Detailed descriptions for these procedures are provided in the Supplemental Data.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/5/469/DC1/.

Acknowledgments

We thank Carolyn Clark for comments on the manuscript, Xianxin Hua for helpful discussions on TGFβ signaling experiments, and Michael Jacobetz, Hongwei Yu, and Catrina King for technical assistance. Supported in part by NCI R25-CA87812 (S.R.H.), NCI P50-CA-62924 (R.H.H.), NIH R01 DK60694 and National Pancreas Foundation (T.B.D., A.K.R.), and NIH R01 CA101973, NIH U01CA084291, and AACR-PanCAN Career Development Award (D.A.T.).

Received: December 10, 2004
Revised: March 16, 2005
Accepted: April 25, 2005
Published: May 16, 2005

References

Aguirre, A.J., Bardeesy, N., Sinha, M., Lopez, L., Tuveson, D.A., Horner, J., Redston, M.S., and DePinho, R.A. (2003). Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. Genes Dev. *17*, 3112–3126.

Allison, D.C., Piantadosi, S., Hruban, R.H., Dooley, W.C., Fishman, E.K., Yeo, C.J., Lillemoe, K.D., Pitt, H.A., Lin, P., and Cameron, J.L. (1998). DNA content and other factors associated with ten-year survival after resection of pancreatic carcinoma. J. Surg. Oncol. *67*, 151–159.

Artandi, S.E., Chang, S., Lee, S.L., Alson, S., Gottlieb, G.J., Chin, L., and

DePinho, R.A. (2000). Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature *406*, 641–645.

Berman, D.M., Karhadkar, S.S., Maitra, A., Montes De Oca, R., Gerstenblith, M.R., Briggs, K., Parker, A.R., Shimada, Y., Eshleman, J.R., Watkins, D.N., and Beachy, P.A. (2003). Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature *425*, 846–851.

Birch, J.M., Alston, R.D., McNally, R.J., Evans, D.G., Kelsey, A.M., Harris, M., Eden, O.B., and Varley, J.M. (2001). Relative frequency and morphology of cancers in carriers of germline TP53 mutations. Oncogene *20*, 4621–4628.

Brat, D.J., Lillemoe, K.D., Yeo, C.J., Warfield, P.B., and Hruban, R.H. (1998). Progression of pancreatic intraductal neoplasias to infiltrating adenocarcinoma of the pancreas. Am. J. Surg. Pathol. *22*, 163–169.

Burris, H.A., 3rd, Moore, M.J., Andersen, J., Green, M.R., Rothenberg, M.L., Modiano, M.R., Cripps, M.C., Portenoy, R.K., Storniolo, A.M., Tarassoff, P., et al. (1997). Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: A randomized trial. J. Clin. Oncol. *15*, 2403–2413.

Caldas, C., Hahn, S.A., da Costa, L.T., Redston, M.S., Schutte, M., Seymour, A.B., Weinstein, C.L., Hruban, R.H., Yeo, C.J., and Kern, S.E. (1994). Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. Nat. Genet. *8*, 27–32.

Cavallaro, U., and Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat. Rev. Cancer *4*, 118–132.

Cho, Y., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: Understanding tumorigenic mutations. Science *265*, 346–355.

Day, J.D., Digiuseppe, J.A., Yeo, C., Lai-Goldman, M., Anderson, S.M., Goodman, S.N., Kern, S.E., and Hruban, R.H. (1996). Immunohistochemical evaluation of HER-2/neu expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasms. Hum. Pathol. *27*, 119–124.

de Lange, T. (2002). Protection of mammalian telomeres. Oncogene *21*, 532–540.

Di Como, C.J., Gaiddon, C., and Prives, C. (1999). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Mol. Cell. Biol. *19*, 1438–1449.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.

Eliyahu, D., Raz, A., Gruss, P., Givol, D., and Oren, M. (1984). Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature *312*, 646–649.

Finlay, C.A., Hinds, P.W., and Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell *57*, 1083–1093.

Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol. Cell. Biol. *21*, 1874–1887.

Goggins, M., Offerhaus, G.J., Hilgers, W., Griffin, C.A., Shekher, M., Tang, D., Sohn, T.A., Yeo, C.J., Kern, S.E., and Hruban, R.H. (1998). Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology. Poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+. Am. J. Pathol. *152*, 1501–1507.

Gottardi, C.J., Wong, E., and Gumbiner, B.M. (2001). E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. J. Cell Biol. *153*, 1049–1060.

Hahn, S.A., Seymour, A.B., Hoque, A.T., Schutte, M., da Costa, L.T., Redston, M.S., Caldas, C., Weinstein, C.L., Fischer, A., Yeo, C.J., et al. (1995). Allelotype of pancreatic adenocarcinoma using xenograft enrichment. Cancer Res. *55*, 4670–4675.

Hahn, S.A., Greenhalf, B., Ellis, I., Sina-Frey, M., Rieder, H., Korte, B., Gerdes, B., Kress, R., Ziegler, A., Raeburn, J.A., et al. (2003). BRCA2 germline mutations in familial pancreatic carcinoma. J. Natl. Cancer Inst. *95*, 214–221.

Hansel, D.E., Kern, S.E., and Hruban, R.H. (2003). Molecular pathogenesis of pancreatic cancer. Annu. Rev. Genomics Hum. Genet. *4*, 237–256.

Harley, C.B., Kim, N.W., Prowse, K.R., Weinrich, S.L., Hirsch, K.S., West, M.D., Bacchetti, S., Hirte, H.W., Counter, C.M., Greider, C.W., et al. (1994). Telomerase, cell immortality, and cancer. Cold Spring Harb. Symp. Quant. Biol. *59*, 307–315.

Hinds, P., Finlay, C., and Levine, A.J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J. Virol. *63*, 739–746.

Hingorani, S.R., Petricoin, E.F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M.A., Ross, S., Conrads, T.P., Veenstra, T.D., Hitt, B.A., et al. (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell *4*, 437–450.

Hruban, R.H., Wilentz, R.E., and Kern, S.E. (2000). Genetic progression in the pancreatic ducts. Am. J. Pathol. *156*, 1821–1825.

Hruban, R.H., Adsay, N.V., Albores-Saavedra, J., Compton, C., Garrett, E.S., Goodman, S.N., Kern, S.E., Klimstra, D.S., Kloppel, G., Longnecker, D.S., et al. (2001a). Pancreatic intraepithelial neoplasia: A new nomenclature and classification system for pancreatic duct lesions. Am. J. Surg. Pathol. *25*, 579–586.

Hruban, R.H., Iacobuzio-Donahue, C., Wilentz, R.E., Goggins, M., and Kern, S.E. (2001b). Molecular pathology of pancreatic cancer. Cancer J. *7*, 251–258.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr. Biol. *4*, 1–7.

Jemal, A., Tiwari, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J., and Thun, M.J. (2004). Cancer statistics, 2004. CA Cancer J. Clin. *54*, 8–29.

Kardon, D.E., Thompson, L.D., Przygodzki, R.M., and Heffess, C.S. (2001). Adenosquamous carcinoma of the pancreas: A clinicopathologic series of 25 cases. Mod. Pathol. *14*, 443–451.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Res. *51*, 6304–6311.

Kern, S.E., Kinzler, K.W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991). Identification of p53 as a sequence-specific DNA-binding protein. Science *252*, 1708–1711.

Kleihues, P., Schauble, B., zur Hausen, A., Esteve, J., and Ohgaki, H. (1997). Tumors associated with p53 germline mutations: A synopsis of 91 families. Am. J. Pathol. *150*, 1–13.

Klimstra, D.S., and Longnecker, D.S. (1994). K-ras mutations in pancreatic ductal proliferative lesions. Am. J. Pathol. *145*, 1547–1550.

Leach, S.D. (2004). Mouse models of pancreatic cancer: The fur is finally flying! Cancer Cell *5*, 7–11.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1997). Genetic instability in colorectal cancers. Nature *386*, 623–627.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. Nature *396*, 643–649.

Lillemoe, K.D., Yeo, C.J., and Cameron, J.L. (2000). Pancreatic cancer: State-of-the-art care. CA Cancer J. Clin. *50*, 241–268.

Montgomery, E., Wilentz, R.E., Argani, P., Fisher, C., Hruban, R.H., Kern, S.E., and Lengauer, C. (2003). Analysis of anaphase figures in routine histologic sections distinguishes chromosomally unstable from chromosomally stable malignancies. Cancer Biol. Ther. *2*, 248–252.

Murphy, K.M., Brune, K.A., Griffin, C., Sollenberger, J.E., Petersen, G.M., Bansal, R., Hruban, R.H., and Kern, S.E. (2002). Evaluation of candidate genes MAP2K4, MADH4, ACVR1B, and BRCA2 in familial pancreatic cancer: Deleterious BRCA2 mutations in 17%. Cancer Res. *62*, 3789–3793.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847–860.

Olivier, M., Eeles, R., Hollstein, M., Khan, M.A., Harris, C.C., and Hainaut, P. (2002). The IARC TP53 database: New online mutation analysis and recommendations to users. Hum. Mutat. *19*, 607–614.

Olivier, M., Goldgar, D.E., Sodha, N., Ohgaki, H., Kleihues, P., Hainaut, P., and Eeles, R.A. (2003). Li-Fraumeni and related syndromes: Correlation between tumor type, family structure, and TP53 genotype. Cancer Res. *63*, 6643–6650.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D., and Rotter, V. (1984). Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature *312*, 649–651.

Prives, C. (1998). Signaling to p53: Breaking the MDM2-p53 circuit. Cell *95*, 5–8.

Rangarajan, A., and Weinberg, R.A. (2003). Opinion: Comparative biology of mouse versus human cells: Modelling human cancer in mice. Nat. Rev. Cancer *3*, 952–959.

Rozenblum, E., Schutte, M., Goggins, M., Hahn, S.A., Panzer, S., Zahurak, M., Goodman, S.N., Sohn, T.A., Hruban, R.H., Yeo, C.J., and Kern, S.E. (1997). Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res. *57*, 1731–1734.

Scarpa, A., Capelli, P., Mukai, K., Zamboni, G., Oda, T., Iacono, C., and Hirohashi, S. (1993). Pancreatic adenocarcinomas frequently show p53 gene mutations. Am. J. Pathol. *142*, 1534–1543.

Schreiber, F.S., Deramaudt, T.B., Brunner, T.B., Boretti, M.I., Gooch, K.J., Stoffers, D.A., Bernhard, E.J., and Rustgi, A.K. (2004). Successful growth and characterization of mouse pancreatic ductal cells: Functional properties of the Ki-RAS(G12V) oncogene. Gastroenterology *127*, 250–260.

Schutte, M., Hruban, R.H., Geradts, J., Maynard, R., Hilgers, W., Rabindran, S.K., Moskaluk, C.A., Hahn, S.A., Schwarte-Waldhoff, I., Schmiegel, W., et al. (1997). Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res. *57*, 3126–3130.

Sieber, O.M., Heinimann, K., and Tomlinson, I.P. (2003). Genomic instability–the engine of tumorigenesis? Nat. Rev. Cancer *3*, 701–708.

Smogorzewska, A., Karlseder, J., Holtgreve-Grez, H., Jauch, A., and de Lange, T. (2002). DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. Curr. Biol. *12*, 1635–1644.

Tarapore, P., and Fukasawa, K. (2002). Loss of p53 and centrosome hyperamplification. Oncogene *21*, 6234–6240.

Thayer, S.P., di Magliano, M.P., Heiser, P.W., Nielsen, C.M., Roberts, D.J., Lauwers, G.Y., Qi, Y.P., Gysin, S., Fernandez-del Castillo, C., Yajnik, V., et al. (2003). Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature *425*, 851–856.

Warshaw, A.L., and Fernandez-del Castillo, C. (1992). Pancreatic carcinoma. N. Engl. J. Med. *326*, 455–465.

Yamamoto, H., Itoh, F., Nakamura, H., Fukushima, H., Sasaki, S., Perucho, M., and Imai, K. (2001). Genetic and clinical features of human pancreatic ductal adenocarcinomas with widespread microsatellite instability. Cancer Res. *61*, 3139–3144.

Yeo, C.J., Cameron, J.L., Lillemoe, K.D., Sohn, T.A., Campbell, K.A., Sauter, P.K., Coleman, J., Abrams, R.A., and Hruban, R.H. (2002a). Pancreaticoduodenectomy with or without distal gastrectomy and extended retroperitoneal lymphadenectomy for periampullary adenocarcinoma, part 2: Randomized controlled trial evaluating survival, morbidity, and mortality. Ann. Surg. *236*, 355–366.

Yeo, T.P., Hruban, R.H., Leach, S.D., Wilentz, R.E., Sohn, T.A., Kern, S.E., Iacobuzio-Donahue, C.A., Maitra, A., Goggins, M., Canto, M.I., et al. (2002b). Pancreatic cancer. Curr. Probl. Cancer *26*, 176–275.