RESEARCH PAPER

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Inactivation of *Brca2* cooperates with *Trp53*^{R172H} to induce invasive pancreatic ductal adenocarcinomas in mice

A mouse model of familial pancreatic cancer

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Key words: Brca2, p53, DNA damage, pancreatic ductal adenocarcinoma, mPanIN, genetically engineered model, familial pancreatic cancer

Abbreviations: mPanIN, murine pancreatic intraepithelial neoplasia; ADM, acinar-to-ductal metaplasia; PDAC, pancreatic ductal adenocarcinoma; LSL, lox-STOP-lox

An inactivating germline mutation in *BRCA2* is the most common known genetic basis for familial pancreatic cancer (FPC), accounting for 5–10% of inherited cases. A genetically engineered mouse model of pancreatic ductal adenocarcinoma (PDAC) arising on the backdrop of *Brca2* deficiency is likely to elucidate valuable diagnostic and therapeutic insights for FPC. Both *Brca2* alleles were conditionally deleted during development within the pancreatic epithelium by generating *Pdx1*-Cre; *Brca2*$^{f/f}$ (CB) mice; in addition, triple transgenic *Pdx1*-Cre; *Brca2*$^{f/f}$; LSL-*Trp53*^{R172H} (CBP) mice were generated, in order to determine the impact of p53 deregulation on Brca2-deficient carcinogenesis. Both CB and CBP mice developed non-invasive ductal precursor lesions (murine pancreatic intraepithelial neoplasia or mPanIN), although these were observed at an earlier time point (5 versus 8 months) and with higher prevalence in CBP mice. A minority of CB mice (15%) developed invasive and metastatic PDAC at a latency of 15 months or greater; in contrast, CBP mice of comparable age uniformly developed PDAC with variable histological features. Mortality in the absence of neoplasia in CB and CBP mice was associated with profound loss of pancreatic parenchyma, consistent with progressive elimination of Brca2-deficient cells. Widespread DNA damage, as evidenced by overexpression of the phosphorylated histone H_2AX^{Ser139}, was observed in the non-neoplastic exocrine pancreas, as well as in the mPanIN and PDAC lesions of *Brca2*-deficient mice, independent of p53 status. Loss of Brca2 function predisposes the exocrine pancreas to profound DNA damage, and the frequency of invasive neoplasia is accentuated by the concomitant deregulation of p53.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an almost uniformly lethal disease and is among the most dismal of human malignancies to date.[1] In recent years, genetically engineered mouse models that recapitulate the multistep progression of the cognate human disease have been successfully created.[2,3] These models have been crucial for a better understanding of the cellular and molecular pathogenesis of PDAC, and for the preclinical development of diagnostic and therapeutic targets that can be translated into the clinic.[4] Most of the existing models recapitulate the genetics of sporadic (i.e., non-familial) PDAC, largely because the genetic basis for familial pancreatic cancer (FPC) remains a matter of investigation. Till date, germline mutations that predispose an individual to developing PDAC in their lifetime have been identified in only a minority (~20%) of FPCs overall, and include most commonly, inactivating mutations of *BRCA2* and other members of the Fanconi Anemia gene family such as *PALB2* (~5–10%), as well as *STK11/LKB1*, *CDKN2A/p16*, *PRSS1* and the DNA mismatch repair genes.[5-7] An interest in understanding the genetic basis for FPC arises not only because it provides an avenue for developing a tangible gene test for unaffected family members, but also as a strategy to implement molecularly targeted therapies. In fact, such synthetic lethal pharmacological approaches have been used with considerable success in familial breast and ovarian cancers that arise on the

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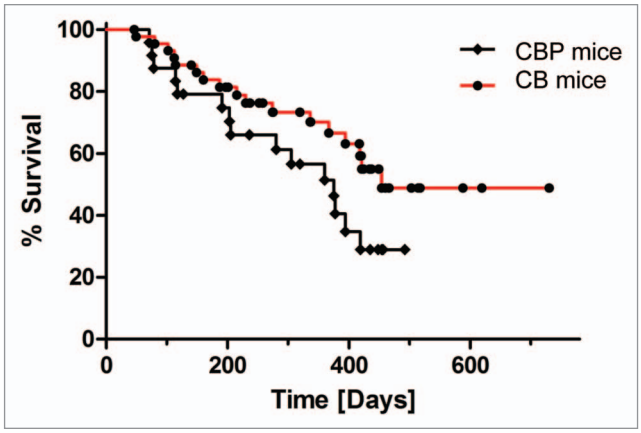

Figure 1. Kaplan-Meier survival analysis of CB and CBP mice. Kaplan-Meier survival analysis of CB and CBP mice cohorts reveals a trend towards shorter survival in the latter, but it is not statistically significant.

backdrop of *BRCA1/2* deficiency,[8] and as recently demonstrated from one of the institutions in this study (JHSOM), this synthetic lethal approach can also be successfully applied to familial PDAC, with striking remission of advanced disease.[9]

The motivation for this study was to develop a mouse model that recapitulated the genetics of the most common known alteration in human FPC, i.e., bi-allelic loss of *BRCA2* in the pancreas, in a patient with an existing hemizygous germline deletion. *Apriori* studies in human pancreata have confirmed that loss of the second *BRCA2* allele is, in fact, present in the precursor lesions and cancers that arise in the setting of germline *BRCA2* deficiency.[10] We were able to conditionally delete both *Brca2* alleles in the murine pancreatic epithelium by Cre-mediated recombination during development within the *Pdx1* expression domain. The resulting *Pdx1*-Cre; $Brca2^{f/f}$ (henceforth called CB) mice develop invasive and metastatic adenocarcinomas in a minority of animals (~15%) at relatively long latencies (at or greater than 15 months of age). Notably, the disease penetrance at the comparable ages is markedly enhanced (100% of cohort) when p53 function is concomitantly deregulated in the setting of Brca2 deficiency in *Pdx1*-Cre; $Brca2^{f/f}$; LSL- $Trp53^{R172H}$ (henceforth called CBP) mice. Both CB and CBP mice that succumb in the absence of tumorigenesis demonstrate profound loss of pancreatic parenchyma, suggesting exocrine pancreatic insufficiency as the basis for their mortality. Both CB and CBP mice also demonstrate evidence of widespread DNA damage in the pancreas, consistent with the role of Brca2 as a genome maintenance protein.[11] Our data indicate that elimination of DNA-damage prone Brca2-deficient cells is the preferred course of action in the mammalian pancreas, but deregulation of p53 function in CBP mice likely attenuates this process, and allows surviving cells to progress to invasive cancer at a higher frequency. The relatively long latency for tumor formation in both CB and CBP mice are in accordance with the late-onset PDAC occurring in patients with germline *BRCA2* mutations, and underscore the distinction between a genome maintenance gene and a traditional tumor suppressor like *CDKN2A* or *SMAD4*. These findings have important ramifications for FPC arising in individuals with germline *BRCA2* mutations, and provides an animal model for testing various therapeutic modalities for disease prevention or elimination in FPC.[12]

Results

Abrogation of Brca2 function leads to depletion of pancreatic parenchyma and early mortality in the absence of invasive neoplasia. Both CB and CBP cohorts were associated with early mortality in subsets of mice, with death occurring as early as two months, and continuing with variable latencies throughout the period of observation. Overall, the median survival of the CB mice (N = 25) and CBP mice (N = 33) were 454 and 375 days, respectively, a difference that did not reach statistical significance (p = 0.085) (**Fig. 1**). Although logistically it was not possible to perform timely necropsy on all mice that experienced spontaneous mortality, on multiple occasions when the pancreata were retrieved in a condition suitable for histopathology, we did not find evidence of invasive neoplasia, but rather observed a profound loss of exocrine pancreatic parenchyma, with near total depletion of acinar elements (**Fig. 2**). The pancreata in such mice often demonstrated extensive adipose (fatty) replacement (**Fig. 2A**), with residual islets prominently embedded in the fat. On occasion, a large portion of the pancreas was replaced by hemorrhagic cysts, the lining of which were comprised of low cuboidal epithelium (**Fig. 2C**). These are likely degenerated cysts, arising on the backdrop of pancreatic exocrine dropout, as suggested by the innocuous nature of the lining epithelium as well as the absence of oncogenic signaling pathways observed in neoplastic tissues (see below). In occasional CB mice that survived for relatively longer periods of time (>1 year or beyond), we observed a remarkable replacement of the pancreatic parenchyma by confluent non-invasive precursor lesions (murine pancreatic intraepithelial neoplasia or mPanIN), with essentially no discernible acinar tissue within these mPanIN strips (**Fig. 2D**). Rigorous histopathological examination in these mice with spontaneous mortality failed to reveal evidence of invasive neoplasms or other organ system pathologies, suggesting that the cause of death was likely to be exocrine insufficiency caused by the striking depletion of acinar tissue.

Murine pancreatic intraepithelial neoplasia (mPanINs) develop in the absence of Brca2 function and their onset is accelerated by p53 deregulation. In addition to the survival cohort described above, we generated separate observation cohorts of CB and CBP mice that underwent euthanasia and necropsy at timed intervals (monthly, beginning at 2 months, up to 10 months of age). Pancreata were harvested in these mice with the specific intent of cataloging ductal precursor lesions or invasive neoplasia, and an effort was made to have at least five mice for each genotype at each of the monthly harvests. As seen in **Figure 3**, both CB and CBP mice demonstrated the full spectrum of mPanIN lesions, although these were initiated at an earlier time point (5 vs. 8 months, respectively) in the absence of p53 function (**Fig. 3E**). In addition to full-fledged mPanINs, lesions of acinar ductal metaplasia (ADM) were also observed in the surrounding pancreata (**Fig. 3C and D**), a not unexpected finding given these

are considered "precursors to the precursors" in the multistep progression model of murine PDAC. Of the 45 mice in each cohort undergoing histopathological examination, none of the CB mice and only one (2%) of the CBP mice (aged 10 months) had evidence of invasive cancer.

An immunohistochemical stain for Brca2 confirmed absence of expression in the mPanIN epithelium, as well as in the epithelium lining of the cystic lesions described above (**Fig. 4**), consistent with epithelial specific developmental *Pdx1* expression. In contrast, robust expression was retained in the pancreatic mesenchyme, where Cre-mediated recombination is not expected.

PDAC development occurs in CB and CBP mice at comparable latencies but deregulation of p53 enhances the frequency of invasive neoplasia. No invasive neoplasms were seen in either the CB or CBP mice (with a single exception in the observation cohort) up to the age of 15 months. Of 13 CB mice that were followed between 15 months and 24 months, two (15%) developed invasive and metastatic PDAC. At necropsy, widespread intra-abdominal metastases were evident (**Fig. 5A**), and explants from the tumors readily grew as subcutaneous xenografts in immunocompromised mice (**Fig. 5B**). Histologically, the neoplasms were comprised of moderately differentiated adenocarcinomas, with evidence of intracellular mucin vacuoles within neoplastic cells, a feature often observed in human PDACs (**Fig. 5C and D**).[13] Notably, we did not find evidence of acceleration in the onset of invasive cancers in CBP mice, although there was a sharp increase in their frequency between 15 and 17 months of age, with five of five (100%) of mice succumbing to widely metastatic tumors during this time frame (**Fig. 6**). In addition to local invasion into neighboring tissues, the tumors also metastasized to the liver, diaphragm and kidneys. At the terminal stages, it was not uncommon to find associated cachexia and malignant ascites, both features observed in advanced human PDAC. Hemorrhagic cysts were observed in two mice. Histologically, a variety of morphological features were observed (**Fig. 7**). Thus, two of the tumors were comprised of moderately differentiated adenocarcinomas, wherein the neoplastic glands were embedded in a robust desmoplastic response, akin to what is observed in human PDAC (**Fig. 7A and B**). Two cases had a biphenotypic appearance, with both adenocarcinoma and sarcomatoid carcinoma components (**Fig. 7C**); areas of sharp transition between the two components were readily visible (**Fig. 7D**). The poorly differentiated sarcomatoid carcinoma was comprised of pleomorphic cells with brisk mitotic activity (**Fig. 7E**). Finally, one of the tumors was an anaplastic carcinoma with bizarre giant cells, also a subtype that is occasionally seen in human PDAC (**Fig. 7F**).[13]

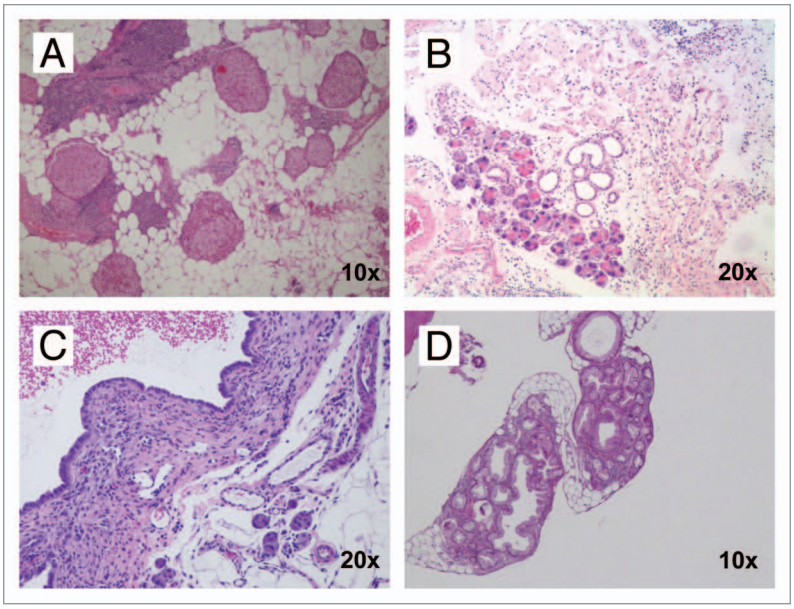

Figure 2. Histopathology of exocrine pancreatic insufficiency in CB and CBP mouse pancreata in the absence of invasive neoplasia. (A) Profound loss of exocrine pancreatic parenchyma in a CB mouse at 6 months age demonstrating extensive adipose (fatty) replacement and residual islets with minimal functioning exocrine tissue (H&E stain, 10x magnification). (B) Minimal residual exocrine parenchyma (acinar tissue) in a six month old CBP mouse (H&E stain, 20x magnification). (C) Degenerated cystic lesions observed in both CB and CBP mice, lined by flattened cuboidal epithelium and often containing hemorrhagic fluid (H&E stain, 20x magnification). (D) Strips of murine pancreatic intraepithelial neoplasia (mPanIN) are observed in a background of adipose (fatty) replacement and near total absence of viable acinar tissue in a 15-month-old CB mouse (H&E stain, 10x magnification).

Brca2 depletion leads to increased DNA damage. The Brca2 protein functions in "genome maintenance", mainly due to its central role in DNA double-strand break repair through homologous recombination.[11] Phosphorylation of histone H_2AX at the Ser139 residue represents an early response to DNA damage.[14,15] Therefore, in order to assess the extent of DNA damage we stained pancreatic tissues obtained from *Brca2*-deficient pancreata for pH_2AX^{Ser139} (**Fig. 8**). In age-matched control mice that retained hemizygous Brca2 function (*Pdx1*-Cre; *Brca2*$^{f/wt}$), we observed minimal pH_2AX^{Ser139} expression (**Fig. 8A**). In contrast, profound upregulation of pH_2AX^{Ser139} was observed in CB mice, specifically within the acinar component (**Fig. 8B and C**). Unexpectedly, we found minimal expression of pH_2AX^{Ser139} in the ductal epithelium (**Fig. 8C**), which suggests that, despite the absence of Brca2 function, this epithelial compartment might be recalcitrant to DNA damage, or be exposed to fewer genotoxic stressors than the acinar compartment. In invasive cancers arising in these mice (a CBP mouse tumor is illustrated in **Fig. 8D**), there was continued elevated expression of pH_2AX^{Ser139}, consistent with ongoing DNA damage.

Neoplastic lesions arising in the setting of Brca2 deficiency lack secondary Kras mutations but express Hedgehog (Hh) ligand. Somatic mutations of the *KRAS* gene are one of the most common genetic alterations in PDAC, and their presence even in non-invasive precursor lesions renders them as an "early" abnormality.[16] Biologically relevant mouse models of mPanINs and PDAC were first generated by expression of a mutant *Kras*^{G12D} allele from its endogenous locus,[17,18] further underscoring the importance of this oncogene to ductal neoplasia. Therefore, we examined whether the mPanIN and PDACs arising in CB and

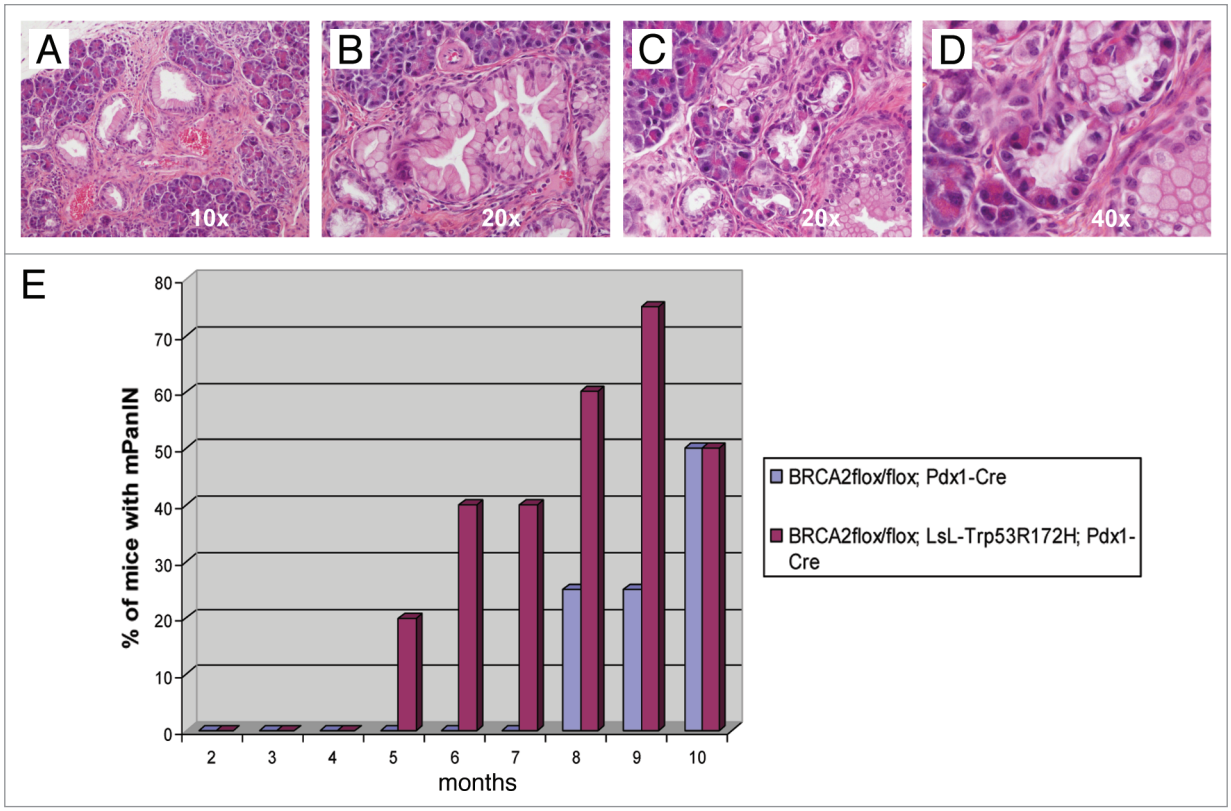

Figure 3. Histopathology of non-invasive precursor lesions in CB mice. Examples of low-grade (A) and high-grade (B) mPanIN lesions arising in the pancreas of a CB mouse at 11 months of age. Several areas with acinar-to-ductal metaplasia were also found (C); higher magnification (D). (H&E stains). (E) CBP mice (red columns) have an earlier onset of mPanIN compared to CB mice (blue columns). By 10 months of age, both cohorts have comparable frequency of mPanIN lesions.

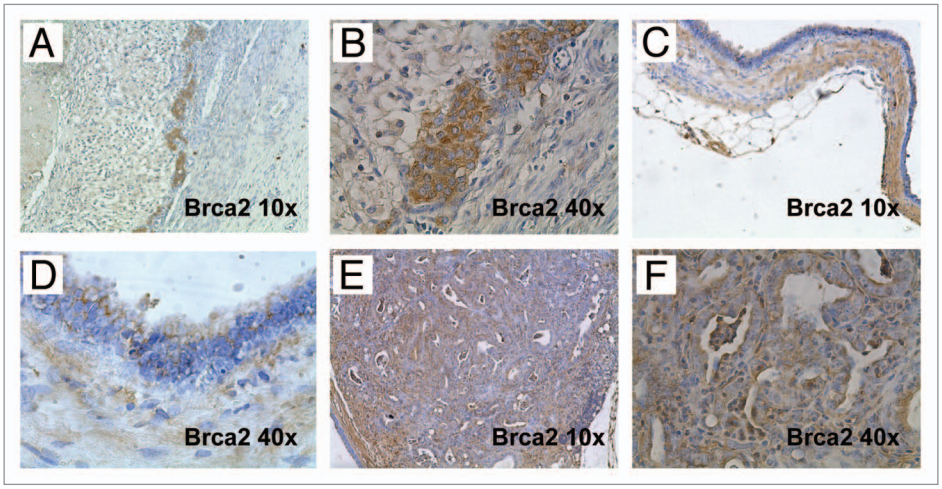

Figure 4. Brca2 immunohistochemistry confirms loss of protein expression in lesional pancreatic epithelium. (A and B) Brca2 immunohistochemistry confirms robust cytoplasmic expression in granulosa cells of the ovary, a known positive control (10x and 40x magnification). (C and D) Minimal to absent Brca2 expression is observed within the lining epithelium of the degenerated cysts, a prototype of which is illustrated in **Figure 2** (10x and 40x magnification). Note that the subjacent mesenchymal cells retain Brca2 expression. (E and F) Minimal to absent Brca2 expression is observed within mPanIN lesions arising in a "CPB" mouse (10x and 40x magnification).

CBP mice might harbor secondary *Kras* mutations. Sanger sequencing of microdissected lesions from a subset of mPanINs and PDAC failed to demonstrate mutations of codons 12, 13 or 61 of the Kras gene (data not shown).

Finally, we assessed whether the neoplastic epithelium expressed the Hh ligand, sonic Hh (Shh), which is a feature of most human PDACs,[19,20] using the epithelium lining of the aforementioned degenerated cysts as controls (**Fig. 9**). We observed that while nuclear Pdx1 was expressed by both epithelial types, only the neoplastic epithelium expressed Shh ligand.

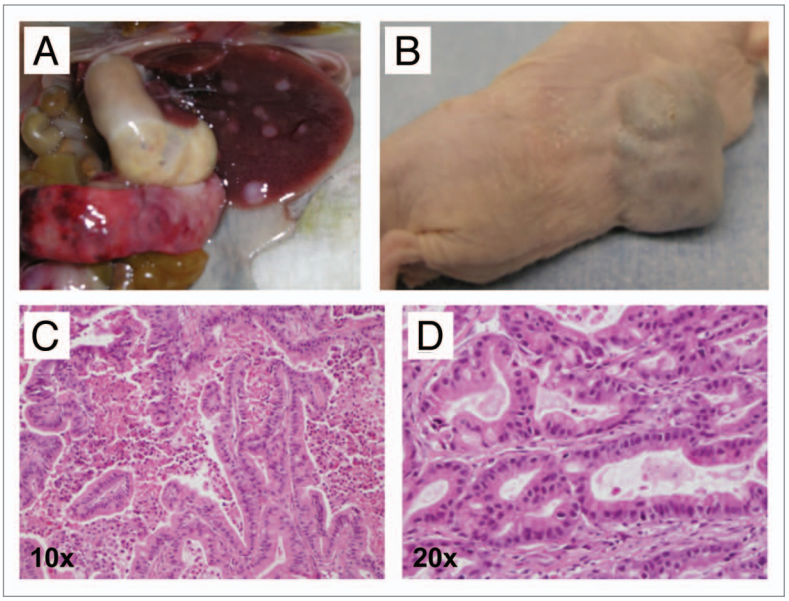

Figure 5. A minority of CB mice develop invasive and metastatic PDAC at relatively long latency (15 months or greater). (A) Example of a metastatic PDAC arising in a 24-month-old CB mouse. Multiple metastatic lesions in liver and spleen are readily visible. (B) Serial transplantation of tumor tissue into athymic mice resulted in growth as subcutaneous xenografts, a property of cancer cells. (C) Histologically, the primary and metastatic tumors were comprised of moderately differentiated PDAC (H&E stain, 10x magnification). (D) Moderately differentiated PDAC, at a higher magnification (H&E stain, 20x magnification). Note mucin vacuoles within neoplastic glands.

Discussion

Germline mutations of *BRCA2* account for ~5–10% of cases with an inherited predisposition to PDAC.[7,21] In addition, while somatic *BRCA2* mutations are uncommon in sporadic PDAC (~1%), this frequency rises to ~10% of cancers within certain ethnic backgrounds, such as individuals of Ashkenazi-Jewish heritage. Besides the obvious implications vis-à-vis genetic counseling and cancer screening for *BRCA2* germline mutation carriers,[22] it is now established that tumors with bi-allelic loss of *BRCA2* (or related Fanconi Anemia pathway genes) also respond dramatically to certain classes of anti-neoplastic agents, such as mitomycin C, cisplatin and the poly (ADP-ribose) (PARP) inhibitor, Olaparib.[8,23] In fact, the utility of this "synthetic lethal" strategy has recently been demonstrated in a rather dramatic manner in a case of advanced PDAC.[9] The creation of a genetically engineered model of *BRCA2* deficiency in the pancreas would provide an in vivo platform for designing improved diagnostic and therapeutic strategies for this genetically defined cancer subset.

Herein, we describe a mouse model of "familial pancreatic cancer" generated on the backdrop of conditional *Brca2* deficiency, with or without concomitant deregulation of p53 function. Our studies demonstrate that the most prevalent response to bi-allelic deletion of *Brca2* (and the secondarily accrued DNA damage) might be elimination of the affected epithelial cells, which accounts for the profound loss of exocrine pancreatic parenchyma observed in mice that succumb without developing invasive cancer. Loss of pancreatic parenchyma is observed in both CB and CBP mouse cohorts, suggesting that a p53-independent cell death pathway, possibly associated with mitotic catastrophe, triggers this response.[24,25] In a mouse pancreas, pan-epithelial *Cre*-mediated recombination during development within the *Pdx1* expression domain results in a large pool of *Brca2*-deficient cells. Needless to add, this scenario is not recapitulated in an individual with a germline *BRCA2* defect, wherein the somatic "second hit" occurs in a more restricted nidus of cells. Therefore, unlike in the current mouse model, pancreatic insufficiency is not a phenotype commonly observed in humans with a germline *BRCA2* mutation. Nonetheless, a *BRCA2*-deficient cell faces considerable selection pressure for elimination in vivo, which likely explains the relatively low-penetrance (no more than 5–10%) for lifetime PDAC incidence in *BRCA2* mutation carriers.[21,26]

In mice with sufficient preservation of pancreatic function, we observed emergence of mPanIN lesions, beginning at 5 months in CBP mice and in 8 months in the CB cohort. Eventually, invasive and metastatic PDAC developed in both genotypes, and was observed at comparable latencies (beginning at ~15 months of age), although the CBP mice had a greater frequency of tumor formation beyond 15 months. The relatively long latency to tumorigenesis is also observed in germline *BRCA2* carriers, whose median age of onset for PDAC is no different from that in sporadic (non-familial) patients.[7,21] The comparable latencies between the two cohorts underscore the role of Brca2 as a genome maintenance protein, rather than a "classic" tumor suppressor controlling cell cycle parameters, and the need for accumulation of additional genetic events that must occur prior to neoplastic transformation. What role does the $Trp53^{R172H}$ allele play in neoplastic progression? One possibility is that the elimination of *Brca2*-deficient cells that occurs with intact p53 function is attenuated (albeit not blocked) with p53 deregulation, such that a larger pool of cells with damaged DNA is available for propagation. Another postulate, not mutually exclusive, is that the "gain-of-function" p53 protein encoded by the $Trp53^{R172H}$ allele facilitates genomic instability, and adds to the genotoxic burden of Brca2 loss.[27,28] For example, the karyotype of PDACs arising in compound heterozygous *Pdx1*-Cre;LSL-$Kras^{G12D}$; LSL-$Trp53^{R172H}$ mice ("KPC" mice) is consistent with widespread

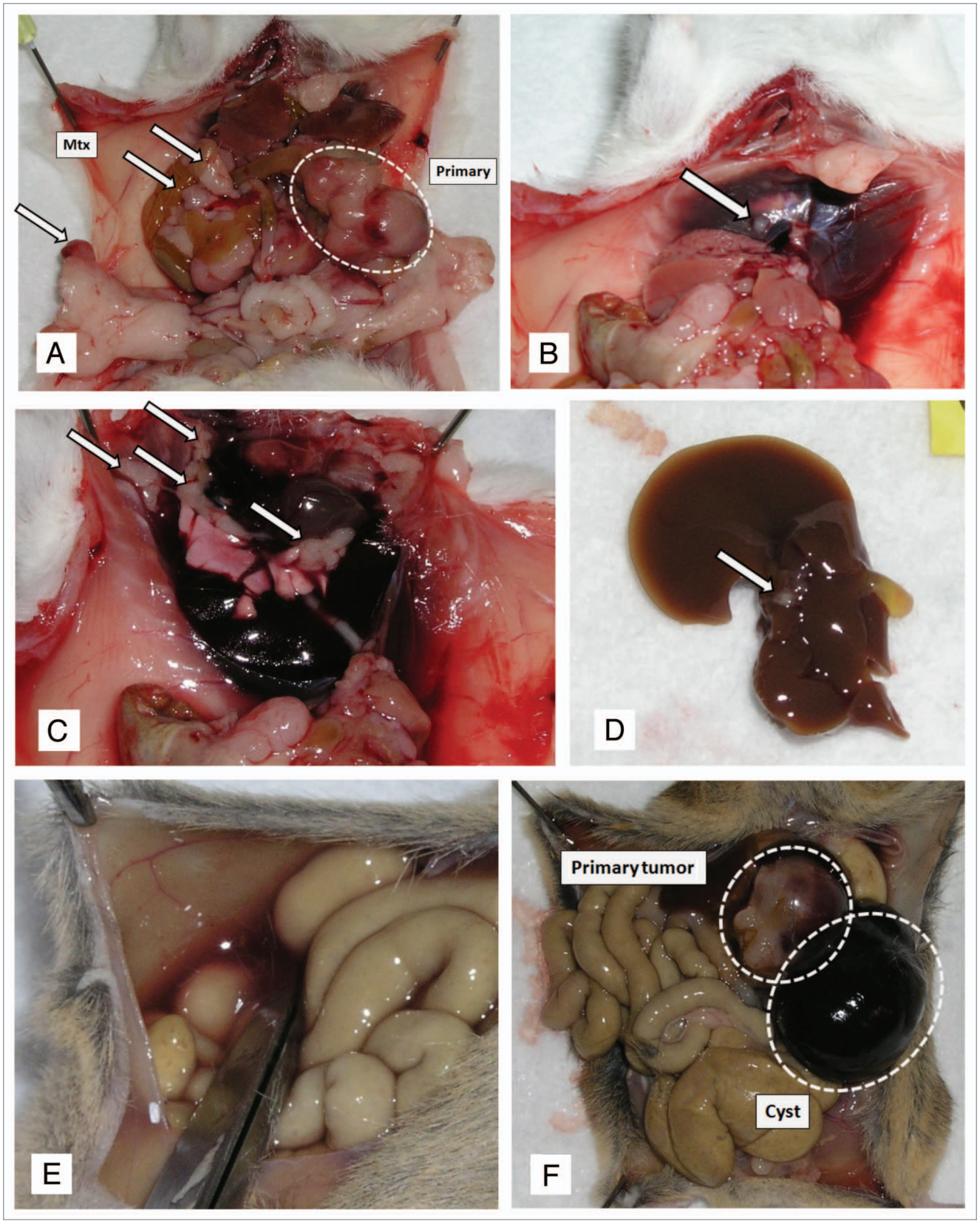

Figure 6. Macroscopic images of metastatic pancreatic cancers arising in CBP mice. (A) A primary pancreatic cancer (white dotted line) and numerous grossly visible metastatic lesions (white arrows) to distant organ sites, including peritoneum, diaphragm (B), lungs (C) and liver (D). At these advanced stages, malignant ascites was observed in some of the affected mice (E). In addition, some animals developed hemorrhagic intra-pancreatic cystic lesions, likely due to bleeding (F).

chromosomal instability,[29] a feature not typically observed in other genetic cooperation models for PDAC.[18,30] Irrespective of the precise mechanism, the concomitant deregulation of p53 and Brca2 results in a greater frequency of invasive PDAC than what is observed in CB mice alone.

In the absence of Brca2 function, we find robust expression of the phosphorylated histone pH_2AX^{Ser139}, a surrogate measure of DNA damage, within the exocrine pancreata, as well as in the resulting neoplastic alterations. Surprisingly, while acinar cells demonstrate widespread pH_2AX^{Ser139} expression, the native ductal epithelium shows minimal labeling. This leads one to speculate whether the major, if not entire, contribution to the neoplastic phenotype is derived from non-ductal elements, most likely the acinar tissues. Notably, prior studies, including from our group,[31,32] have shown the ability of mature acinar cells to spontaneously develop mPanINs and/or PDAC in the appropriate oncogenic context, and therefore, the development of "ductal" adenocarcinoma from non-ductal elements in the pancreas is not entirely unexpected. A better understanding of the cell-of-origin of *Brca2*-deficient pancreatic neoplasia will require rigorous lineage tracing experiments and the use of compartment-specific *Cre* "driver" lines. Another feature of interest was the absence of secondary *Kras* gene mutations at codons 12, 13 or 61 in the resulting mPanINs and PDACs of CB and CBP mice. The emerging consensus has been that expression of a mutant *Kras* allele might be a requirement for developing PDACs along the mPanIN multistep progression cascade, with any cooperating genetic lesion (such as loss of *Cdkn2a/p16* or *Smad4*, or misexpression of *Gli2* oncoprotein etc.),[18,30,33-35] essentially altering the natural history and penetrance of the "*Ras*-induced" invasive neoplasia phenotype. However, we were unable to demonstrate *Kras* mutations in microdissected mPanIN or PDAC lesions, suggesting that, on occasion, mutations of this oncogene may be dispensable (see further discussion below). It is important to note that we do not have the information to exclude somatic mutations in downstream Ras effector genes, such *BRAF*, *PI3KCA*, *AKT*, etc., which are altered in minor subsets of human PDACs,[36,37] in the murine lesions.

Finally, this discussion would not be complete without an acknowledgement of two elegant reports on *Brca2*-deficient pancreatic neoplasia that were recently published, while our own work was nearing completion.[38,39] In the study by Skoulidis et al. the authors generated a model with expression of a truncated *Brca2* (*Brca2*^{Tr}) allele in all somatic tissues, and found that, irrespective of p53 status, pancreatic haploinsufficiency of *Brca2* was sufficient to cooperate with mutant *Kras*^{G12D} and induce PDAC in mice. The impact of bi-allelic *Brca2* deletion, on the

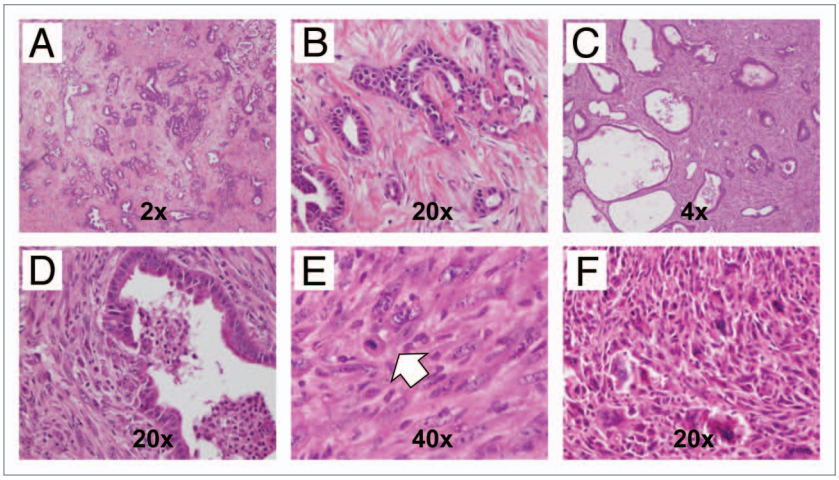

Figure 7. Variable histopathological features of PDAC arising in CBP mice. (A) Primary PDAC with extensive stromal desmoplasia, resembling the cognate human disease is observed in this CBP mouse (H&E stain, 2x magnification). (B) At higher magnification, infiltrating neoplastic glands in a bed of desmoplastic stroma can be seen (H&E stain, 20x magnification). (C) An example of a biphasic tumors with both adenocarcinoma and sarcomatoid components (H&E stain, 4x magnification). (D) At higher magnification of the tumor in (C), the confluence between the two components is highlighted (H&E stain, 20x magnification). (E) Higher magnification of the sarcomatoid carcinoma component also confirms the presence of atypical pleomorphic nuclei and mitotic figures (arrow) (H&E stain, 40x magnification). (F) An anaplastic carcinoma with prominent giant cells arising in a CBP mouse (H&E stain, 20x magnification).

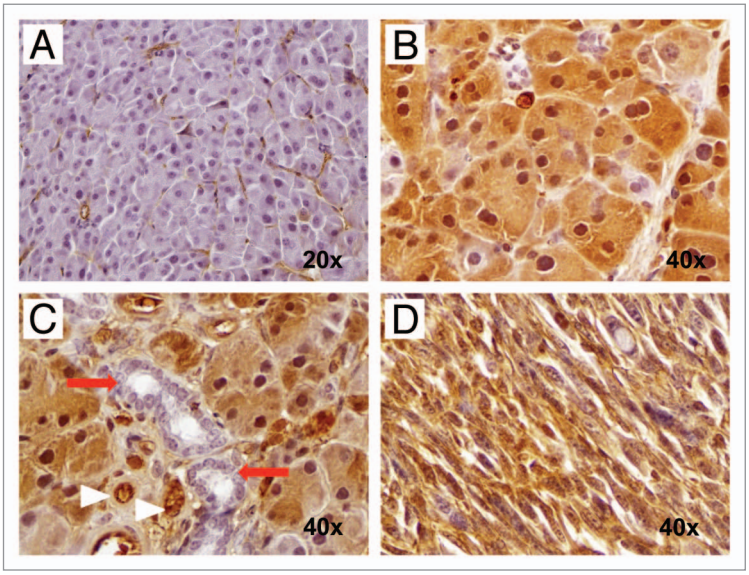

Figure 8. Inactivation of Brca2 promotes DNA damage in the exocrine pancreas. (A) Immunohistochemical staining for pH_2AX^{Ser139} shows minimal labeling within acinar cells in pancreatic tissue sections derived from hemizygous *Pdx1*-Cre; *Brca2*$^{flox/wt}$ mice (negative control). (B and C) Complete abrogation of Brca2 function in CB mice results in markedly increased pH_2AX^{Ser139} immunolabeling in pancreatic acinar cells (B and C), but not in the ductal epithelia (red arrows). Note robust expression of pH_2AX^{Ser139} in two highly atypical acinar cell nuclei (white arrowheads). (D) Marked upregulation of pH_2AX^{Ser139} labeling in the sarcomatoid carcinoma component of a PDAC arising in a CBP mouse.

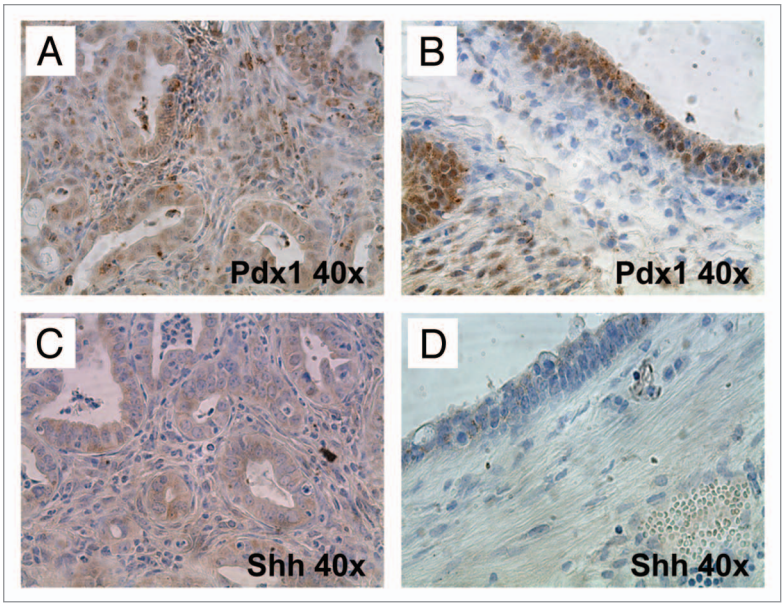

Figure 9. Hedgehog ligand is expressed by neoplastic glandular epithelium but not by the epithelium of non-neoplastic cysts. (A) and (B) Nuclear Pdx1, a marker of pancreatic progenitor cells not expressed in the mature ductal epithelium, is expressed within both neoplastic glands (A) as well as the lining epithelium of non-neoplastic cysts (B). (C and D) In contrast, Hh ligand, known to be overexpressed in most human PDAC, is expressed only by neoplastic glands (C) and not by the non-neoplastic cystic epithelium (D).

other hand, was largely influenced by the functional status of p53. Thus, in the presence of intact p53 function, most of the recombined epithelial cells are eliminated leading to pancreatic insufficiency, and PDAC is only observed in surviving mice after a relatively long latency, a finding that essentially mimics our own observation in the CB cohort. On the contrary, bi-allelic *Brca2* loss of function, when occurring concomitantly with mutant *Kras* expression plus p53 deregulation, results in rapid progression to PDAC. This latter observation is in contrast to our own study, where the latency of tumor onset is not significantly affected by p53 status, and almost certainly reflects the absence of ongoing "oncogene-induced DNA damage" in the CBP mice that would be caused by co-expression of mutant *Kras*.[40,41] As stated above, germline *BRCA2* mutation carriers are not prone to develop PDAC at an earlier age of onset than sporadic cancers, a phenomenon that contrasts with well known "Knudsonian" syndromes like familial retinoblastoma or familial adenomatous polyposis, amongst others. The basis for this observation has not been fully examined, but one possibility could be the later onset acquisition of, or even absence, of cooperating *KRAS* mutations in this subset of PDACs.

The second study, by Rowley et al. also reported a mouse model of *Brca2*-deficient PDAC, with either *Trp53* or *Kras*^{G12D} as cooperating mutations, utilizing a *Cre*-expressing driver line identical to the current manuscript, and a floxed *p53* allele, in contrast to the gain-of-function R172H allele in our study. In mice with bi-allelic deletions of both *Brca2* and *Trp53* (the functional equivalent of our CBP mice), they observed invasive PDAC of variable histological features, occurring with relatively long latency, essentially identical to our own findings. Another notable commonality between the studies was the low frequency of secondarily acquired *Kras* gene mutations in the resulting PDAC lesions (only 2 of 13, 15%, examined in the Rowley report), while none were present in our smaller cohort of CBP mice. This again begs the question as to the status of the *KRAS* gene in human PDACs arising on the backdrop of *BRCA2* deficiency, and if their absence or low frequency could account for the later onset tumors seen in such individuals. A putative "antagonistic" relation between *Brca2* deficiency and mutant *Kras* is also bolstered by the observation that, in the presence of intact p53 function, the incidence of mPanINs and PDAC was actually lower compared to mice expressing mutant *Kras* alone, when both genes were altered in the pancreatic epithelium.[39] The one discordance between our study and that of Rowley et al. was the observation that CB mice also developed mPanINs, albeit at a later time point than CBP mice, and in a minority of instances (15%), the lesions progressed to full blown PDAC. In the previous report, mice with loss of Brca2 function alone had no preneoplastic ductal lesions or cancers. While the exact basis for this discordance remains unclear, it is possible that the later onset mPanINs and PDAC in CB mice result from secondary abrogation of p53 function, or dysfunction of other DNA damage checkpoint proteins like ATM and Chk2, thus allowing surviving DNA damaged cells to bypass mitotic catastrophe.[15]

In conclusion, the current report "dovetails" with two recent publications[38, 39] on successfully generating a mouse model of familial pancreatic cancer (FPC). These reports establish that bi-allelic loss of *Brca2*, possibly alone and certainly in conjunction with p53 deregulation, are capable of inducing the entire spectrum of ductal neoplasia of the pancreas, and that this multistep progression can occur without oncogenic mutations of Kras itself. These models provide an in vivo approach for testing new therapeutic strategies that might be synthetic lethal to a *Brca2* defect, as well as for developing serum based "signatures" of Brca2 deficiency as an early detection biomarker for FPC.

Materials and Methods

Generation of *Pdx1-Cre*; *Brca2*$^{flox/flox}$ (CB) and *Pdx1-Cre*; *Brca2*$^{flox/flox}$; *LsL-Trp53*^{R172H} (CBP) cohorts. Heterozygous *Brca2*$^{flox/wt}$ breeder mice were obtained from the National Cancer Institute's Mouse Models of Human Cancer Consortium (MMHCC) repository. *Lox*P sites are inserted into introns surrounding exon 11 in the *Brca2* locus.[42] The LsL-*Trp53*^{R172H} and *Pdx1*-Cre mice used in this study have been previously described in reference 29. *Brca2*$^{flox/wt}$, LsL-*Trp53*^{R172H} and *Pdx1*-Cre mice were interbred to generate *Brca2*$^{flox/flox}$; LsL-*Trp53*^{R172H} and *Pdx1*-Cre; *Brca2*$^{flox/wt}$ mice, respectively. Next, *Brca2*$^{flox/flox}$; LsL-*Trp53*^{R172H} and *Brca2*$^{flox/wt}$; *Pdx1*-Cre mice were utilized as breeders to generate *Pdx1*-Cre; *Brca2*$^{flox/flox}$ (referred to as CB)

and *Pdx1*-Cre; *Brca2*$^{flox/flox}$; LsL-*Trp53*^{R172H} (referred to as CBP) cohorts. For genotyping, genomic DNA was extracted from tail snips by means of the REDExtract-N-Amp™ Tissue PCR kit (Sigma-Aldrich, Saint Louis, MO), following the standard protocol provided by the manufacturer. For each mouse three independent PCR reactions were carried out to test for the presence of Trp53^{R172H}, floxed Brca2 alleles (hetero- or homozygote) and *Pdx1*-Cre (using Cre-specific primers along with Gabra as positive control), respectively.

Primer sequences for these three reactions were as follows: *Trp53*^{R172H}: mutant_forward-5'-AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A-3'; reverse-5'-CTT GGA GAC ATA GCC ACA CTG-3';[27] *Brca2*: B012-5'-GGC TGT CTT AGA ACT TAG GCT G-3', B013-5'-CTC CAC ACA TAC ATC ATG TGT C-3', B014-5'-CTC ATC ATT TGT TGC CTC ACT TC-3', B015-5'-TGTTGGATAcAAGGcATGTAcAc-3';[43] *Pdx1*-Cre: Cre26-5'-CCT GGA AAA TGC TTC TGT CCG-3', Cre36-5'-CAG GGT GTT ATA AGC AAT CCC-3', GABRA12-5'-CAA TGG TAG GCT CAC TCT GGG AGA TGA TA-3', *GABRA70*-5'-AAC ACA CAC TGG CAG GAC TGG CTA GG-3'.

Tissue sample preparation. Mice were euthanized and tissue samples obtained at necropsy were either snap-frozen in liquid nitrogen and stored at -80°C until further use, or directly fixed in 10% neutral buffered formalin solution for paraffin embedding. H&E staining of formalin-fixed paraffin-embedded tissue sections were performed by the Johns Hopkins Reference Histology Laboratory following established procedures.

Immunohistochemistry. Immunohistochemistry was performed as previously described in reference 43. In brief, formalin-fixed paraffin-embedded tissue sections were deparaffinized using established procedures and quenched with 3% H_2O_2 for 10 min. Next, slides were heated in a steamer in 10 mM citrate buffer (pH 6.0) to unmask the epitopes for 20 min at 95°C, after which they were allowed to cool down to room temperature for 20 min. Slides were blocked with 10% fetal bovine serum solution (Invitrogen, Carlsbad, CA) for 30 min. Anti-Brca2 (Abcam) was used as primary antibody at a dilution of 1:100. Anti-phospho H_2AX^{Ser139} (Upstate/Millipore, Millerica, MA) was used as primary antibody at a dilution of 1:200. Anti-Pdx1 (Chemicon, Temecula, CA) was used at a primary dilution of 1:100, and anti-Shh (R&D Systems, Minneapolis, MN) was used at a dilution of 1:50. Immunolabeling was detected using the PowerVision$^+$ Poly-HRP IHC kit (Immunovision Technologies, Norwell, MA) following the standard protocol. Slides were counterstained with Harris hematoxylin solution. Negative controls (primary antibody replaced by serum from appropriate species) were used in each run.

Direct sanger sequencing. For confirmation of *Kras* codons 12, 13 and 61 somatic mutation status, tissue samples from two invasive PDAC and adjacent normal tissues, respectively, were collected by means of stereomicroscope microdissection. In addition, tissue samples from two mPanIN lesions were collected using laser capture microdissection. DNA extraction and direct Sanger sequencing were done using standard techniques as described elsewhere in reference 31. Primer sequences for exons 12, 13 and 61 of murine *Kras* are described elsewhere in reference 44.

Statistical analysis. Kaplan-Meier analysis was performed using GraphPad Prism for MS Windows, version 5.02.

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