MAINTENANCE OF ACINAR CELL DIFFERENTIATION BY

PTF1A INHIBITS PANCREATIC CANCER INITIATION

by

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ABSTRACT

Pancreatic cancer remains one of the poorest prognoses in medicine, with limited treatment options and 5-year patient survival of only ~7%. Activating mutations in the *Kras* proto-oncogene (*Kras*^{G12D}) are nearly ubiquitous in human pancreatic cancer and are sufficient to initiate precancerous pancreatic intraepithelial neoplasia (PanINs) when expressed in adult acinar cells of mice. *Kras*^{G12D}-driven PanINs normally take months to form, which lead to the hypothesis that acinar differentiation determinants may have an inhibitory effect on PanIN formation and subsequent cancer initiation. While targeted drugs that promote cell differentiation are curative in blood cancers like acute promyelocytic leukemia, there is currently minimal evidence suggesting that cell differentiation could play a role in limiting or stopping solid tumor growth. This dissertation aims to test whether loss of acinar differentiation, mediated through the transcription factor *Ptf1a*, is necessary and/or sufficient to initiate the early stages of pancreatic cancer. Using mouse genetics, we demonstrate that loss of *Ptf1a* alone is sufficient to promote acinar-to-ductal reprogramming and a cancer-like gene expression profile that is conducive to inflammation and *Kras*-dependency. As a result, *Ptf1a*-deficient acinar cells are rapidly transformed into PanINs in the presence of oncogenic *Kras*^{G12D}. Consistent with PanINs acting as precursor lesions to invasive pancreatic cancer, we demonstrate that loss of *Ptf1a* allows for rapid progression to carcinoma *in situ* and mortality from invasive pancreatic cancer in an established mouse model of disease.

These data confirm that loss of acinar cell identity hastens tumor development independently of canonical tumor suppressor loss. In contrast, a novel mouse line that sustains *Ptf1a* expression during pancreatic cancer initiation eliminates formation of $Kras^{G12D}$-driven precancerous PanINs. Perhaps most strikingly, reintroduction of *Ptf1a* into established precancerous PanINs reverts them to primitive acinar cells. Our findings therefore suggest that loss of acinar cell differentiation is required for pancreatic cancer origination and progression. This dissertation is among the first studies demonstrating that a cell differentiation program can prevent and reverse premalignant solid tumor cells *in vivo* and thus introduces a novel paradigm for solid tumor prevention and treatment.

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CHAPTER 1

INTRODUCTION

Cancer, at its essence, is uncontrolled proliferation and migration of cells that have lost a normal identity. What causes cancer initiating cells to emerge, however, is a question that has puzzled researchers for decades. Several hypotheses exist about the origination of cancer cells in different tissues. In organs with high cell turnover, such as the intestine, skin, and blood, accumulation of mutations and epigenetic changes in resident stem cells are thought to lead to their emergence as cancer propagating cells. Given a stem cell's capacity for proliferation, this is an attractive hypothesis and has been experimentally validated. For example, when cancer causing mutations are engineered into the stem cells of the intestinal crypt, they give rise to precancerous polyps, resembling human disease (Barker et al., 2009). This hypothesis, however, does not explain why organs that are largely quiescent and do not have yet-identified stem cells populations, are so prone to cancer. Cancers from one such organ, the liver, for example, are the second leading cause of cancer-related death worldwide (https://www.cdc.gov/cancer/international/statistics.htm). How then could cancer be propagated from other, mature cells types? What confers growth advantages to differentiated cells that transform into cancer? Are mutations in oncogenes or tumor suppressors sufficient to transform differentiated cells and initiate cancer? My dissertation work utilizes the pancreas, another quiescent organ, and pancreatic ductal adenocarcinoma (PDAC) initiation as a model to study how fully differentiated cells can give rise to cancer. Our data suggest that loss of the transcriptional programs that confer cell identity might be the crucial and rate-limiting step leading to the emergence of tumor-initiating cells, regardless of mutation burden.

Why study PDAC as a model cancer? PDAC is among the worst maladies in

medicine; while the disease only accounts for 3.1% of all new cancer cases in the USA, it causes 7.0% of all cancer deaths (seer.cancer.gov). The median survival with chemotherapy treatment is only ~9 months and fewer than 7% of patients live longer than 5 years after diagnosis (Ryan et al., 2014; Von Hoff et al., 2013). Several factors contribute to this poor prognosis: for one, localized tumors are often asymptomatic and this issue is compounded by the lack of screening tests that can detect advanced but pre-invasive lesions. Consequently, patients often present to clinics with metastatic disease, which is incurable. Obtaining sustained remission is rare even in patients with completely resected primary tumors, and approximately 70% of these patients succumb to recurrent illness. While the clinical course of disease is rapid, recent studies suggest that PDAC has an insidious onset: quantitative analysis of the genetic evolution of PDAC suggests it takes over 15 years from initiating mutation (usually in the proto-oncogene *Kras*, discussed below) to formation of metastatic cancer (Yachida et al., 2010). Understanding how the disease originates and evolves over this period could provide avenues for detection and therapeutic intervention, which might stop the disease before it metastasizes and becomes “incurable.”

For the reasons described above, studying PDAC initiation in humans is difficult. However, much insight has been gained from genetically engineered mouse models (GEMMs), which have been refined over the years to recapitulate many of the hallmarks of human PDAC initiation and progression (Murtaugh, 2014). Mouse and human pancreata are similar in histology and function; in both species, the adult pancreatic epithelium is largely quiescent and is comprised of several different cell types, including acinar cells, duct cells, and islet cells. Acinar cells produce and secrete digestive enzymes

such as lipases and proteases, which are carried in an inactive state to the duodenum through a tubular network of duct cells. Together, acinar and duct cells comprise the exocrine pancreas from which PDAC arises. Notably, PDAC is thought to arise through a classic progression sequence, where mutations in the epithelium give rise to pre-cancerous lesions termed pancreatic intraepithelial neoplasia (PanINs) (Hruban et al., 2000; Morris et al., 2010b). PanINs evolve as they accumulate additional mutations and can eventually become invasive. Desmoplasia, a fibroinflammatory reaction to the mutating epithelium, increases with mutational burden and eventually forms the tumor stroma.

The defining mutational event in >90% of human PDAC cases is a single base substitution in the proto-oncogene *KRAS* (*Kras*^{G12D} or *Kras*^{G12V}) (Almoguera et al., 1988; Bailey et al., 2016; Maitra and Hruban, 2008; Waddell et al., 2015). The *KRAS* gene encodes a small GTPase signaling protein, which propagates a pro-proliferative signaling cascade in response to mitogen stimulation. Mutational events in cancer lock the KRAS protein in an activated, GTP-bound state, which allows for continuous propagation of pro-proliferative signaling through ERK and other pathways (di Magliano and Logsdon, 2013). It is worth noting that RAS molecules are notoriously hard to drug, and targeting its major downstream effectors produces unfortunate side-effects in patients (Collins and Pasca di Magliano, 2013; McCormick, 2015). Thus, there is a great interest in understanding how RAS proteins intersect with other, targetable cellular pathways. While most precancerous PanINs lack the full mutational spectrum of invasive PDAC, they almost universally harbor one of these *KRAS* alterations, suggesting that *KRAS* mutation is necessary for the earliest stages of pancreatic cancer initiation (Hosoda et al., 2017;

Maitra and Hruban, 2008). But which exocrine cells sustain an initial genetic "hit" in *KRAS* to initiate PDAC?

In the early 2000s, a first generation of transgene-based GEMMs were developed to identify the cell of origin of PDAC. Because the epithelium of PDAC resembles pancreatic duct cells, it was long hypothesized that ducts must be the cell of origin of PDAC. However, driving $Kras^{G12V}$ expression with the promoter of the duct-specific gene *Cytokeratin-19* (*Krt-19*) had no significant effect on pancreatic morphology, producing only a mild inflammatory response without ductal dysplasia (Brembeck et al., 2003). In contrast, expressing oncogenic $Kras^{G12D}$ under the control of the acinar-specific *Elastase1 (Ela1)* promoter lead to the formation of lesions with mixed acinar and ductal characteristics (Grippo et al., 2003). While these mixed lesions did not directly resemble human PanINs (Grippo et al., 2003; Hruban et al., 2006), these studies were among the first to indicate that duct-like tumor precursors might arise from acinar cells.

The GEMMs described above, however, had shortcomings that prevented definitive conclusions. For one, over-expressing oncogenic *Kras* can lead to cellular senescence – a problematic phenotype when studying cancer (Serrano et al., 1997). Another concern is that the *Ela1* promoter might not be active in transformed cells or PanINs, as cells comprising these structures do not express *Elastase1* or any other acinar-specific genes. Therefore, the expression of oncogenic *Kras* might be transient in the lesions observed by Grippo and colleagues, explaining the mixed acinar and ductal phenotype (Collins et al., 2012; Grippo et al., 2003). These issues were partially solved by the laboratories of Tyler Jacks and Mariano Barbacid with the construction of conditional mutant mouse *Kras* alleles, expressed from the endogenous *Kras* locus

(Guerra et al., 2003; Tuveson et al., 2004). These two alleles have a similar design: both contain a polyadenylation "STOP" cassette, flanked by loxP sites, upstream of a mutation in exon 2 that changes the Gly12 codon to aspartic acid or valine (*Kras*$^{LSL\text{-}12D}$ or *Kras*$^{LSL\text{-}G12V}$ [LSL = loxP-STOP-loxP]). In the absence of Cre-recombinase, the STOP cassette prevents the expression of oncogenic *Kras*. However, when an active Cre enzyme is present, it removes the transcriptional STOP signal and allows for the permanent expression of oncogenic *Kras* at the endogenous level. Importantly, these alleles do not promote the senescence phenotype associated with RAS overexpression (Guerra et al., 2003; Serrano et al., 1997; Tuveson et al., 2004).

To direct expression of *Kras*^{G12D} exclusively to the pancreas, cancer researchers have employed tools from the developmental biology community. Using the *Pdx1* promoter (*Pdx1-Cre*) (Gu et al., 2002) or the endogenous *Ptf1a* locus (*Ptf1a*^{Cre}) (Kawaguchi et al., 2002) to express Cre allows for recombination of the *Kras* locus in progenitor cells of the early embryonic pancreas, thus inducing expression of oncogenic *Kras* throughout the pancreatic epithelium. The combination of *Pdx1-Cre* and *Kras*$^{LSL\text{-}G12D}$ is currently the most commonly used model of PDAC and is often referred to as the "KC" (Kras, Cre) mouse. As its wide usage would suggest, the KC mouse model recapitulates many hallmarks of human disease, most prominently including progressive disease development. Low grade PanIN-1 lesions are first observed in pancreata of KC mice at ~2 months age, albeit infrequently (Aguirre et al., 2003; Hingorani et al., 2003). These lesions become more numerous and dysplastic with age; however, they tend not to progress to *bona fide* PDAC unless additional mutations/deletions in tumor suppressor genes are engineered into the genetic background (Aguirre et al., 2003; Bardeesy et al.,

2006; Hingorani et al., 2003; Hingorani et al., 2005). While the KC mouse is an extremely useful model for PDAC research, it still has shortcomings. For one, pancreatic cancer is primarily a disease of aging; thus, activation of oncogenic *Kras* early in the embryonic pancreas may not recapitulate the true disease course. Second, the expression of oncogenic *Kras* throughout the pancreatic epithelium precludes identifying the cell of origin of PanINs and PDAC. Finally, the ubiquitous activation of $Kras^{G12D}$ in the pancreatic epithelium is unlike human disease, where mutations likely occur sporadically in random, isolated cells.

How could these issues be addressed? The introduction of lineage-specific, inducible Cre lines led to a series of elegant studies revealing that acinar cells are a primary cell of origin of both PanINs and PDAC. To test whether acinar cells could give rise to PanINs, the *Elastase1* promoter (*Ela-CreERT*), or the endogenous *Mist1* ($Mist1^{CreERT2}$) or *Ptf1a* ($Ptf1a^{CreERT}$) loci are used to express a Cre-recombinase fused to a modified form of the estrogen receptor (ER) ligand binding domain. This Cre-ER complex remains inactive in the cytoplasm until tamoxifen (TM) is administered. Upon TM treatment, Cre enters the nucleus and recombines the $Kras^{LSL-G12D}$ locus, inducing expression of oncogenic $Kras^{G12D}$ specifically in adult acini. In 2008, our lab, along with Drs. Steven Leach, Stephen Konieczny and Anirban Maitra used these models to demonstrate that acinar-specific $Kras^{G12D}$ lead to the spontaneous formation of mouse PanINs in <2 months (De La O et al., 2008; Habbe et al., 2008). Consistent with the PanIN-PDAC progression model, the lab of Craig Logsdon went on to establish that acinar-specific activation of $Kras^{G12D}$ and simultaneous deletion of the tumor suppressor *Tp53* led to advanced PanIN formation and death from invasive PDAC within 6 months

(Ji et al., 2009). These studies were the first to definitively show that acinar cells could serve as a cell of origin for PDAC, despite the ductal phenotype of PanINs and carcinoma.

Are duct cells also capable of giving rise to PanINs and subsequent PDAC? To answer this question, the lab of Maike Sander developed a *Sox9-CreER* BAC transgenic line, which could be utilized to express $Kras^{G12D}$ specifically in adult duct cells (Kopp et al., 2011). While they confirmed that $Kras^{G12D}$ expression in acinar cells readily gives rise to PanINs, duct cells (including centroacinar cells that occupy the junction between acinus and duct) were almost completely resistant to transformation by $Kras^{G12D}$, even after more than a year (Kopp et al., 2012). These studies strongly suggest that acinar cells are the principal cell of origin in the PanIN-to-PDAC progression sequence. Interestingly, over the past year, the lab of Jen Bailey demonstrated that duct cells with $Kras^{G12D}$ and homozygous point mutations in *Tp53* ($p53^{R172H}$, a variant often seen in human cancer) can form invasive PDAC (Bailey et al., 2015). These carcinomas arise rapidly and do not seem to be associated with PanINs. But, human PanINs rarely contain even heterozygous *Tp53* mutations, suggesting that the PanIN-to-PDAC model is exclusively an acinar derived entity (Hosoda et al., 2017).

In all GEMMs described above, acinar cell transformation by $Kras^{G12D}$ is relatively inefficient; that is, $Kras^{G12D}$ is necessary but not sufficient for PanIN formation even when ubiquitously expressed in the acinar cell population. In fact, most acinar cells expressing $Kras^{G12D}$ remain resistant to transformation for the entire life of the animal. What then inhibits $Kras^{G12D}$-mediated transformation in acinar cells? One intriguing hypothesis is that the transcriptional programs that maintain acinar cell identity prohibit

the reprogramming of acini into PanINs (Murtaugh, 2014; Rooman and Real, 2012). Central to the acinar differentiation program is the PTF1 complex, which consists of three transcription factors: Rbpj-L, a member of the Suppressor of Hairless transcription factor family; one of several ubiquitously-expressed bHLH E-proteins; and, forming a heterodimer with the E-protein, the bHLH factor pancreas-specific transcription factor 1a, or Ptf1a (Beres et al., 2006; Masui et al., 2010; Rose et al., 2001). Importantly, Ptf1a regulates the expression of itself and other transcriptional mediators, such as *Nr5a2* and *Mist1*, with which it cooperates to control transcription of secretory proteins and zymogens, exocytosis components, and regulators of endoplasmic reticulum stress – functions all crucial to normal acinar cell function and homeostasis (Hess et al., 2016; Holmstrom et al., 2011; Jiang et al., 2016; Kim et al., 2015; Masui et al., 2008).

The hypothesis that this transcriptional network could inhibit *Kras*-mediated transformation is born out of both histological and genomic data. For example, when $Kras^{G12D}$ and Notch signaling synergistically reprogram acinar cells into PanINs, Ptf1a is rapidly lost (De La O et al., 2008). This results in complete reprogramming of the cell, represented by a shift away from acinar-specific gene expression and upregulation of ductal genes and mucin production (Habbe et al., 2008). Additionally, genome-wide association studies identified PDAC risk-associated single nucleotide polymorphisms (SNPs) in *NR5A2*, a protein that promotes acinar-specific gene expression in collaboration with Ptf1a (Holmstrom et al., 2011; Li et al., 2012; Petersen et al., 2010). Consistent with these data, when loss of loss of *Nr5a2* is compounded with $Kras^{G12D}$ activation in acinar cells, PanIN formation is dramatically increased (Flandez et al., 2014; von Figura et al., 2014). Taken together, these data suggest that loss of acinar identity

might be a key factor contributing to *Kras*^{G12D}-mediated PanIN initiation and subsequent PDAC formation. Interestingly, mouse *Nr5a2* is also required for resolution of organ injury following acute pancreatitis, highlighting the tension between acinar cell differentiation and inflammation (von Figura et al., 2014).

Pancreatitis itself is one of the most significant risk factors for PDAC development (Duell et al., 2012; Lowenfels et al., 1993). Several studies, from our lab and others, have translated this human epidemiological data into experimental mechanism; for example, pharmacological induction of pancreatitis in mice with acinar mutant *Kras* greatly accelerates the number and severity of PanINs (De La and Murtaugh, 2009; Guerra et al., 2007; Morris et al., 2010a). Interestingly, progenitor-like gene expression and downregulation of acinar-specific genes is a transient hallmark of pancreatic regeneration following acute injury (Jensen et al., 2005; Karki et al., 2015). This includes downregulation of Ptf1a target genes, and increased expression of the progenitor markers *Pdx1* and *Sox9* as well as upregulation of Notch signaling components (Jensen et al., 2005; Karki et al., 2015; Murtaugh et al., 2003). Could this brief loss of cell identity be co-opted by *Kras*^{G12D} leading to PanIN formation? These findings ultimately suggest that even a brief window of acinar dedifferentiation creates an intermediate cellular phenotype that is uniquely sensitive to *Kras*-mediated transformation.

Based on these findings, we hypothesized that loss of *Ptf1a,* the master regulator of acinar cell identity, is a crucial and rate-limiting step in PanIN/PDAC formation. Through my dissertation project, we have demonstrated that loss of *Ptf1a* alone is sufficient to induce acinar cell reprogramming and a gene expression profile resembling

that of RAS-addicted human cancer cells. Consequently, deletion of *Ptf1a* accelerates both PanIN and invasive PDAC formation in the presence of $Kras^{G12D}$ (Krah et al., 2015). Our unpublished results suggest that sustaining *Ptf1a* expression prevents PanIN formation, and that reintroduction of *Ptf1a* into established PanINs leads to redifferentiation of dysplastic cells. Taken together, the data presented here establish a role for cell identity factors as a novel class of “noncanonical” tumor suppressor genes, and highlight the future potential for differentiation-based therapy in solid tumors.

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CHAPTER 2

DIFFERENTIATION AND INFLAMMATION; 'BEST ENEMIES' IN GASTROINTESTINAL CARCINOGENESIS

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Trends in Cancer

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Review

Differentiation and Inflammation: 'Best Enemies' in Gastrointestinal Carcinogenesis

Nathan M. Krah[1] and L. Charles Murtaugh[1,*]

While recent studies demonstrate that cancer can arise from mutant stem cells, this hypothesis does not explain why tissues without defined stem cell populations are susceptible to inflammation-driven tumorigenesis. We propose that chronic inflammatory diseases, such as colitis and pancreatitis, predispose to gastrointestinal (GI) adenocarcinoma by reprogramming differentiated cells. Focusing on colon and pancreas, we discuss recently discovered connections between inflammation and loss of cell differentiation, and propose that dysregulation of cell fate may be a novel rate-limiting step of tumorigenesis. We review studies identifying differentiation mechanisms that limit tumor initiation and that, upon reactivation, can prevent or revert the cancer cell transformed phenotype. Together, these findings suggest that differentiation-targeted treatments hold promise as a therapeutic strategy in GI cancer.

Inflammation, Stem Cells, and Metaplasia in GI Tumor Initiation

Chronic inflammatory conditions such as ulcerative colitis (UC), **chronic pancreatitis** (CP, see Glossary), and chronic viral hepatitis increase the lifetime risk of cancer development. Why exactly this might be has puzzled researchers and physicians for some time. Given the regenerative capacity of many GI organs, it has been hypothesized that chronic inflammation causes the gradual accumulation of mutations and epigenetic changes in resident stem cells, leading to their emergence as cancer-initiating cells [1]. However, this argument does not fully explain why organs that apparently lack a major stem cell population, such as the pancreas, are so susceptible to inflammation-driven tumorigenesis [2]. Epithelial **metaplasia**, the process by which one cell type appears to turn into another, is a hallmark of many chronic inflammatory diseases that predispose to cancer [3]. The ability of cells to change their differentiation state in response to injury and stress may explain recent discoveries that the phenotype of a tumor cell – from histology to gene expression profile – may not provide an accurate account of its site of origin.

In various organ systems, numerous elegant studies now support the longstanding hypothesis that resident tissue stem cells can directly give rise to cancer [4]. **Colorectal cancer** (CRC), in particular, is generally considered to be a disease of mutated stem cells [5], which is underscored by the high proliferation capacity of the colon. An interesting observation supporting this hypothesis is that the number of stem cell divisions within a given organ appears to correlate with the lifetime cancer risk [6], suggesting that cancer initiation is mostly due to the 'bad luck' of DNA replication errors in stem cells. Chronic inflammation, by promoting additional rounds of stem cell proliferation to facilitate tissue repair, could increase the chance of replication errors and mutation.

Trends

Dedifferentiation induced by GI tract inflammation enhances oncogenic transformation of cells in the intestine and pancreas, and may be a rate-limiting step in tumor initiation.

Colorectal cancer can arise from direct mutation of crypt stem cells, but can also arise via NF-κB mediated dedifferentiation of mature enterocytes.

Pancreatic ductal adenocarcinoma can arise via reprogramming of acinar cells into pancreatic intraepithelial neoplasia (PanIN) or directly from duct cells without a 'pre-cancerous' PanIN-like intermediate.

NF-κB signaling, as well as pathways crucial for endodermal development, play crucial roles in dedifferentiation and subsequent oncogenesis in the colon, pancreas, and liver.

Reintroduction of acinar differentiation determinants to pancreatic cancer cells prevents proliferation and could hold promise as a therapeutic strategy.

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This conclusion is not without controversy, however, because an independent analysis of the same data argues that extrinsic factors play a much larger role in cancer initiation than predicted by the 'bad luck' hypothesis, favoring alternative models of carcinogenesis in the GI tract and elsewhere that are potentially independent of stem cell division [7]. Stem cell-independent tumor development has been directly observed in animal models: for example, in a zebrafish model of melanoma development driven by mutant *BRAF* and *Trp53*, tumors initiate from differentiated melanocytes that reacquire a progenitor-like identity [8]. Indeed, it is debatable whether stem cells even exist in the pancreas and liver: both organs are generally quiescent in adults, with no need for continuous replenishment, and lineage-tracing studies have not yet identified convincing markers of a stem cell population in either organ [9]. Nonetheless, each of these accounts for more new cancer cases in the USA than the indisputably stem cell-driven stomach (http://seer.cancer.gov/).

Consequently, the question emerges: because stem cells engineered with cancer-causing mutations are capable of giving rise to cancer in mouse [5,10], does that necessarily mean that this is the only mechanism by which human cancer can arise? Could changes in differentiation state, cued by intrinsic mutation or local inflammatory signals, represent a physiologically plausible mechanism to initiate cancer in organs both with and without defined stem cell populations? Based on recent work in colon, pancreatic, and liver cancers, we hypothesize that inflammatory signals alter the transcriptional networks controlling differentiation in these organs, reprogramming mature cells toward a progenitor-like state that is uniquely sensitive to tumor initiation.

CRC Can Arise from Both Stem and Non-Stem Cells

Undergoing continuous cellular turnover, the small and large intestine represent a paradigm of stem cell-driven organ homeostasis [11]. These tissues comprise multiple mature cell types, including enterocytes, goblet cells, enteroendocrine cells, and, in the small intestine, Paneth cells. All of these emerge from multipotent stem cells located in the basal crypts (Figure 1). There appear to be two classes of stem cells, separated by several cells diameters and expressing several unique markers, including the Wnt target gene **leucine-rich repeat-containing G protein-coupled receptor 5**, *Lgr5* ('crypt basal cells', the most basally located stem cells and the best-studied to date) and the transcription factor Bmi1 ('+4 cells', so-called for their position relative to the crypt base). Cells exiting the crypt, moving toward the luminal surface of the intestine, first pass through a **transit-amplifying** (TA) phase in which they undergo rapid expansion, followed by differentiation. The restriction of stem cell activity to the basal crypt suggests that this microenvironment represents a niche for multipotency and self-renewal, maintained in part by paracrine Wnt signaling. Departing from this niche, both the TA and mature cells are restricted in their lineage and in their lifespan, and are destined to be shed into the lumen and replenished by new progeny of the stem cells [11,12].

Conditional deletion and lineage-tracing experiments in mice indicate that intestinal neoplasia can propagate directly from genetically mutated $Lgr5^{+}$ or $Bmi1^{+}$ crypt stem cells [5,10]. For example, deletion of the **adenamatous polyposis coli** (*Apc*) tumor-suppressor gene specifically from adult crypt stem cells using an inducible **Cre recombinase** ($Lgr5$–$CreER^{T2}$) results in the rapid formation of β-$catenin^{High}$, $cMyc^{+}$ microadenomas throughout the mouse intestine [5], resembling the genetic cancer syndrome familial adenomatous polyposis. These results support the **bottom-up hypothesis** of CRC initiation and progression in which polyp growth initiates from a crypt stem cell and then propagates toward the lumen, eventually predisposing to CRC (Figure 1, bottom panel). The long life of the crypt stem cell makes it particularly susceptible to a high burden of cancer-causing mutations because the same mutations occurring in TA or differentiated cells would be predicted to be lost with the cells themselves.

Glossary

Adenomatous polyposis coli (*Apc*): an intestinal tumor suppressor gene that encodes a negative regulator of Wnt/β-catenin signaling. Inherited mutations in human *APC* cause familial CRC predisposition, while sporadic mutations often represent the first genetic 'hits' in sporadic CRC.

Bottom-up hypothesis: the idea that CRC is initiated from a mutated crypt stem cell and propagated from the bottom of the crypt toward the lumen.

Caerulein: oligopeptide similar to cholecystokinin (CCK); stimulates pancreatic secretion and, at supraphysiological levels, induces intrapancreatic activation of digestive enzymes and subsequent pancreatitis.

Chronic pancreatitis (CP): longstanding inflammation of the exocrine pancreas, causing malabsorption and abdominal persistent pain, and significantly increasing the risk of PDAC.

Colorectal cancer (CRC): cancer originating from the epithelium of the colon or rectum. It is the most commonly diagnosed cancer of the gastrointestinal (GI) tract, with over 140 000 cases presenting each year in the USA.

Cre recombinase: bacteriophage enzyme that catalyzes site-specific DNA recombination between nearby *loxP* sites. Commonly utilized for tissue-specific recombination in mouse genetics.

Intraductal papillary mucinous neoplasia (IPMN): putative PDAC precursor lesion that grows within pancreatic ducts and is characterized by thick fluid production. Recent studies suggest that IPMNs arise from cells of the ductal epithelium.

Kras proto-oncogene: an oncogene precursor that is mutated to an 'active' form in 90–95% of PDAC and 30–50% of CRC cases. Once in an active (GTP-bound) conformation, KRAS propagates growth factor signals mediated by RAF/MEK/ERK and PI3K/AKT, among other pathways.

Leucine-rich repeat-containing G protein-coupled receptor 5 (*Lgr5*): a Wnt target gene, encoding a cell-surface receptor, that is expressed exclusively in adult stem cells of the intestine, stomach, and hair follicle.

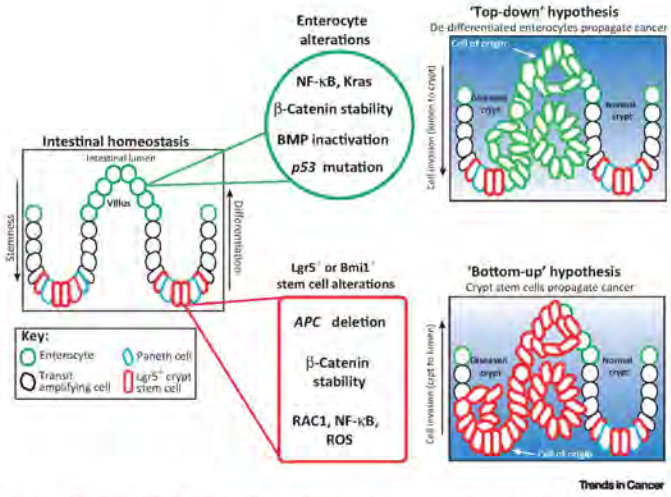

Figure 1. Top-Down Versus Bottom-Up Hypotheses of Colorectal Cancer (CRC) Initiation. During intestinal homeostasis, Lgr5$^+$ or Bmi1$^+$ basal crypt stem cells proliferate and give rise to transit-amplifying (TA) cells (black outline) and, subsequently, differentiated enterocytes and other mature cell types (green). Alterations in non-stem cells, such as simultaneous NF-κB activation and β-catenin stabilization, can produce adenomas, suggesting that CRC can initiate from the top of the villus and grow down into the crypt (top right). This 'top-down' hypothesis proposes that differentiated or committed cells acquire stem-like characteristics to drive tumor growth from the luminal side of the colon. According to the more-traditional 'bottom up' model, mutations occurring directly in Lgr5$^+$ or Bmi1$^+$ crypt stem cells, such as loss of *Apc*, initiate adenoma and CRC formation. The 'bottom-up' hypothesis suggests that mutations accumulate in crypt stem cells as they continually proliferate, and then can serve as the effective cell of origin in CRC; they propagate disease from the base of the crypt, growing toward the lumen (bottom right).

As early as the 1960s, however, physicians observed that early adenomatous polyps are often found at the top of the colonic crypts, suggesting that mature cells in the villus or mucosal layer may dedifferentiate, proliferate, and replace the normal mucosa from the luminal side [13–15] (Figure 1, top panel). Supporting this **top-down hypothesis** of CRC initiation, a recent study demonstrated that CRC initiates from epithelial cells outside the stem cell niche in a mouse model of hereditary mixed polyposis syndrome (HMPS), a familial CRC susceptibility syndrome. HMPS is caused by a 40 kb duplication upstream of the BMP antagonist gene *Grem1* that leads to its ectopic expression in the intestinal epithelium [16]. Misexpression of epithelial *Grem1* in mice results in 'top-down' intestinal polyp formation, originating from an *Lgr5*-negative progenitor cell population that forms outside the crypts [17]. Notably, these Grem1-induced, *Lgr5*-negative progenitor-like cells appear to be dramatically sensitized to CRC initiation, as indicated by a synergistic increase in polyp numbers on an *Apc* mutant background [17]. These results suggest that dysregulation of differentiation by altered microenvironmental signaling, in this case loss of BMP activity, can allow the persistence or reacquisition of stem-like properties such that cells outside of the stem cell niche can serve as tumor initiating cells. Notably, these tumor-initiating cells need not have all the properties of normal stem cells (e.g., *Lgr5* expression); instead, their phenotype may reflect stabilization of a normally transient intermediate cell fate or else the adoption of a new, non-physiological gene expression program that wild-type cells cannot access.

Whether fully differentiated cells ultimately serve as cells of origin for the top-down polyps seen in *Grem1*-misexpressing mice is as yet unknown, in part owing to the scarcity of Cre deletor lines

Metaplasia: from Greek, 'change in form', refers to the reversible change of one tissue type into another. This can occur through multiple mechanisms including transdifferentiation of mature cells, alterations to stem cells such that they produce inappropriately differentiated offspring, or the death of one tissue type and replacement by adjacent cells of another type.
Nuclear factor κB (NF-κB): family of transcription factors rapidly activated by diverse mediators of injury and infection, often regulating downstream inflammatory responses.
Pancreatic ductal adenocarcinoma (PDAC): the most common cancer of the pancreas, often comprising cells with duct-like morphology. About 46 000 cases are diagnosed in the USA each year and the 5 year survival rate is ~6%.
Pancreatic intraepithelial neoplasia (PanIN): putative precursor lesion of PDAC. Recent studies suggest PanINs arise from reprogramming of mature acinar cells.
Top-down hypothesis: the idea that CRC initiates from a differentiated cell on the luminal surface of the colon and subsequently invades the crypt.
Transit-amplifying (TA) cell: daughter cell of a stem cell, destined to undergo a burst of proliferation followed by differentiation. Often committed to a limited range of cell fates.

that specifically mark non-stem cells in the intestine. Recent studies have begun to address this issue, and reveal that 'stemness' in the intestine is a product of both intrinsic and extrinsic factors, including inflammatory signals that can reprogram differentiation and induce tumor-initiating properties [15]. For example, the Greten laboratory has demonstrated that expression of oncogenic versions of the ***Kras* proto-oncogene** or activation of **nuclear factor κB** (NF-κB), in combination with Wnt/β-catenin activation, confers tumor-initiating properties on otherwise quiescent and differentiated intestinal villi [18]. In this system NF-κB acts downstream of oncogenic *Kras* as a target of *Kras*-induced inflammatory signaling. Using an *Xbp1s-CreERT2* deletor mouse, which allows Cre-mediated recombination specifically outside the *Lgr5*$^+$ stem cell domain, this group found that coactivation of Wnt/β-catenin and NF-κB could induce dedifferentiation of villus cells and rapid polyp development. Interestingly, mutation of the tumor-suppressor *Trp53* promotes NF-κB-dependent inflammation in the mouse intestine [19,20], suggesting that this key inhibitor of CRC and other cancers may act in part by limiting inflammation and subsequent dedifferentiation.

Notwithstanding these results, is it plausible that non-stem cells, with their inherently limited lifespan, could serve as cells of origin for CRC under physiological conditions? Of note, clone-marking studies in the intestine indicate that a subset of TA cells, particularly those restricted to the goblet cell lineage, can persist for months after leaving the crypt [12]. The location of such cells would make them a logical source for top-down polyp generation, particularly if mechanisms exist to further extend their lifespan. Importantly, recent studies indicate that inflammation and tissue damage can override the normal commitment process in the intestine, and rekindle stem cell potential in otherwise lineage-restricted TA populations [21]. For example, enterocyte-restricted TA cells, expressing the alkaline phosphate intestinal (*Alpi*) gene, are capable of reacquiring stem-like properties when Lgr5$^+$ cells are specifically ablated [22]. An earlier study by this group found that secretory lineage-restricted TA cells, marked by Delta-like-1 (*Dll1*) expression, could reconstitute Lgr5$^+$ stem cells after radiation-induced crypt damage [23], although dedifferentiation in this model appears to be less efficient than is observed with *Alpi*$^+$ cells. These studies suggest that TA and recently differentiated cells can act as a stem cell reserve in response to injury, as has been observed in several *Drosophila* organs [24]. Whether this process is driven by NF-κB is unknown; however, these injury models do not appear to be associated with widespread inflammation. In contrast to the observations made with *Xbp1s-CreERT2*, above, *Alpi*$^+$ cells appear to be resistant to transformation *in vivo*, even by the combination of *Apc* loss and *Kras*^{G12D} activation [22]. However, the oncogenic potential of these cells was not tested following Lgr5$^+$ stem cell ablation, or in the context of inflammatory injury. It will be important to determine if NF-κB and other inflammatory pathways can induce tumors from Alpi$^+$ or Dll1$^+$ cells by reprogramming their differentiation state.

These findings support a model in which tumors developing within inflammatory microenvironments, such as that of colitis, arise from dedifferentiation of TA or mature cells rather than from stem cells. While more work is needed to determine whether dedifferentiation occurs during intestinal regeneration, suggestive findings indicate significant cell fate rearrangement during this process. In human ulcerative colitis, for example, expression of the intestinal stem cell marker *OLFM4* expands into the superficial epithelium overlying lesions [18]. Meanwhile, mice subjected to experimental colitis exhibit almost complete loss of Lgr5$^+$ cells in the distal colon, followed by reappearance of stem cells as the injury is resolved [25]. Whatever their origin, these newly formed stem cells could promote regeneration by supporting tissue remodeling and epithelial barrier restoration [21], while at the same time representing an expanded pool of cells susceptible to tumor initiation.

Going forward, it will be interesting to interrogate the mechanisms of reprogramming in the intestine, including how pro-tumorigenic signaling pathways such as NF-κB, Kras, and

Wnt/β-catenin interact with pro-differentiation transcription factors, such as Hnf1a, Hnf1b, and Klf4 [26,27]. It will also be important to determine whether there is any relationship between mutational 'drivers' and the cell of origin. A recent study indicates that CRC arising in patients with preexisting inflammatory bowel disease has a unique mutational spectrum compared to sporadic CRC, including a lower prevalence of *APC* mutations and a higher prevalence of mutations in *SOX9*, a transcription factor that, in the intestine, is expressed in crypts and required for normal differentiation [28,29]. It should be possible to determine whether *Sox9*-knockout intestines exhibit a pro-tumorigenic response to colitis, and whether this reflects cell fate plasticity in TA or mature cells.

Pancreatic Cancer Is Initiated from Differentiated Epithelial Cells

Unlike the intestine, the pancreas is a quiescent organ with no obvious need for a dedicated stem cell population. Numerous lineage-tracing studies of both exocrine and endocrine cells support a model in which each cell type is maintained by self-renewal rather than by stem cell replenishment [9]. The pace of self-renewal is accelerated by injury, but influx of newly differentiated cells appears to be minimal or non-existent even during regeneration. For example, animal models of acute pancreatitis exhibit widespread destruction of digestive enzyme-producing acinar cells, which are replaced upon injury resolution by proliferation of surviving differentiated cells [30]. In considering the potential origins of **pancreatic ductal adenocarcinoma** (PDAC), therefore, attention has focused on differentiated cells.

Activation of the *KRAS* proto-oncogene occurs in ~90% of all human PDAC cases [31,32] and mouse models reveal that $Kras^{G12D}$ drives the formation and maintenance of both PDAC and pre-cancerous **pancreatic intraepithelial neoplasia** (PanIN) [33]. In the 'KC' ($Kras^{G12D}$, Cre) mouse model, activation of a $Kras^{G12D}$ allele in the embryonic pancreas by *Pdx1–Cre* or *Ptf1a/p48–Cre* causes focal PanIN formation [34] (Figure 2). When subjected to additional genetic 'hits' in tumor-suppressor genes, such as *Trp53* or *Cdkn2a* (*p16-INK4A/p19-ARF*), mice develop invasive PDAC [35–37] (Figure 2). Because the *Pdx1* and *p48/Ptf1a* promoters drive Cre expression in nearly every pancreatic epithelial cell beginning *in utero* (Figure 2), it is not possible to determine the PDAC cell of origin using these models. While duct cells might seem to be an obvious source for duct-like PanINs and PDAC, driving $Kras^{G12D}$ expression specifically within ducts generates very few, if any, PanINs or alternative PDAC precursors, such as **intraductal papillary mucinous neoplasm** (IPMN) [38,39] (Figure 3). Conversely, when $Kras^{G12D}$ is expressed exclusively in adult acinar cells, PanINs, and [with mutation of *Trp53* or *Cdkn2a* (*p16-INK4A/p19-ARF*)] PDACs are generated with nearly 100% penetrance [38,40–42] (Figure 3).

While these findings suggest that acinar cells are susceptible to transformation, it is noteworthy that most acinar cells are not detectably altered by $Kras^{G12D}$ expression, with only a small minority giving rise to PanINs. The fact that acinar cells preserve their normal identity in the face of oncogenic *Kras* leads to the hypothesis that pro-differentiation factors restrain transformation by preserving cell identity [33,43]. Interestingly, **caerulein**-induced pancreatitis, in which acinar cells transiently upregulate progenitor-like gene expression [44,45], greatly accelerates PanIN development from *Kras*-mutant acinar cells [46–48]. The effects of caerulein pancreatitis are normally transient in wild-type acinar cells, but acinar cells expressing mutant *Kras* are incapable of regenerating normally after this acute injury, and the disease evolves into a chronic form similar to that associated with increased PDAC risk in humans [2,49]. Interestingly, a high-fat diet can also promote PanIN formation from Kras-mutant acini in a COX-2-dependent manner, perhaps modeling the PDAC-promoting inflammatory effects of obesity in humans [50]. By contrast, PanIN formation is not accelerated from $Kras^{G12D}$ mutant duct cells following caerulein-induced pancreatitis [33]. These findings suggest that acinar but not duct cells initiate PDAC in response to the combination of mutant *Kras* and pancreatitis, and further suggest that human pancreatitis-associated PDAC may arise from acinar cells.

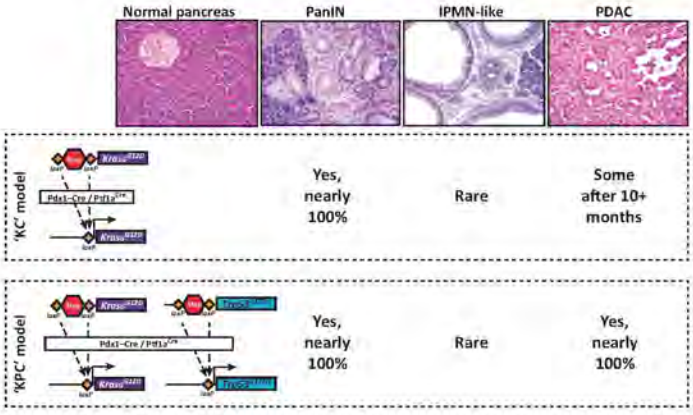

Figure 2. Mouse Models of Pancreatic Ductal Adenocarcinoma (PDAC) Replicate Human Pathology. To activate cancer-causing alleles specifically within the mouse pancreatic epithelium, Cre recombinase is expressed under the control of the *Pdx1* or *Ptf1a/p48* promoter, both of which are active in embryonic pancreatic progenitor cells. In the 'KC' (*Kras/Cre*) model, one copy of the endogenous *Kras* allele is mutated to change amino acid 12 from glycine to aspartic acid, locking the Kras protein in its GTP-bound (active) form. A *loxP*–STOP–*loxP* cassette (LSL) is placed upstream of this mutation and, before recombination, inhibits transcription of the mutant allele. When Cre is expressed it excises the STOP cassette, activating $Kras^{G12D}$ in all epithelial cells of the early pancreas. This produces fully-penetrant pancreatic intraepithelial neoplasia (PanIN) formation, rare intraductal papillary mucinous neoplasia (IPMN) formation, and occasional PDAC (in ~50% of aged animals). In the 'KPC' (*Kras/p53/Cre*) model of invasive PDAC, the same oncogenic *Kras* allele is activated along with a *Trp53* 'gain-of-function' (neomorphic) allele. The R172H point mutation in this *Trp53* allele models a mutation frequently found in human PDAC. Expression of both $Kras^{G12D}$ and $p53^{R172H}$ using *Pdx1–Cre* results in fully-penetrant PanIN and PDAC development in young animals.

As in the intestine, pancreatic injury is associated with changes in differentiation that facilitate recovery. In particular, the expression and secretion of acinar digestive enzymes that drive tissue damage are downregulated during experimental pancreatitis, preventing further damage and allowing regeneration [44,45]. This is accompanied by activation of inflammatory pathways such as NF-κB and JAK/STAT; in the context of oncogenic *Kras* expression, these pathways can stimulate tumor initiation via dedifferentiation. For example, $Kras^{G12D}$ cooperates with inflammatory stimuli to induce NF-κB activation in acinar cells; in turn, the upstream NF-κB activators IKK2 and NEMO are necessary to maintain high levels of signaling downstream of $Kras^{G12D}$, creating a proinflammatory positive feedback loop that is required for acinar reprogramming and for PanIN and PDAC development [49,51,52]. It should be noted that a very recent study identified an unexpected PanIN-suppressive role of the NF-κB family member *RelA*, in other words the opposite knockout phenotype to that of *Ikk2* and *Nemo* [53]. *RelA* appears to be essential for the activation of the senescence-associated secretory phenotype (SASP), in which high oncogene activity stimulates release of proinflammatory cytokines to both enforce local senescence and promote clearance of potential tumor-initiating cells. These results suggest that IKK2 and Nemo have important targets apart from RelA, either other NF-κB family members or entirely novel factors that are essential for reprogramming of acinar cells.

What are the pro-differentiation factors that might act to preserve acinar cell identity in the face of oncogenic *Kras*? In adult acini, a network of transcription factors cooperate to promote the expression of acinar-specific digestive enzymes and maintain cell differentiation [30]. Among

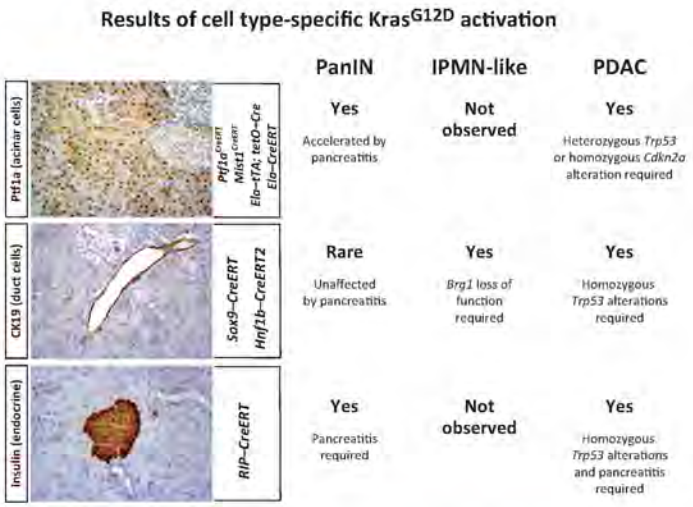

Figure 3. Outcome of *Kras*^{G12D} Activation in Different Adult Pancreatic Cell Types. Expression of *Kras*^{G12D} specifically in acinar cells (Ptf1a^{+} nuclei, top panel) using *Ptf1a*^{CreER}, *Mist1*^{CreERT}, elastase (*Ela*)–*tTA tetO–Cre*, or *Ela–CreERT*, results in focal PanIN formation, which can be greatly accelerated and enhanced by pancreatitis. When compounded with mutations in either *p53* (heterozygous) or *p16-INK4a/p19-ARF* (homozygous), PDAC develops within 2–8 months. Activating *Kras*^{G12D} using a duct-specific *Sox9–CreERT* or *Hnf1b–CreER*^{T2} transgene (CK19^{+} duct cells, middle panel) only leads to rare PanIN formation even in the presence of pancreatitis. Combining duct-specific *Kras*^{G12D} expression and *Brg1* deletion, however, induces lesions that are reminiscent of IPMNs, while combining ductal *Kras*^{G12D} with homozygous *p53*$^{R172H/R172H}$ induces rapid PDAC development without intermediate PanIN precursors. Finally, one study has found that combining *Kras*^{G12D} expression in β-cells (insulin^{+} cells, bottom panel) with pancreatitis is sufficient to induce PanIN formation, while the combination of β cell-specific *Kras*^{G12D}, pancreatitis, and homozygous *Trp53* deletion can produce PDAC.

these are MIST1 (also known as BHLHA15), NR5A2 (also known as LRH-1), and the multiprotein PTF1 complex. The core of this complex is the bHLH transcription factor PTF1A, which is essential to maintain acinar cell differentiation through adulthood [54–57]. Indeed, conditional deletion of *Ptf1a*, *Mist1*, or *Nr5a2* greatly accelerates *Kras*^{G12D}-mediated PanIN formation, causing rapid acinar reprogramming as well as widespread inflammation similar to that seen in caerulein-treated KC mice [54,58–60]. In addition, genetic deletion of *Nr5a2* or *Ptf1a* in the context of acute pancreatitis is sufficient to reprogram the acinar epithelium to a ductal fate, with increased (wild-type) RAS signaling and persistent inflammation [54,59]. These findings indicate that the acinar transcriptional network is necessary for regeneration and may restrain the positive feedback between activated *Kras* and inflammation that drives PDAC initiation [61].

Therefore, we have proposed that two distinct levels of tumor suppression exist during the evolution of PDAC. Under homeostatic conditions, a mutually reinforcing transcriptional network maintains acinar differentiation and suppresses both intrinsic and extrinsic programs that promote PDAC initiation [54,58–60]. *Kras* mutations are tolerated at the cellular level, but may furnish the raw material for later inflammation-induced carcinogenesis. Local or widespread injury to the pancreas acts in *Kras*-mutant acinar cells to silence pro-differentiation factors and facilitate a dramatic switch to a duct-like, precancerous phenotype. The initial formation of

PanINs thus represents evasion of this first, epigenetic, tumor-suppression mechanism. The subsequent progression of PanINs to invasive cancer is opposed by a second, canonical mechanism of tumor suppression (e.g., *TRP53* and *CDKN2A* mutation). The fact that epidemiological risk factors for PDAC such as pancreatitis and obesity appear to impact on the acinar reprogramming process highlights the importance of this novel tumor-suppressor mechanism.

Are Acinar Cells the Only Cell of Origin for PDAC?

While acinar cells are the most plausible cell of origin for PanINs in mice, one study reported that insulin-producing β-cells could give rise to PanINs in the context of combined $Kras^{G12D}$ activation and pancreatitis [62] (Figure 3). Because islets remain generally intact and functional in most PDAC and pancreatitis models, this finding may reflect the properties of a unique subset of β cells. Interestingly, the RIP–CreERT mouse line used to express $Kras^{G12D}$ in the endocrine compartment has previously been shown to recombine 3–6% of acinar cells in addition to islet cells [63], and this could account for the PanIN formation observed in these studies. Whether non-exocrine cells may contribute to pancreatitis-associated PDAC in human patients remains unknown, and these results require follow-up studies to exclude technical artifacts.

Interestingly, other recent studies suggest that exocrine duct cells can also give rise to PDAC independently of the conventional PanIN progression model. To test whether duct cells could give rise to invasive PDAC, Bailey and colleagues generated a novel inducible Cre line to express $Kras^{G12D}$ and $Trp53^{R172H}$ specifically in the pancreatic ductal epithelium. Whereas expressing $Kras^{G12D}$ and a single copy of $Trp53^{R172H}$ in ducts had little effect, compounding $Kras^{G12D}$ mutation with homozygous expression of $Trp53^{R172H}$ led to lethality from PDAC only 8 weeks after induction (Figure 3). Intriguingly, duct-derived PDAC in $Kras^{G12D}$ $Trp53^{R172H/R172H}$ mice developed without associated PanIN precursors, suggesting that invasive carcinoma arises directly from these mutated cells [64]. While these results confirm that ducts are relatively resistant to transformation, they also demonstrate that these cells can initiate PDAC in the context of homozygous *Trp53* mutations. However, because even heterozygous *TP53* mutations are uncommon in human PanINs [65], these results also imply that the conventional PanIN–PDAC sequence represents a uniquely acinar-derived entity.

Recent studies from the Hebrok laboratory additionally suggest that the PDAC precursor lesion IPMN arises from mutated duct cells. For example, deletion of *Brg1*, a component of the SWI/SNF chromatin remodeling complex (also called SMARCA2) in a 'KC' ($Kras^{G12D}$ activated throughout the pancreas) background produces lesions similar to IPMNs [66] (Figure 3). Deleting *Brg1* in adult acinar cells inhibits *Kras*-induced PanIN development, while its deletion in $Kras^{G12D}$-expressing adult duct cells drives IPMN-like dysplasia, which is otherwise never seen with duct-specific *Kras* activation. Further studies demonstrated that *Brg1* is necessary to maintain duct cell differentiation and thus attenuate the formation of IPMN-like lesions [67]. Intriguingly, the antagonism between differentiation and tumor initiation observed in acinar cells also appears to apply in ducts. While duct differentiation is less well studied than that of acinar cells, the transcription factor *Sox9* is important in the pancreas for developmental specification of the duct lineage, and is thereafter restricted in expression to these cells [68,69]. *Sox9* is downregulated in the IPMN-like lesions that develop from *Brg1*-deleted, $Kras^{G12D}$-expressing ducts, and ectopic expression of *Sox9* prevents lesion development and preserves duct differentiation [67].

In contrast to ducts, ectopic *Sox9* expression in acinar cells promotes metaplasia, and *Sox9* is required for acinar-derived PanIN formation [38]. In addition, as noted above, deletion of *Brg1* within $Kras^{G12D}$-expressing acini restrains PanIN formation [66]. These observations suggest divergent roles for *Brg1* and *Sox9* in the initiation of PanINs and IPMNs, and highlight the importance of understanding the cell of origin of PDAC precursors. They also raise the possibility

that the mutational profile of human PDAC may indicate its origin: approximately one-third of PDAC samples harbor somatic mutations affecting the SWI/SNF complex [70,71]. Do these mutations identify tumors of ductal origin, and would those tumors fail to respond to hypothetical drugs targeting Sox9 or Brg1? Moving forward, we propose that understanding the differential role of these and other genes in tumor initiation will provide new and selective treatment approaches.

It should be noted that the reprogramming that occurs in pancreatic tumor initiation is not as simple as a switch between mature cell fates. PanIN lesions, in particular, are known to express markers not only of pancreatic ducts but also of stomach and bile duct epithelium [72,73]. Thus, pancreatic cells that lose their capacity for normal differentiation may adopt non-physiological gene expression programs, possibly analogous to the aberrant, *Lgr5*-negative progenitor cells that populate HMPS colon polyps [17]. In addition to regulating mature cell functions, an important function of differentiation regulators such as *Ptf1a*, *Brg1*, and *Sox9* may be to close-off aberrant cell fates that, while accommodating to the growth of the cell, are harmful to the life of the organism.

Can Dedifferentiation and Redifferentiation Be Exploited as Therapeutic Targets?

We propose that acinar and ductal differentiation determinants form an early, epigenetic barrier to PDAC initiation, and it will be important to determine whether reactivating their expression could halt PDAC progression even after genetic mutation of canonical tumor suppressors. Most of the work to date has focused on the initiation of tumorigenesis, leaving open the question of whether fully malignant cells are susceptible to redifferentiation. On this front, Itkin-Ansari and colleagues recently demonstrated that expressing the bHLH transcription factor E47 (a component of the PTF1 complex) in human PDAC cell lines actively triggers G0/G1 arrest, induces the expression of acinar differentiation markers, and inhibits tumorigenesis in mouse xenografts. Thus, PDAC cells retain an important degree of plasticity and, given the proper conditions, can be reprogrammed back to a benign phenotype [74]. This concept is also supported by *in vivo* mouse genetic experiments in which sustained *Mist1* expression was found to prevent $Kras^{G12D}$-induced acinar cell reprogramming and PanIN formation [75]. Taken together, these findings suggest that enhancing acinar transcription factor activity could serve as differentiation-based therapy to treat or prevent human PDAC. Going forward, it will be necessary to test whether activation of PTF1A and other acinar network components can reverse PDAC progression and metastatic lesions *in vivo*.

Although less is known about the transcriptional basis for dedifferentiation in the intestine, the signaling pathways driving regeneration and tumorigenesis in this organ are remarkably overlapping with those implicated in the pancreas. These include EGFR, Hippo/YAP, JAK/STAT, Notch, and NF-κB signaling [21,30]. The common role of NF-κB in intestinal and pancreatic tumorigenesis is particularly striking: in both organs, this proinflammatory transcription factor is activated by the small GTPase Rac1, acting through elevated reactive oxygen species (ROS) in an evolutionarily ancient regenerative pathway [76–78]. In other cell types, including endothelium and adipocytes, NF-κB inhibits differentiation-specific gene expression by titrating away essential transcriptional coactivators [79,80]. It will be interesting to determine whether such a model accounts for the importance of this pathway in CRC and PDAC, and whether the requirement for Rac1 and NF-κB in preclinical models of these cancers reflects a role in cellular reprogramming.

Concluding Remarks

While the intestine maintains its epithelium via stem cell replenishment, other organs with regenerative capacity, such as the pancreas and liver, do not have well-defined stem cell populations that contribute to epithelial cell turnover. These differences in tissue maintenance have led to diverging hypotheses about how cancer might initiate in these different organs.

Outstanding Questions

Will determining cell of origin inform clinical treatment decisions? Do tumors that arise via different cellular mechanisms have different expression profiles? Different 'Achilles heels'? Different potential targets?

Do alternative cells of origin have different routes to tumorigenesis?

Are intestinal stem cells generated *de novo* during recovery from colitis or do cells that reactivate Lgr5 expression post-injury derive from pre-existing Lgr5$^+$ stem cells?

Why are Alpi$^+$ TA cells able to form tumor organoids in culture, but not tumors when directly mutated *in vivo*? Could forced dedifferentiation or inflammatory stimuli drive these cells to produce tumors?

What is the role of *p53* in determining tumor cell of origin and tumor progression pathway (e.g., PanIN-dependent vs -independent PDAC)?

Do precancerous adenomas (colon) and PanINs/IPMNs (pancreas) limit tumor progression by restraining potentially oncogenic cells? Is the inflammatory and stromal response associated with dedifferentiation and reprogramming favorable to the host in this regard (Box 2)?

Can differentiation-based therapy revert carcinomous cells to a benign state *in vivo*? What is the most efficient way to screen for drugs that would have this benefit? Could gene therapy be beneficial?

Are there stem cell populations within the pancreas and liver? What cues during injury activate these cells? Can these cell populations also give rise to cancer?

Box 1. Reprogramming and Tumorigenesis in the Liver

The past several years have seen remarkable progress in understanding the cellular basis of regeneration in the liver, next-door-neighbor to the pancreas and intestine [8]. As in the exocrine pancreas, hepatocytes and biliary cells of the liver are predominantly maintained by slow self-renewal, although hepatocyte proliferation occurs preferentially in a pericentral domain exposed to endothelial Wnt ligands [81]. This placid picture changes dramatically after injury, however. First, although new hepatocytes continue to self-renew, the locale of proliferation changes to a periportal domain [82]. Second, new biliary cells are generated by reprogramming of hepatocytes, also in this periportal domain [83]. Biliary cells themselves, by contrast, can self-renew after injury but do not contribute to hepatocytes [8].

The two deadly cancers of the liver, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC), are thought to represent transformed counterparts of normal hepatocytes and biliary cells. Indeed, lineage-tracing studies indicate that mouse HCC arises from hepatocytes [84,85]. The origins of CC are more complex and present remarkable parallels to those of PDAC. In one study, carcinogen-induced CC was found to develop strictly from hepatocytes, but not from biliary cells [86]. A separate study found that deletion of the hepatocyte differentiation factor *Hnf4a* enhances CC development [87], paralleling the role of acinar differentiation factors in suppressing PDAC initiation. Another study, however, found that biliary cells could contribute to CC, but only after homozygous deletion of *Trp53* [88], analogous to the *Trp53*-suppressed contribution of pancreatic duct cells to PDAC [64]. Taken together, these data suggest that cellular reprogramming contributes to at least one form of liver cancer, possibly accounting for the increased risk of human HCC and CC associated with chronic inflammation.

Indeed, intestinal stem cells engineered with cancer-causing mutations are able to give rise to tumors; however, inflammatory signaling can induce a cancer-susceptible state via reprogramming of non-stem and differentiated cells in the colon and pancreas alike. Recent studies support a similar model in the liver, where cell fate reprogramming has been found to occur in both regeneration and tumorigenesis (Box 1).

This model does not exclude an effect for stem cells in the tumor-promoting effects of inflammation. In the intestine, for example, one can imagine a scenario in which one allele of *Apc* is inactivated in a stem cell, while loss of the second allele in a more-differentiated daughter cell results in adenoma initiation only when inflammatory cues are present. While true stem cells have yet to be identified in the pancreas, it is possible that a subset of differentiated cells are specialized for a longer proliferative lifetime, and might present a target for mutation accumulation analogous to stem cells in the crypt. Whether the initial mutational events occur in stem or non-stem cells, we propose that the emergence of cancer-initiating activity in differentiated cells is due to positive feedback between intrinsic changes, such as *Apc* and *Kras* mutations, and

Box 2. Does Inflammation Help To Restrain Precancerous Lesions?

The observation that PDAC arises rapidly in the $Kras^{G12D}$ $Trp53^{R172H/R172H}$ ductal model, without precursor lesions and in the absence of extrinsic injury [64], suggests the intriguing hypothesis that the formation of pre-cancerous lesions, such as PanINs (in PDAC) and polyps (in CRC), can actually be part of a protective host response that physically isolates potential carcinoma cells. Mathematical models of human pancreatic cancer suggest that the progression from initial tumor-driving mutation (most likely *KRAS*) to invasive carcinoma takes over a decade, an interval generally attributed to the accumulation of additional mutations [89]. However, a recent study demonstrates that mouse and human PDAC cells, following organoid culture and orthotopic transplantation, initially form PanIN-like structures, lacking the dysplastic features of carcinoma, and only progress to invasive PDAC after a delay of several months [90]. Thus, even fully endowed with cancer-causing mutations sufficient to kill human patients, PDAC cells can be constrained to a preinvasive phenotype by the local microenvironment. Interestingly, the invasiveness of orthotopic transplanted mouse organoids may depend on the *p53* status of the tumor cells, mirroring what is seen in the $Kras^{G12D}$ $Trp53^{R172H/R172H}$ ductal model. Specifically, cells with homozygous *p53* mutations become invasive within weeks, bypassing the hypothetical constraint of PanINs, while cancerous cells that are heterozygous for *p53* initially form PanINs and are contained for months. Previous work suggests that the fibroinflammatory reaction associated with PanINs and PDAC may act to inhibit, rather than to promote, PDAC progression [91]. This may be mediated in part by the dual role of inflammatory cytokines in promoting pre-tumor senescence and tumor growth, as revealed in the pancreas by deletion of *RelA* [53]. A hypothetical tumor-suppressive role for inflammation may explain why PanINs take decades to progress from initial genetic 'hit' to invasive and metastatic carcinoma [89]. Together, these findings raise the possibility that inflammation may have both positive and negative effects on tumorigenesis, and that PanINs and other preneoplastic lesions may serve as host-protective "dead ends" in which mutation-bearing cells are encapsulated and denied the opportunity to invade and metastasize.

extrinsic signals from inflammatory cells. These alterations destabilize or limit the complete maturation of differentiated cells and render them susceptible to oncogenic transformation. Even in the absence of chronic disease such as colitis and pancreatitis, shared risk factors between CRC and PDAC, such as obesity and smoking, are also known to promote systemic inflammation, which could stimulate cellular reprogramming in cooperation with genetic changes.

The studies discussed here leave numerous important issues unresolved and awaiting future work (see Outstanding Questions). Among the most central is the question of how to translate these findings to improved clinical outcomes. Defining the epigenetic changes that divert quiescent or short-lived cells to immortal tumor precursors may identify new targets for therapy based on restoring the memory of the cell of origin and thus neutralizing the effect of otherwise irreversible genetic lesions.

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CHAPTER 3

THE ACINAR DIFFERENTIATION DETERMINANT PTF1A INHIBITS INITIATION OF PANCREATIC DUCTAL ADENOCARCINOMA

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RESEARCH ARTICLE

The acinar differentiation determinant PTF1A inhibits initiation of pancreatic ductal adenocarcinoma

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Abstract Understanding the initiation and progression of pancreatic ductal adenocarcinoma (PDAC) may provide therapeutic strategies for this deadly disease. Recently, we and others made the surprising finding that PDAC and its preinvasive precursors, pancreatic intraepithelial neoplasia (PanIN), arise via reprogramming of mature acinar cells. We therefore hypothesized that the master regulator of acinar differentiation, PTF1A, could play a central role in suppressing PDAC initiation. In this study, we demonstrate that PTF1A expression is lost in both mouse and human PanINs, and that this downregulation is functionally imperative in mice for acinar reprogramming by oncogenic KRAS. Loss of *Ptf1a* alone is sufficient to induce acinar-to-ductal metaplasia, potentiate inflammation, and induce a KRAS-permissive, PDAC-like gene expression profile. As a result, *Ptf1a*-deficient acinar cells are dramatically sensitized to KRAS transformation, and reduced *Ptf1a* greatly accelerates development of invasive PDAC. Together, these data indicate that cell differentiation regulators constitute a new tumor suppressive mechanism in the pancreas.
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Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis, with a 5-year survival rate around 5% and a cure rate approaching zero. The most up-to-date chemotherapy regimens extend life only minimally (*Ryan et al., 2014*), and patients undergoing resection of ostensibly local tumors almost invariably succumb to recurrent disease. While this observation suggests that PDAC is usually metastatic at the time of diagnosis, recent studies suggest that tumors require over 20 years to evolve from precancerous pancreatic intraepithelial neoplasia (PanIN) to invasive carcinoma (*Yachida et al., 2010*). Thus, in principle, there is a large window of time for effective and early detection, prevention, and treatment, provided appropriate methods are in place. Therefore, defining the cell type of origin and characterizing the process of PanIN-PDAC evolution within the physiologic context of key risk factors (e.g., chronic pancreatitis, type 2 diabetes, or genetic cancer syndromes [*Ryan et al., 2014*]) is crucial to finding effective therapies.

The vast majority of human PanINs and PDAC contain activating mutations in the *KRAS* oncogene, which have been shown in mice to represent driver mutations for PanIN initiation, maintenance, and progression to PDAC (reviewed in *Pasca di Magliano and Logsdon, 2013*). Notably, the progression of PanINs to PDAC is accompanied by additional mutations in tumor suppressor genes, such as *INK4A*, *CDKN2A*, *TRP53* (commonly referred to as *P53*), and *DPC4/SMAD4* (*Ryan et al., 2014*). While

eLife digest Pancreatic cancer is one of the most lethal forms of cancer, with fewer than 20% of people surviving for longer than twelve months after diagnosis. Two types of genetic mutation play important roles in pancreatic cancer. First, genes called oncogenes can be activated by mutations to drive unscheduled cell division. Second, the genes for tumor suppressors—proteins that prevent cells from dividing when they should not—can be switched off due to other mutations. Together, these mutations cause cells to over-proliferate and disrupt the structure of the pancreas.

In a healthy pancreas, several different cell types perform various roles: acinar cells produce proteins that digest food, ductal cells carry these proteins to the intestine, and β cells produce insulin. Certain proteins are responsible for telling each of these cells what tasks to perform, which defines their so-called differentiation state. The protein PTF1A is crucial for establishing the differentiation state of acinar cells. In the most common form of pancreatic cancer, acinar cells are reprogrammed to become ductal cells. Moreover, pancreatic cancer cells contain much lower levels of PTF1A than normal pancreatic cells.

To explore the connection between PTF1A and pancreatic cancer, Krah et al. deleted the gene for PTF1A in mice. This led to acinar cells being reprogrammed to become ductal cells. Additionally, when an oncogene mutation was activated at the same time as the gene for PTF1A was deleted, Krah et al. observed the rapid formation of large numbers of malignant pancreatic tumors in the mice. PTF1A therefore protects against pancreatic cancer by acting as a tumor suppressor and keeping acinar cells in their healthy, differentiated state.

Unlike other tumor suppressors, however, PTF1A levels are reduced in cancer cells by a mechanism that does not involve a genetic mutation. Therefore, a future challenge is to determine how the amount of PTF1A protein is reduced, and in the longer term, to explore if it is possible to reverse cancer progression by forcing cancer cells back into their original differentiation state.

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the initiation and progression of PDAC has understandably been difficult to study in human patients or to model in human tissue (**Boj et al., 2015**), much has been learned from the 'KC' mouse model in which a Cre-inducible oncogenic Kras allele ($Kras^{LSL\text{-}G12D}$) causes focal PanIN formation when activated universally in the pancreas (**Aguirre et al., 2003**; **Hingorani et al., 2003**; **Murtaugh, 2014**). Until recently, PDAC was primarily thought to originate from pancreatic ductal cells because of the cancer's duct-like epithelial phenotype. However, recent studies indicate that PanINs (**De La et al., 2008**; **Habbe et al., 2008**; **Kopp et al., 2012**) and PDAC (**Ji et al., 2009**) can be initiated by activating oncogenic $Kras^{G12D}$ expression specifically within mature acinar cells, while $Kras^{G12D}$ activation in adult duct cells or centroacinar cells has little or no effect. Interestingly, even in the KC mouse model, where embryonic Cre recombinase activity directs $Kras^{G12D}$ expression to nearly every cell of the mature pancreas, only a small number of acinar cells eventually give rise to PanINs. The mechanism by which most acinar cells remain refractory to $Kras^{G12D}$-mediated transformation has not been elucidated. An attractive hypothesis is that the factors that induce and maintain acinar cell differentiation state play a crucial role in inhibiting the acinar cell reprogramming step that serves to initiate PDAC formation and progression (**Rooman and Real, 2012**; **Bailey et al., 2014**; **Murtaugh, 2014**).

Consistent with acinar cells as the cell of origin in PDAC, and acinar cell identity being a protective mechanism against $Kras^{G12D}$-mediated transformation, recent genome-wide association studies identified PDAC risk-associated single-nucleotide polymorphisms in the non-coding region of the gene encoding the acinar differentiation transcription factor *NR5A2*, also known as *LRH-1* (**Petersen et al., 2010**). These findings have been confirmed in mouse studies, where pan-pancreatic loss of *Nr5a2* significantly sensitizes pancreatic cells to *KRAS*-induced PanIN initiation. Additionally, pancreatic *Nr5a2* is necessary to regenerate the acinar compartment following caerulein-induced pancreatitis (**Flandez et al., 2014**; **von Figura et al., 2014b**). These studies begin to define how acinar cell differentiation programs may act as an important defense in a progressively severe sequence of events: loss of the mature acinar phenotype, PanIN initiation, and formation of PDAC.

In adult pancreata, NR5A2 maintains acinar cell identity by cooperating with the acinar-specific pancreas-specific transcription factor 1 (PTF1) complex, which has binding motifs upstream of essentially all acinar differentiation products, such as *Cpa1*, *Cela1*, and *Cel* (**Holmstrom et al., 2011**).

The central specificity component of PTF1 is the cell type-restricted basic helix-loop-helix protein, PTF1A (also known as p48). PTF1A plays two distinct roles during pancreatic organogenesis. First, it is necessary for the growth and morphogenesis of the early pancreatic epithelium, working to impart multipotency and second, its upregulation and lineage-specific interaction with RBPJL promotes acinar differentiation and regulates acinar cell-specific gene expression in adulthood (**Krapp et al., 1998**; **Rose et al., 2001**; **Kawaguchi et al., 2002**; **Masui et al., 2007**, **2010**; **Holmstrom et al., 2011**). Homozygous mutations in human *PTF1A* that disrupt its function or expression cause pancreatic agenesis, supporting its role in pancreas development (**Sellick et al., 2004**; **Weedon et al., 2014**). The severity of this phenotype, however, precludes analysis of PTF1A function in mature human acinar cells. Importantly, in the adult pancreas, PTF1A drives its own expression and that of other PTF1 components via a positive autoregulatory loop (**Masui et al., 2008**). Consistent with the central role of this transcription factor in defining and maintaining acinar cell identity, we have shown that PTF1A is downregulated in acinar cells transformed by $Kras^{G12D}$ and Notch activation (**De La et al., 2008**). Beyond these observations, however, a definitive role of PTF1A in regulating the pathogenesis of PDAC and other adult pancreatic pathology has not yet been described. Based on the studies described above, we hypothesized that loss of PTF1A is a necessary and sufficient step in acinar cell reprogramming, the initiation of PanINs, and the progression of PDAC.

In this study, we demonstrate that downregulation of PTF1A is a decisive and rate-limiting step in acinar-to-ductal metaplasia (ADM), PanIN initiation, and PDAC progression. Our findings suggest that PTF1A acts in a dosage-sensitive manner to safeguard the pancreatic acinar population against both oncogene activity and environmental insults, such as damage caused by pancreatitis. Our study is the first to establish that an endogenous, autoregulatory differentiation program protects mature pancreatic cells from cancer initiation.

Results

PTF1A expression is lost during KRAS-induced transformation of acinar cells and in human PanINs

We have previously demonstrated that *Ptf1a* expression is lost when activated Notch and $Kras^{G12D}$ work synergistically to reprogram acinar cells into PanINs (**De La et al., 2008**). Given that *Ptf1a* is a central regulator of acinar cell gene expression, we hypothesized that this transcription factor should also be downregulated when acinar cells are transformed by oncogenic $Kras^{G12D}$ alone, as well as in human PanINs. To test this hypothesis, we activated $Kras^{G12D}$ specifically in acinar cells using a tamoxifen-inducible Cre expressed by the endogenous *Ptf1a* locus ($Ptf1a^{CreER}$) (**Kopinke et al., 2012**; **Pan et al., 2013**). Like the widely used $Ptf1a^{Cre}$ allele (**Kawaguchi et al., 2002**), $Ptf1a^{CreER}$ is a 'knock-in/knock-out' allele, and therefore, these mice are functionally heterozygous for *Ptf1a*. We induced $Kras^{G12D}$ expression at 6 weeks of age and harvested pancreata 9 months later. While most acini appeared histologically normal and resistant to KRAS-mediated transformation (**Figure 1A**), there was intermittent PanIN formation throughout the pancreas (**Figure 1B**), as previously reported (**Kopp et al., 2012**). By immunohistochemistry (IHC), normal acinar cells in these tissues exhibited robust nuclear PTF1A (**Figure 1C**); however, PTF1A was strongly decreased or absent in all acinar-derived PanIN lesions (**Figure 1D**). To extend these studies to human pancreatic cancer initiation, we stained pathological specimens (n = 4) containing both normal acinar tissue (**Figure 1E**) and PanIN lesions (**Figure 1F**). As observed in the $Kras^{G12D}$ mouse model, normal acini exhibited a strong PTF1A nuclear signal (**Figure 1G**), but PTF1A was largely absent from epithelial cell nuclei within PanINs (**Figure 1H**). In a small fraction of human PanINs, low levels of PTF1A were observed in a subset of epithelial cells (**Figure 1—figure supplement 1**). Residual PTF1A expression is consistent with the finding that approximately one-third of human PDAC samples express low levels of acinar-specific genes (**Collisson et al., 2011**).

Deletion of *Ptf1a* causes acinar-ductal metaplasia and dramatically enhances KRAS-driven acinar cell transformation

In order to determine whether PTF1A downregulation was a functionally important step in PanIN initiation, or a side effect of acinar cell transformation itself, we used an inducible system to delete *Ptf1a* both in the absence and presence of oncogenic $Kras^{G12D}$. In this model, we combined the $Ptf1a^{CreER}$ allele, which does not express PTF1A protein, with a 'floxed' *Ptf1a* allele, to generate *Ptf1a*

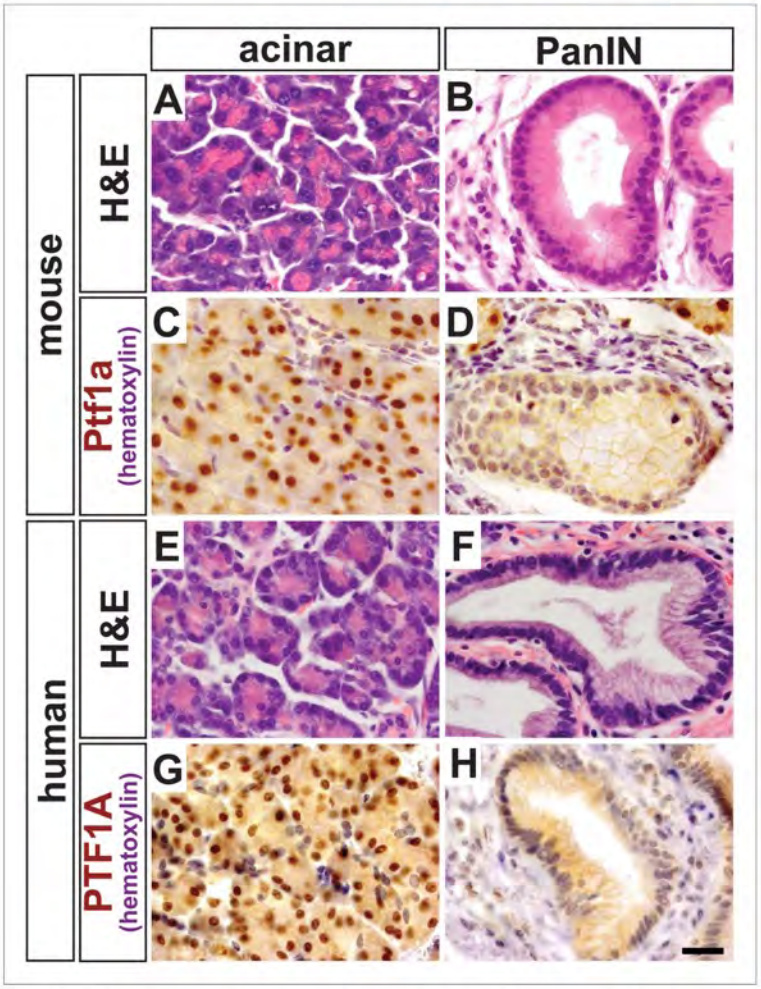

Figure 1. PTF1A is downregulated in PanINs from mice and humans. (**A**, **B**) H&E staining of normal acinar and pancreatic intraepithelial neoplasia (PanIN) tissue of *Ptf1a*^{CreERT}; *Kras*$^{LSL\text{-}G12D}$ pancreata. (**C**, **D**) PTF1A immunohistochemistry (IHC) of mouse acinar and PanIN tissue. (**E**, **F**) H&E staining of human acinar and PanIN tissue. (**G**, **H**) PTF1A immunostaining of normal acinar and PanIN tissue of human. Scale bar: 25 μm.

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Figure supplement 1. PTF1A expression in rare epithelial cells of human PanINs.

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conditional knock-out (cKO) mice of the genotype *Ptf1a*$^{CreERT/lox}$. We also crossed *Kras*$^{LSL\text{-}G12D}$ onto this *Ptf1a* cKO background. Negative control littermates were *Ptf1a* heterozygous (*Ptf1a*$^{CreERT/+}$) without oncogenic *Kras*. An additional control group, representing baseline PanIN initiation in the presence of wild-type PTF1A, consisted of *Ptf1a*$^{CreERT/+}$; *Kras*$^{LSL\text{-}G12D}$ littermates (henceforth referred to as *Kras*^{G12D} mice). All inducible-Cre mice also contained a *R26R*^{EYFP} reporter (**Srinivas et al., 2001**), which allowed monitoring of the frequency of Cre-mediated recombination and lineage-tracing of the fate of recombined acinar cells. **Table 1** summarizes the genotypes of mice used throughout this study; ***Figure 2—figure supplement 1*** schematically depicts the alleles in each genotype.

In initial studies, 6- to 8-week-old mice were administered tamoxifen (TM) at 0.17 mg/g body weight, and pancreata were harvested 9 months later (***Figure 2A***). Compared with control samples, *Ptf1a* cKO pancreata exhibited intermittent ADM throughout the pancreas (***Figure 2B,C***). Metaplastic 'ductules' of *Ptf1a* cKO expressed Cytokeratin-19 (CK19), similar to normal ducts of control; however, *Ptf1a* cKO ductules appeared more dilated than control ducts (***Figure 2F,G***). *Ptf1a* cKO ductules also expressed the duct cell-restricted transcription factor SOX9 (***Figure 2J,K***), indicating a shift from an acinar to a duct-like differentiation state (**Kopp et al., 2012**). However, these metaplastic ductules did not have the histological morphology of PanINs (***Figure 2C***), nor did they stain positively for the PanIN-specific markers Claudin-18 (CLDN18) by IHC (**Westmoreland et al., 2012**) (***Figure 2O***) or acidic mucins by Alcian Blue histochemistry (**Hingorani et al., 2003**; **Kopp et al., 2012**) (***Figure 2S***). Interestingly, ADM in *Ptf1a* cKO mice was associated with no or scant inflammatory infiltrates, and the surrounding areas did not stain positively with Sirius Red (***Figure 2W***), a histochemical stain that highlights fibrotic collagen matrix (**Neuschwander-Tetri et al., 2000**).

We next tested if inactivation of *Ptf1a* sensitized acinar cells to oncogenic KRAS-mediated transformation and PanIN initiation. While intermittent PanIN formation was observed in *Kras*^{G12D} mice (***Figure 2D***), pancreata from *Ptf1a* cKO; *Kras*^{G12D} mice were uniformly composed of extensively distributed PanINs embedded in fibrotic stroma, with almost no remaining normal acinar tissue (***Figure 2E***). PanINs in both *Kras*^{G12D} and *Ptf1a* cKO; *Kras*^{G12D} mice were positive for the duct marker Cytokeratin-19 (***Figure 2H,I***) and the duct-cell transcription factor SOX9 (***Figure 2L,M***), as well as the PanIN markers CLDN18 (***Figure 2P,Q***) and Alcian Blue acidic mucin staining (***Figure 2T,U***). Interestingly, only the *Ptf1a* cKO; *Kras*^{G12D} pancreata exhibited abundant Sirius Red staining, indicating widespread fibrotic injury (***Figure 2X,Y***). Taken together, these data indicate that loss of *Ptf1a* sensitizes acinar cells to ADM and dramatically increases their susceptibility to oncogenic KRAS transformation and PDAC initiation.

Loss of *Ptf1a* expression is a rate-limiting step for PanIN initiation

Given the severity and robustness of PanIN formation in *Ptf1a* cKO; *Kras*^{G12D} mice 9 months after TM administration, we next determined if loss of *Ptf1a* had a more acute effect on acinar cell transformation. To address this issue, 6- to 8-week-old mice were administered TM (0.17 mg/g) and pancreata were harvested 2 or 6 weeks thereafter (***Figure 3A***). To ensure that Cre-mediated recombination rates were comparable between genotypes, we determined the percentage of acinar cells expressing the *R26R*^{EYFP} reporter at 2 weeks post-TM administration. We found similar acinar recombination rates of 21–25% between genotypes (***Figure 2—figure supplement 1***). As the efficiency of Cre-mediated recombination can vary between different target loci (**Liu et al., 2013**), we additionally compared the extent and distribution of PTF1A ablation to that of *R26R*^{EYFP} activation. 3 days after TM administration (0.17 mg/g), there was a ~20% decrease in the number of PTF1A+ cells

Table 1. Nomenclature of mouse mutants used in this study

Short-hand notation	Ptf1a alleles	Kras allele	Reporter allele
Control	*Ptf1a*$^{CreERT/+}$	–	*R26R*$^{EYFP/+}$
Ptf1a cKO	*Ptf1a*$^{CreERT/lox}$	–	*R26R*$^{EYFP/+}$
Kras^{G12D}	*Ptf1a*$^{CreERT/+}$	*Kras*$^{LSL\text{-}G12D/+}$	*R26R*$^{EYFP/+}$
Ptf1a cKO; *Kras*^{G12D}	*Ptf1a*$^{CreERT/lox}$	*Kras*$^{LSL\text{-}G12D/+}$	*R26R*$^{EYFP/+}$

cKO, conditional knock-out.

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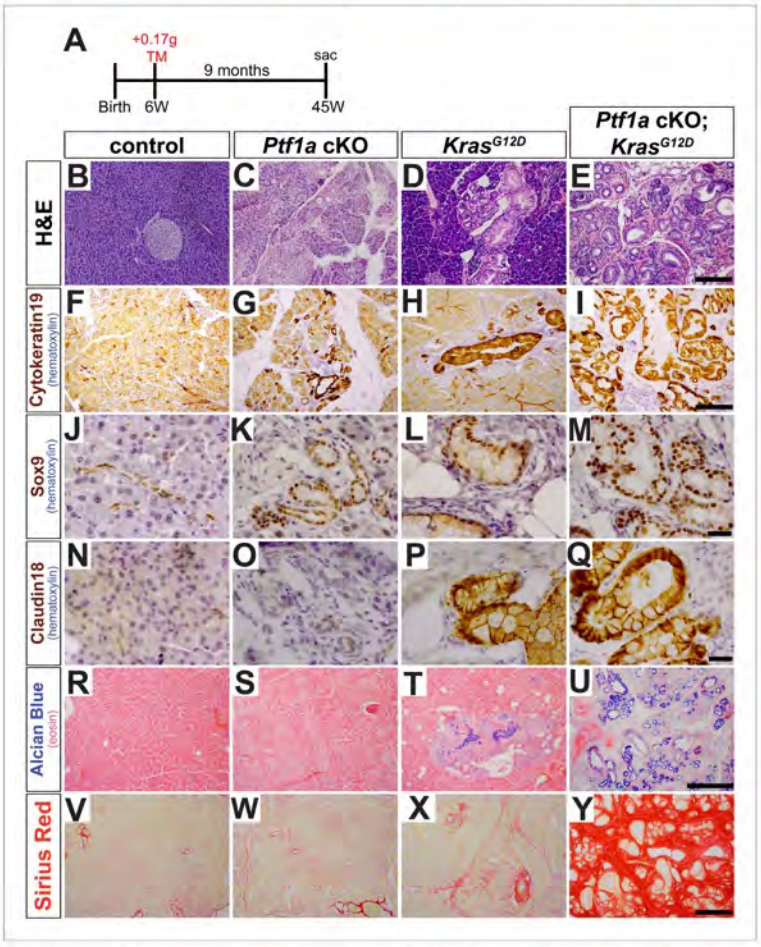

Figure 2. Loss of *Ptf1a* promotes acinar-to-ductal metaplasia and sensitizes acinar cells to KRAS-mediated transformation. (**A**) Mice of indicated genotypes were administered TM (0.17 mg/g) to induce recombination, and sacrificed 9 months later. (**B**–**E**) H&E staining of pancreata from mice of indicated genotypes. (**F**–**M**) IHC for the duct markers CK19 and SOX9, indicating upregulation in both acinar-to-ductal metaplasia (ADM) and PanINs. (**N**–**Q**) IHC for the PanIN marker, CLDN18, highlighting intermittent PanIN formation in $Kras^{G12D}$ mice and widespread lesion development in *Ptf1a* conditional knock-out (cKO); $Kras^{G12D}$. (**R**–**U**) Alcian Blue staining, indicating PanIN lesions in $Kras^{G12D}$ and *Ptf1a* cKO; $Kras^{G12D}$ pancreata. (**V**–**Y**) Sirius Red staining, highlighting local and widespread fibrosis in $Kras^{G12D}$ and *Ptf1a* cKO; $Kras^{G12D}$ mice, respectively. Scale bars: (**B**–**E**) 200 μm; (**F**–**I**) 200 μm; (**J**–**Q**) 25 μm; (**R**–**U**) 500 μm; (**V**–**Y**) 200 μm.
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The following figure supplements are available for figure 2:

Figure supplement 1. Schematic of mouse alleles used in this study.
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Figure supplement 2. $Ptf1a^{CreERT}$ deletion efficiency following tamoxifen treatment.
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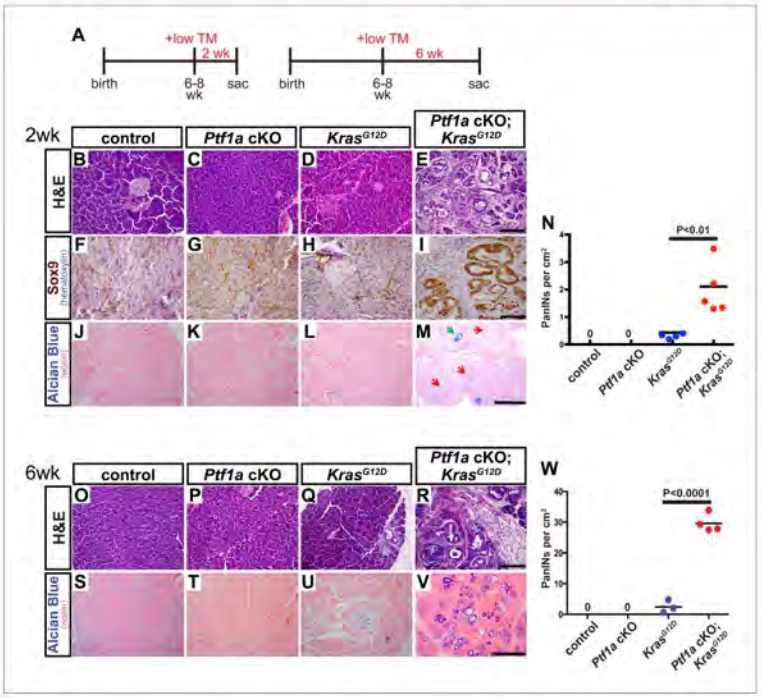

Figure 3. Loss of *Ptf1a* is a rate-limiting step in PanIN initiation. (**A**) Mice of specified genotypes were administered 0.17 mg/g body weight TM to induce Cre-mediated recombination and were sacrificed either 2 or 6 weeks later. (**B**–**E**) H&E staining of pancreata from mice of indicated genotypes 2 weeks after TM administration. (**F**–**I**) IHC for the ductal transcription factor SOX9, indicating upregulation in ADM and PanINs of *Ptf1a* cKO; $Kras^{G12D}$ pancreata. (**J**–**M**) Alcian blue staining, indicating PanIN lesions in *Ptf1a* cKO; $Kras^{G12D}$ pancreata. In panel (**M**), green arrow indicates an Alcian Blue+ lesion, while red arrows indicate ADM that is Alcian Blue-negative. (**N**) Quantification of the genotype-dependent PanIN burden; *Ptf1a* cKO; $Kras^{G12D}$ pancreata possessed significantly more PanINs at 2 weeks post-TM than $Kras^{G12D}$ mice ($p < 0.01$). (**O**–**R**) H&E staining of pancreata from mice of indicated genotypes 6 weeks after TM administration. (**S**–**V**) Alcian Blue staining, highlighting PanIN lesions in pancreata from $Kras^{G12D}$ mice and *Ptf1a* cKO; $Kras^{G12D}$ mice. (**W**) Quantification of PanINs at 6 weeks post-TM. *Ptf1a* cKO; $Kras^{G12D}$ pancreata had ~15-fold more Alcian Blue+ PanINs at this time point than $Kras^{G12D}$ ($p < 0.0001$). Scale bars: (**B**–**E**) 200 μm; (**F**–**I**) 100 μm; (**J**–**M**) 500 μm; (**O**–**R**) 200 μm; (**S**–**V**) 500 μm.

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The following figure supplements are available for figure 3:

Figure supplement 1. Microenvironmental remodeling in *Ptf1a* cKO; $Kras^{G12D}$ pancreata.

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Figure supplement 2. Acinar-ductal reprogramming in 3D culture.

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detected by immunofluorescence (***Figure 2—figure supplement 2***). Importantly, the majority (~75%) of EYFP+ cells were PTF1A-negative at this dose of TM (***Figure 2—figure supplement 2***), indicating that activation of EYFP provides an approximate surrogate for deletion of *Ptf1a*.

Interestingly, this level of *Ptf1a* deletion alone did not produce ADM or other histologically detectable effects at 2 weeks post-TM, compared to control mice (***Figure 3B,C***). While $Kras^{G12D}$ pancreata exhibited few or no PanINs at this time point, there was widespread induction of ADM,

leukocyte infiltration, fibrosis, and PanIN initiation in *Ptf1a* cKO; $Kras^{G12D}$ pancreata (***Figure 3D,E*** and ***Figure 3—figure supplement 1***). We further confirmed that acinar-derived ADM and PanINs were being reprogrammed to a duct-like fate based on expression of the ductal transcription factor SOX9. While only normal ducts expressed SOX9 in control pancreata, PanINs and ADM in *Ptf1a* cKO; $Kras^{G12D}$ were SOX9$^+$ at 2 weeks post-TM (***Figure 3F–I***). These data are consistent with a recent study indicating that *Sox9* is necessary but not sufficient for the earliest stages of mouse PanIN initiation (***Kopp et al., 2012***).

To quantify lesion burden, we stained pancreata from all genotypes with Alcian Blue to highlight acidic mucin-rich PanINs (***Figure 3J–M***). Following a counting procedure established in our lab (***De La et al., 2008***), we observed a ~sixfold increase in the frequency of PanINs in *Ptf1a* cKO; $Kras^{G12D}$ mice compared to mice expressing $Kras^{G12D}$ alone (***Figure 3N***). This is likely an underestimation of overall phenotypic change, since ADM, which precedes PanIN formation, does not stain with Alcian Blue. Based on histological inspection, ADM is widespread in *Ptf1a* cKO; $Kras^{G12D}$ mice at 2 weeks post-TM, but negligible in $Kras^{G12D}$ pancreata (***Figure 3D,E,L,M***).

Initiation and progression of PDAC involves interactions between KRAS-active epithelial cells and their stromal microenvironment, with local inflammation being commonly associated with more rapid tumorigenesis (***Gukovsky et al., 2013***). We observed that PanINs developing after 2 weeks in *Ptf1a* cKO; $Kras^{G12D}$ mice, identified by CLDN18 staining, were consistently surrounded by CD45$^+$ leukocytes, indicating interactions between transformed epithelial cells and inflammatory cells (***Figure 3—figure supplement 1***). Because activation of pancreatic stellate cells is a hallmark of PDAC, we assessed the activation state of these cells using the marker α-smooth muscle actin (SMA). While SMA-positive cells were observed around blood vessels in pancreata of all genotypes, lobules of *Ptf1a* cKO; $Kras^{G12D}$ pancreata affected by ADM and PanIN initiation exhibited widespread SMA$^+$ fibroblasts surrounding ADM and PanIN lesions (***Figure 3—figure supplement 1***). Staining with the fibrosis marker Sirius Red confirmed that PanIN-associated fibroblasts of *Ptf1a* cKO; $Kras^{G12D}$ pancreata were actively secreting collagenous matrix (***Figure 3—figure supplement 1***), indicating activation of stellate cells only 2 weeks after Cre-mediated recombination. The activation of stellate cells and fibrotic phenotype observed in *Ptf1a* cKO; $Kras^{G12D}$ pancreata (***Figure 3—figure supplement 1***) is likely a reaction to the high level of acinar cell transformation rather than a direct reaction to *Ptf1a* deletion itself, as *Ptf1a* cKO pancreata with ADM do not stain with Sirius red (***Figure 2V***).

In order to determine the acinar cell-intrinsic consequences of *Ptf1a* deletion, we used a 3D culture system in which acini can undergo metaplasia into ductal cysts in response to mutant *Kras* or EGF receptor (EGFR) ligand stimulation, without the influence of other cell types (***Means et al., 2005***; ***Ardito et al., 2012***). To induce widespread, acinar-specific *Kras* activation and/or *Ptf1a* deletion, we treated mice with three daily doses of tamoxifen at 0.25 mg/ml, a treatment paradigm that we found to drive widespread recombination (see below). Acinar cell clusters from control, *Ptf1a* cKO, $Kras^{G12D}$, and *Ptf1a* cKO; $Kras^{G12D}$ were isolated at 3 days after the final TM dose, prior to the appearance of any histological abnormalities, and embedded in a collagen matrix, as previously described (***Means et al., 2005***; ***Ardito et al., 2012***). Neither control nor *Ptf1a* cKO acinar clusters underwent spontaneous cyst conversion, in the absence of added growth factors, implying that loss of *Ptf1a* is not sufficient for acinar-ductal reprogramming. As expected, acini of both genotypes generated CK19+ ductal cysts in response to the EGFR ligand TGFα (data not shown). By contrast, $Kras^{G12D}$ activation was sufficient for generation of acinar-derived cysts; importantly, *Ptf1a* cKO; $Kras^{G12D}$ acini formed significantly larger cysts than those derived from $Kras^{G12D}$ pancreata (***Figure 3—figure supplement 2***). These results are consistent with our in vivo data and suggest that acinar cell loss of *Ptf1a* enhances KRAS-mediated transformation independent of effects on the stromal microenvironment.

A generally similar synergy between $Kras^{G12D}$ and *Ptf1a* cKO was observed in vivo at the 6-week post-tamoxifen time point. *Ptf1a* cKO pancreata remained histologically unchanged compared to control, as at 2 weeks post-TM, while intermittent PanIN-1 lesions were observed in $Kras^{G12D}$ pancreata (***Figure 3O–Q***). *Ptf1a* cKO; $Kras^{G12D}$ pancreata, by contrast, were completely overrun by PanINs at this time point (***Figure 3R***), most of which stained positively with Alcian Blue (***Figure 3V***). Quantifying PanIN lesions by Alcian Blue staining, we observed a >15-fold increase in *Ptf1a* cKO; $Kras^{G12D}$ compared to mice expressing $Kras^{G12D}$ alone (***Figure 3W***). As we did not score more than one lesion per individual anatomic lobule, to avoid double-counting large or discontinuous lesions, this number likely underestimates the overall PanIN burden in *Ptf1a* cKO; $Kras^{G12D}$ pancreata given the

likelihood of multiple initiation events per lobule. Altogether, the dramatic acceleration of PanIN development upon *Ptf1a* deletion suggests that downregulation of this TF is a rate-limiting step for KRAS-driven pancreatic tumorigenesis.

Extensive deletion of *Ptf1a* promotes rapid but incomplete acinar-ductal metaplasia

As we were surprised that a moderate level of acinar cell recombination (~25%) failed to produce an overt, short-term phenotype in *Ptf1a* cKO pancreata (***Figure 3***), we tested if more pervasive deletion of *Ptf1a* would produce a more robust reprogramming phenotype. Control and *Ptf1a* cKO mice were administered a higher dose of TM (0.25 mg/g) by oral gavage on three consecutive days (a net 4.5-fold higher dose than previously) and were harvested 2 weeks later (***Figure 4A***). Quantification of EYFP+ acinar cells following this TM regimen demonstrated a recombination frequency of ~65% (***Figure 4—figure supplement 1***). Additionally, we quantified the number of PTF1A-deficient acinar cells at 3 days after the final TM gavage, and found that only ~15% of all pancreatic cells retained nuclear PTF1A, compared with ~82% in TM-untreated controls (***Figure 2—figure supplement 2***). As with low-dose TM, described above, the majority (>90%) of EYFP+ cells were PTF1A-negative at 3 days post-TM, confirming that EYFP expression highlights acinar cells deleted for *Ptf1a* (***Figure 2—figure supplement 2***). The apparently greater extent of PTF1A ablation, relative to EYFP activation, may imply the existence of *Ptf1a*-deleted cells within the EYFP-negative population; such an observation would be consistent with previous evidence of locus-specific Cre deletion efficiencies (***Liu et al., 2013***).

2 weeks following high-dose TM, *Ptf1a* cKO pancreata were less than half the mass of their control counterparts (***Figure 4B***). Immunofluorescence revealed that while nearly all EYFP+ acinar cells expressed the acinar marker carboxypeptidase A1 (CPA1) in controls, this marker was lost from approximately 15% of EYFP+ cells in *Ptf1a* cKO tissues, indicating loss of the normal differentiation state (***Figure 4C–E***). Histologically, *Ptf1a* cKO pancreata exhibited extensive acinar disorganization and dilation as well as sporadic upregulation of CK19 within acinar structures, suggestive of early stages of ADM (***Figure 4F–I***). CK19+ acinar cells (defined by EYFP co-expression) were consistently surrounded by CD45+ leukocytes (***Figure 4—figure supplement 2A–C***), consistent with an intimate association between metaplasia and inflammatory cell recruitment (***Liou et al., 2013***; ***Murtaugh and Keefe, 2015***). Nonetheless, *Ptf1a* cKO pancreata did not exhibit a general pancreatitis phenotype (***Figure 4—figure supplement 2A–C***) nor did they exhibit a detectable increase in epithelial cell apoptosis (***Figure 4—figure supplement 2D–F***). In addition, we found that treatment of wild-type mice with high-dose TM was not sufficient to induce pancreatic inflammation (***Figure 4—figure supplement 3***), suggesting that the stronger phenotype of high-dose *Ptf1a* cKO mice, relative to low-dose, was not due to stimulation of ADM by non-specific tissue damage.

Loss of PTF1A was accompanied by upregulation of SOX9 by the majority of EYFP+ cells, indicating partial reprogramming to a duct-like state (***Figure 4J–M***). Surprisingly, we also observed a significant (~fourfold) increase in the fraction of Ki67+ epithelial cells in *Ptf1a* cKO pancreata compared with control, suggesting that loss of PTF1A results in deregulation of proliferation as well as differentiation (***Figure 4—figure supplement 2G–I***). Taken together, these data indicate that *Ptf1a* is required to maintain acinar gene expression and quiescence, as well as prevent metaplasia to a duct-like state, potentially by inhibiting upregulation of SOX9.

Loss of *Ptf1a* activates KRAS-dependency and fibroinflammatory pathways

In order to investigate further the mechanism of ADM after loss of *Ptf1a*, we performed RNA-seq on whole pancreata from three control and three *Ptf1a* cKO mice, each of which received three doses of TM (0.25 mg/g) to induce maximal recombination 2 weeks prior to RNA extraction. Initial analysis of RNA-seq data sets by *edgeR* (***Robinson et al., 2010***), setting a false discovery rate (FDR) threshold of 0.05, identified significant changes in expression of over 3000 total genes (***Figure 5A***). Consistent with our immunostaining (***Figure 4***), among the most significantly downregulated mRNAs were *Ptf1a* (18.4-fold) and *Cpa1* (5.45-fold), while *Sox9* was significantly upregulated (4.61-fold) in *Ptf1a* cKO pancreata (***Figure 5A***). Additional downregulated mRNAs

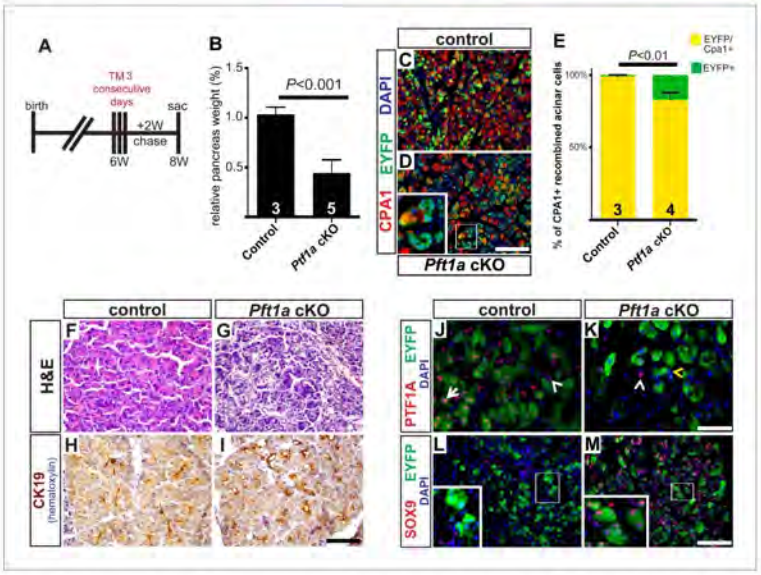

Figure 4. Widespread loss of *Ptf1a* promotes rapid acinar-to-ductal metaplasia. (**A**) Control and *Ptf1a* cKO mice were administered TM (0.25 mg/g) on three consecutive days and sacrificed following a 2-week chase period. (**B**) Pancreas mass, measured as a percent of body weight, was significantly decreased in *Ptf1a* cKO mice 2 weeks after TM administration. (**C**, **D**) Immunofluorescence for the acinar enzyme carboxypeptidase A1 (CPA1) (red) and Cre reporter *R26R*^{EYFP} (green). Nuclei are labeled with DAPI (blue). Inset highlights EYFP+, CPA1-negative acinar cells forming duct-like structures in *Ptf1a* null pancreata. (**E**) Quantification of CPA1 expression by EYFP+ (Cre-recombined) cells in control and *Ptf1a* cKO pancreata (control n = 3, *Ptf1a* cKO n = 4, $p < 0.01$). (**F**, **G**) H&E staining of control and *Ptf1a* cKO pancreata 2 weeks after high-dose TM administration. (**H**, **I**) IHC for the duct marker CK19 highlighting areas of ADM in *Ptf1a* cKO pancreata. (**J**, **K**) Immunofluorescence for PTF1A (red) and the Cre reporter *R26R*^{EYFP} (green). White arrow indicates an EYFP+ cell expressing PTF1A in control; white arrowheads indicate non-recombined PTF1A+ cells; yellow arrowhead indicates a recombined, PTF1A-negative cell undergoing metaplasia in *Ptf1a* cKO. (**L**, **M**) Immunofluorescence for the duct transcription factor SOX9 (red) and the Cre reporter *R26R*^{EYFP} (green). Insets highlight restricted expression of SOX9 in controls and upregulation of SOX9 within EYFP+ acinar cells of *Ptf1a* cKO. Scale bars: (**C**, **D**) 100 μm, (**F**–**I**) 200 μm, (**J**, **K**) 50 μm, (**L**, **M**) 100 μm.

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The following figure supplements are available for figure 4:

Figure supplement 1. Cre-mediated recombination rates following high-dose tamoxifen treatment.

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Figure supplement 2. Loss of Ptf1a promotes pancreatic epithelial transdifferentiation and proliferation.

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Figure supplement 3. High-dose tamoxifen administration does not induce pancreatitis.

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included a wide variety of digestive enzymes and other secreted proteins characteristic of the exocrine acinar phenotype, consistent with the long-standing hypothesis that they are directly regulated by PTF1A (***Rose et al., 2001***; MacDonald et al., in preparation).

Given our finding that loss of *Ptf1a* strongly potentiates KRAS-induced PanIN initiation (***Figures 2, 3***), we analyzed the expression of genes previously implicated in KRAS signaling and PDAC development. Interestingly, tumor suppressors classically associated with PDAC, such as *p53* (*Trp53*), *Cdkn2a/Ink4a*, *Pten*, *Brca2*, and *Smad4*, were not significantly downregulated in the absence of *Ptf1a* (data not shown),

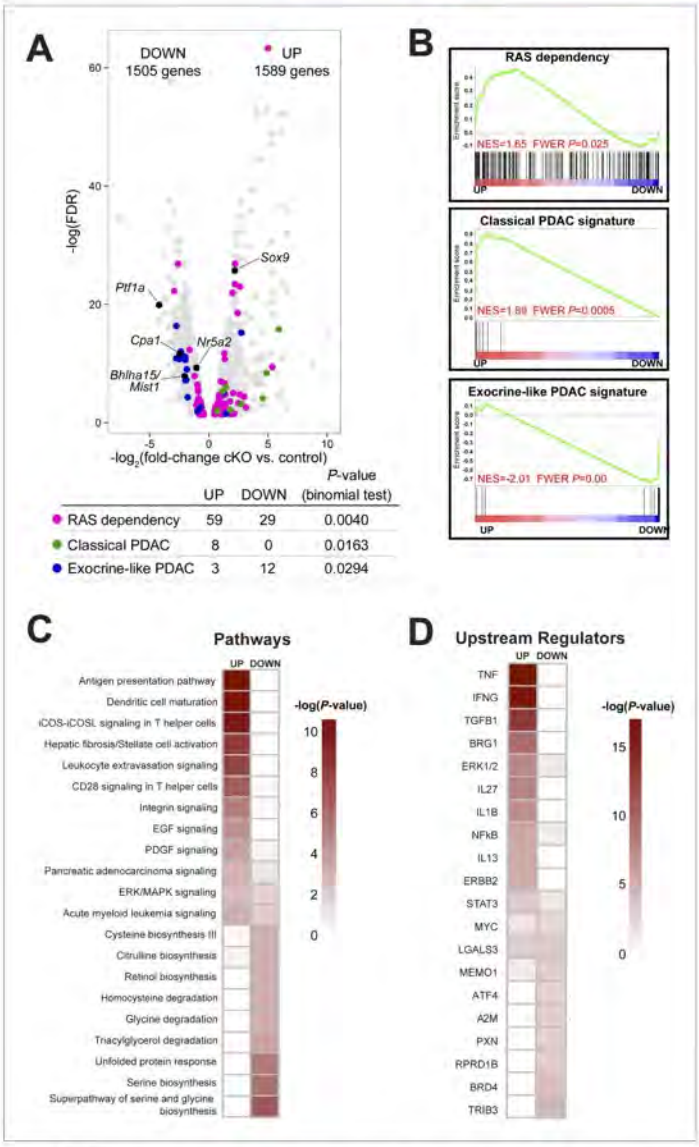

Figure 5. Ptf1a suppresses fibroinflammatory pathways and oncogenic KRAS associated gene signatures. (**A**) Volcano plot showing differentially expressed genes (false discovery rate [FDR] <0.05; gray) in *Ptf1a* cKO pancreata, relative to control. Individual genes are labeled and highlighted in black. Genes belonging to signatures characteristic of RAS dependency, classical and exocrine-like pancreatic ductal adenocarcinoma (PDAC) are

Figure 5. continued on next page

Figure 5. Continued

highlighted in pink, green, and blue, respectively. Table below indicates p-values from binomial test for enrichment of gene signatures within up- or down-regulated genes. (**B**) Gene Set Enrichment Analysis (GSEA) enrichment plots of differentially expressed genes between *Ptf1a* cKO and control indicating positive enrichment of RAS dependency and classical PDAC signatures and negative enrichment of exocrine-like PDAC signature genes. (**C**, **D**) Ingenuity Pathway Analysis (IPA, Qiagen Redwood City, www.qiagen.com/ingenuity) was used to identify differentially expressed pathways and upstream regulators in *Ptf1a* cKO pancreata. (**C**) Heat map of pathways that are significantly increased and decreased upon *Ptf1a* deletion. (**D**) Heat map of upstream pathways and regulators predicted to drive the observed changes in gene expression. Color scale is indicative of the -log p-value (significance). All analyses are based on a ±2.0-fold expression threshold. Full details of the data set and analyses can be found in the supplementary data files.

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leading to the notion that the susceptibility of *Ptf1a* cKO pancreata to KRAS involves a novel mechanism distinct from canonical tumor suppression pathways. By contrast, we found that two acinar-specific transcription factors previously implicated in suppressing PanIN development, *Bhlha15* (commonly referred to as *Mist1*) and *Nr5a2* (***Shi et al., 2009b***; ***Flandez et al., 2014***; ***von Figura et al., 2014b***), were downregulated in *Ptf1a* cKO mice, consistent with PTF1A acting at or near the top of a regulatory hierarchy responsible for maintaining acinar identity and suppressing tumorigenesis (***Figure 5A***).

In human cell lines derived from pancreatic and other cancers, dependence on KRAS signaling correlates with expression of specific gene signatures, including genes whose activity is required to sustain RAS activity and malignancy (***Singh et al., 2009***; ***Loboda et al., 2010***). We found that previously identified RAS dependence genes were significantly enriched, by binomial test, among mRNAs upregulated in *Ptf1a* cKO tissue (***Figure 5A***). Within this signature were some of the most highly upregulated mRNAs in our data set, such as *Tspan1* (32.4-fold increase), *Slc1a2* (62.2-fold increase), *Fut2* (41.4-fold increase), and *Egr1* (6.2-fold increase) (***Supplementary files 1, 2***). The preferential upregulation of RAS dependency genes in *Ptf1a* cKO was confirmed by Gene Set Enrichment Analysis (GSEA) (***Subramanian et al., 2005***) (***Figure 5B***), and suggests that loss of PTF1A results in a phenotypic shift toward a KRAS-permissive phenotype. RAS dependency is characteristic of human PDAC cell lines and primary tumors with a 'classical', duct-enriched gene expression profile (***Collisson et al., 2011***). We find that the classical PDAC signature is also preferentially upregulated upon *Ptf1a* deletion, while the distinct 'exocrine-like' PDAC signature, largely comprising acinar-specific secreted proteins, is downregulated (***Figure 5A,B***). These results therefore strongly suggest not only that PTF1A maintains acinar differentiation, including expression of genes marking an acinar-like subset of human PDAC, but also that PTF1A suppresses an alternative gene expression program that facilitates KRAS signaling activity.

To identify biological pathways that were activated or attenuated by *Ptf1a* deletion, we analyzed this RNA-seq data set using Qiagen's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.ingenuity.com) (***Thomas and Bonchev, 2010***; ***Kramer et al., 2014***). We analyzed canonical pathways using three different thresholds of gene expression (1.5-fold up/downregulation, 2.0-fold up/downregulation, and 3.0-fold up/downregulation; ***Supplementary files 3–5***). At an upregulation threshold of 2.0, deletion of *Ptf1a* significantly affected over 300 pathways, several of which have an established role in PDAC initiation. These included T-helper cell-signaling pathways (***McAllister et al., 2014***), stellate-cell activation and fibrosis (***Sherman et al., 2014***), and epidermal growth factor (EGF) signaling (***Ardito et al., 2012***; ***Navas et al., 2012***) (***Figure 5C***). A general 'pancreatic adenocarcinoma signaling' pathway was also upregulated, consisting primarily of genes involved in PI-3-kinase and JAK/STAT signaling. We also used IPA Upstream Regulator Analysis to predict upstream signaling mediators that could explain the changes in gene expression within our data set (***Kramer et al., 2014***). The predicted upregulated mediators were consistent across multiple expression thresholds and included TNF-α, TGF-β, IL-1β, NFκB, and the SWI/SNF component Smarca4/Brg1 (***Figure 5D***). All of these signaling pathways have been implicated in PDAC initiation and progression (***Bardeesy et al., 2006***; ***Adrian et al., 2009***; ***Khasawneh et al., 2009***; ***Maniati et al., 2011***; ***Daniluk et al., 2012***; ***Maier et al., 2013***; ***Gore et al., 2014***; ***von Figura et al., 2014a***). Thus, we propose that loss of *Ptf1a* alters cell state at multiple levels, ultimately promoting gene expression and signaling activities that are supportive of KRAS transformation.

Caerulein-induced pancreatitis is sufficient to reprogram *Ptf1a*-deficient acinar cells

Among the upstream mediators activated in the *Ptf1a* cKO model are TNF-α and NFkB, both of which promote ADM and inflammation in pancreatitis and amplify KRAS activity in pancreatic tumorigenesis (**Maniati et al., 2011**; **Daniluk et al., 2012**; **Huang et al., 2013**; **Maier et al., 2013**; **Sendler et al., 2013**). As *Ptf1a* deletion upregulates other pathways characteristic of pancreatic injury, such as stellate-cell activation, TGF-β signaling, and dendritic cell maturation (**Bedrosian et al., 2011**; **Erkan et al., 2012**), we were interested to determine if loss of *Ptf1a* would sensitize acinar cells to injury-induced reprogramming even without oncogenic *KRAS*.

To test this hypothesis in vivo, we deleted *Ptf1a* via high-dose TM administration (three doses of 0.17 mg/g), which induced a recombination rate of ~65% (**Figure 4—figure supplement 1**). At 1 week post-TM, acute pancreatitis was induced by two consecutive days of treatment with the secretagogue caerulein, as previously described (**Jensen et al., 2005**; **Keefe et al., 2012**), and pancreata were harvested 1 week later (**Figure 6A**). As a control for caerulein injections, additional TM-treated *Ptf1a* cKO and control mice were administered saline vehicle alone. As previously reported, control mice recovered from caerulein treatment and were indistinguishable from saline-injected controls after 1 week (**Figure 6B–D**). In contrast, *Ptf1a* cKO mice subjected to caerulein-induced pancreatitis exhibited widespread acinar atrophy, persistent inflammation, fibrotic stroma, and the appearance of mucinous metaplastic structures (**Figure 6E,F**). These abnormal ductules were Alcian Blue-reactive, similar to PanINs (**Figure 6G**), although staining for the PanIN-specific markers CLDN18 and MUC5AC was observed in only rare and isolated lesions (**Figure 6—figure supplement 1A,B**). Consistent with the overall distorted histology (**Figure 6E**) and atrophy (**Figure 6H**) of caerulein-treated *Ptf1a* cKO mice, no normal amylase+ acinar clusters could be detected in these pancreata, in contrast to controls (**Figure 6I–L**). Acinar-derived EYFP+ cells in caerulein-treated *Ptf1a* cKO pancreata were instead integrated within CK19+ duct-like structures, suggesting that pancreatitis synergizes with loss of *Ptf1a* to cause a rapid loss of acinar gene expression and complete reprogramming to a duct-like fate (**Figure 6I–L**).

As our findings in *Ptf1a* cKO; $Kras^{G12D}$ mice indicate that loss of PTF1A enhances the transforming activity of mutant KRAS, we were interested to determine if development of mucinous metaplasia involved enhanced signaling through endogenous RAS. The MEK-ERK pathway is a major regulator of KRAS-induced acinar reprogramming (**Collins et al., 2014**), and we found that nearly all metaplastic lesions of caerulein-treated *Ptf1a* cKO mice exhibited robust nuclear phospho-ERK staining (**Figure 6—figure supplement 1C–F**). Phospho-ERK was undetectable in saline-treated *Ptf1a* cKO mice, or control mice under either treatment. Taken together, these data demonstrate that PTF1A is necessary for acinar-cell redifferentiation and resolution of tissue injury following acute pancreatitis. In the absence of PTF1A, a persistent inflamed microenvironment may have tumor promoter-like activity, enhancing KRAS-MEK-ERK signaling to induce transformation (**Gukovsky et al., 2013**; **Murtaugh, 2014**).

Ptf1a heterozygosity promotes PDAC by increasing the frequency of initiating events

The above studies rely on genetic deletion of *Ptf1a*, a process without clear parallel in human disease: somatic mutations of *PTF1A* are not observed in human PDAC, according to the Catalogue of Somatic Mutations in Cancer (COSMIC) database (cancer.sanger.ac.uk). *PTF1A* is more likely to be downregulated by an epigenetic mechanism, for example, via attenuation of the positive autoregulatory loop by which PTF1A maintains its own expression and that of its partner transcription factors (**Masui et al., 2008**). Impaired expression of PTF1-network components, lowering the threshold for KRAS-mediate reprogramming and transformation, might explain the dosage-sensitive requirement for *Nr5a2* in preventing PanIN formation (**Flandez et al., 2014**; **von Figura et al., 2014b**). To determine if the role of *Ptf1a* itself is dosage-sensitive, we generated mice of the 'KC' genotype, using the *Pdx1-Cre* driver to activate $Kras^{LSL\text{-}G12D}$ throughout the pancreas (**Aguirre et al., 2003**; **Hingorani et al., 2003**; **Murtaugh, 2014**), and which were either heterozygous for a germ line deletion of *Ptf1a* (*Pdx1-Cre*; $Kras^{LSL\text{-}G12D}$; $Ptf1a^{\Delta/+}$) or remained homozygous *Ptf1a* wild type. We harvested pancreata at 1 month of age, at which time PanIN formation is usually minimal in KC mice, and quantified PanIN burden by Alcian Blue staining. Mice heterozygous for *Ptf1a* had increased

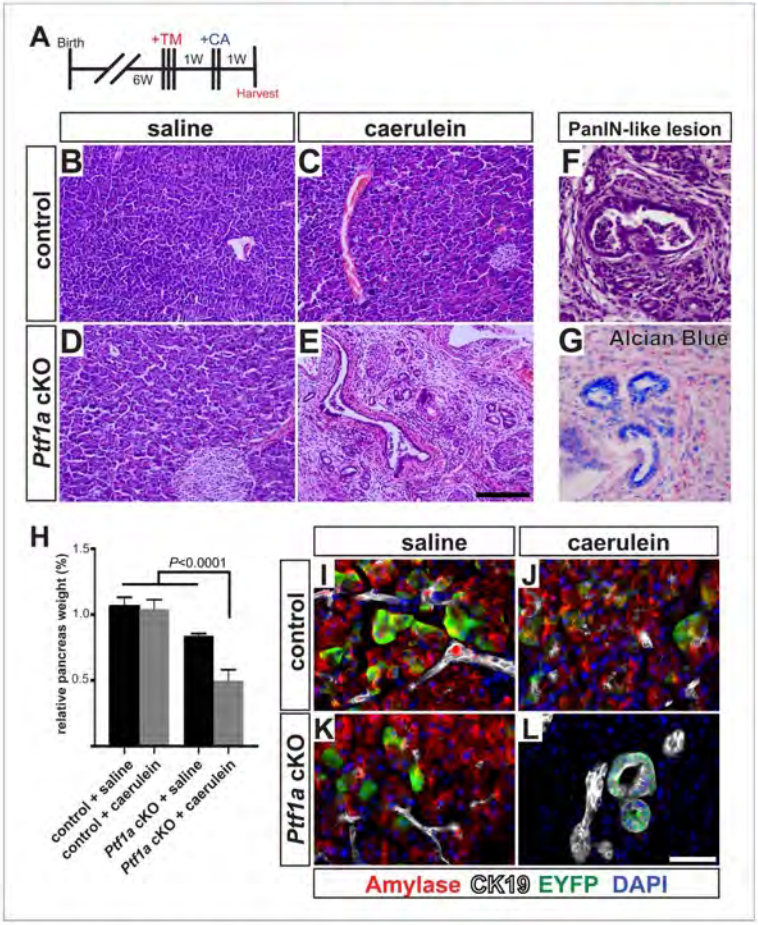

Figure 6. *Ptf1a* is necessary for acinar cell regeneration and suppression of dysplasia following induced pancreatitis. (**A**) 6- to 8-week-old control and *Ptf1a* cKO mice were administered three doses of TM (0.17 mg/g) on consecutive days. 1 week later, mice were administered eight hourly injections of caerulein or saline vehicle, on two consecutive days. Mice were sacrificed 1 week following caerulein treatment. (**B**–**E**) H&E staining on control and *Ptf1a* cKO pancreata (n = 4–5 per group) 1 week following caerulein treatment. (**F**) H&E stain highlighting a PanIN-like lesion in caerulein-treated *Ptf1a* cKO. (**G**) Alcian Blue-positive lesions in caerulein-treated *Ptf1a* cKO. (**H**) Relative pancreas size, measured as a percent of body weight, among treatment groups (n = 4–5 per group, $p < 0.01$). (**I**–**L**) Immunofluorescence for amylase (red), CK19 (white), and the Cre reporter *R26R*^{EYFP} (green), in pancreata of control and *Ptf1a* cKO treated with saline or caerulein. EYFP+ cells of caerulein-treated cKO have downregulated amylase and contribute to CK19+ PanIN-like structures. Scale bars: (**B**–**E**) 200 μm, (**I**–**L**) 50 μm.

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The following figure supplement is available for figure 6:

Figure supplement 1. Mucinous metaplasia associated with hyperactive MEK-ERK signaling in caerulein-treated Ptf1a cKO pancreata.

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PanINs at this early stage, compared to *Ptf1a*$^{+/+}$ littermates (***Figure 7A–C***). This result is consistent with a dosage-sensitive function for PTF1A, such that reduced levels or activity already begin to destabilize acinar differentiation in the face of oncogenic insults.

In humans, increased PanIN burden in early life is associated with familial risk of PDAC, suggesting that mutations driving genetic predisposition to PDAC act at the level of tumor initiation (***Brune et al.,***

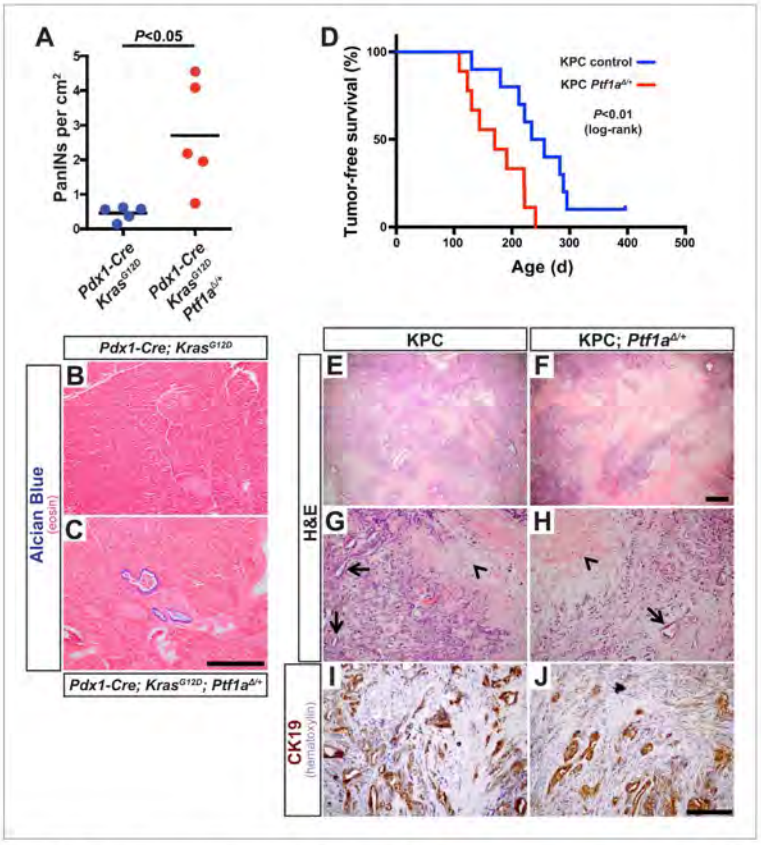

Figure 7. *Ptf1a* heterozygosity increases the frequency of PanINs and allows for rapid progression of PDAC. (**A**) Quantification of PanINs in pancreata from 1-month-old *Pdx1-Cre; Kras*^{G12D} and *Pdx1-Cre; Kras*^{G12D}*; Ptf1a*$^{\Delta/+}$ mice ($n = 5$ per genotype, $p < 0.05$). (**B**, **C**) Representative Alcian Blue and Eosin staining from 1-month-old mice of indicated genotypes. (**D**) Kaplan–Meier analysis from KPC mice (*Pdx1-Cre; Kras*^{G12D}*; p53*$^{lox/+}$*; Ptf1a*$^{+/+}$, blue line) and KPC; *Ptf1a*$^{\Delta/+}$ mice (red line) (Log-Rank test $p < 0.01$). (**E**–**H**) H&E staining on tumors from both KPC and KPC:*Ptf1a*$^{\Delta/+}$ mice at low and high magnification. (**G**, **H**) Arrows indicate ductile epithelial cells and arrowheads indicate areas of necrosis. (**I**, **J**) IHC for CK19 on tumor specimens from mice of indicated genotypes. Scale Bars: (**B**, **C**) 500 μm, (**E**, **F**) 500 μm, (**G**–**J**) 200 μm.

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The following figure supplement is available for figure 7:

Figure supplement 1. Liver metastases in KPC mice heterozygous for *Ptf1a*.

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2006; Shi et al., 2009a). We therefore hypothesized that decreased *Ptf1a* dosage would promote cancer susceptibility by increasing the rate of PanIN initiation. Therefore, we utilized the well-characterized 'KPC' model of mouse PDAC in which heterozygous loss of *p53* (official gene symbol *Trp53*) is added to the *Pdx1-Cre; Kras*$^{LSL\text{-}G12D}$ genotype (Hingorani et al., 2005; Rhim et al., 2012). As above, KPC mice (*Pdx1-Cre; Kras*$^{LSL\text{-}G12D}$; *p53*$^{flox/+}$) were generated on either *Ptf1a*$^{+/+}$ or *Ptf1a*$^{\Delta/+}$ backgrounds, and animals were monitored for tumor-free survival. The results of Kaplan–Meier analysis showed that *Ptf1a*-heterozygous KPC mice developed PDAC much earlier than *Ptf1a*$^{+/+}$ counterparts (Figure 7D, Log-rank test, $p < 0.01$). We observed prominent metastases to the liver in 3/9 *Ptf1a*$^{\Delta/+}$ KPC mice, but none in *Ptf1a*$^{+/+}$ KPC controls (Figure 7—figure supplement 1). Importantly, despite the earlier onset of PDAC in KPC mice with *Ptf1a* heterozygosity, once tumors arose they were histologically indistinguishable between genotypes (Figure 7E–H). They contained classical features of human PDAC, including abundant fibrotic stroma surrounding CK19^{+} epithelial cells (Figure 7I,J) and substantial areas of necrosis. We therefore conclude that decreased *Ptf1a* gene dosage sensitizes pancreata to early KRAS-mediated PanIN initiation and rapid progression to PDAC.

Discussion

Previously, we and others established that acinar-to-ductal reprogramming is a necessary step in PanIN initiation (De La et al., 2008; Habbe et al., 2008; Kopp et al., 2012). Several recent studies extended these findings, demonstrating that several genes required for PanIN and PDAC development appear to act at the level of acinar cell reprogramming (Heid et al., 2011; Ardito et al., 2012; Kopp et al., 2012; Baer et al., 2014; Wu et al., 2014; Zhang et al., 2014). Here, we demonstrate that the loss of a principal regulator of acinar cell identity, PTF1A, is sufficient to prompt rapid and extensive acinar-to-ductal metaplasia even in the absence of other exocrine insults (Figure 4). Additionally, we demonstrate that *Ptf1a*-deficient acinar cells are extremely sensitive to oncogenic transformation, as they undergo rapid and robust KRAS-mediated PanIN formation (Figures 2, 3).

Deletion of *Ptf1a* alone at moderate frequency (~25%) did not produce detectable histological changes in the pancreas over the course of 2–6 weeks (Figure 3). By contrast, we observed rapid de-differentiation of *Ptf1a cKO* acinar cells generated under a high-TM dose regimen that produced >65% deletion (Figure 4). This is an important finding regarding potentially non-cell autonomous protective mechanisms working to offset PanIN/PDAC initiation. We propose two linked hypotheses: first, when *Ptf1a* is lost from individual cells, other acinar-specific transcription factors that prevent reprogramming and co-regulate PTF1 target genes, such as NR5A2 and BHLHA15/MIST1, are sufficient to maintain a differentiated phenotype over the short term. Second, we propose that the persistent differentiation of *Ptf1a cKO* acinar cells is promoted by interactions with neighboring *Ptf1a* WT cells, producing a phenomenon similar to the 'community effect' in embryonic development (Gurdon et al., 1993). However, with increasing TM-driven deletion, the fraction of *Ptf1a* WT acinar cells passes a tipping point, the community effect cannot be sustained, and ductal metaplasia is correspondingly rapid. At a molecular level, the protective effect of wild-type acinar cells could be mediated by their ability to dampen local inflammation, suggested by the upregulation of fibroinflammatory pathways in our RNA-seq analyses (Figure 5). This is also suggested by our finding that *Ptf1a* cKO pancreata exhibit sustained inflammation after acute injury, including the conversion of acinar cells to PanIN-like, Alcian Blue+ ductule structures (Figure 6). These resemble tubular complexes observed in human and mouse chronic pancreatitis (Bockman et al., 1982; Strobel et al., 2007), suggesting that dysregulation of PTF1A expression or function might be involved in the etiology of this disease and its well-known contribution to PDAC risk. We additionally demonstrate that inflammation and loss of *Ptf1a* synergize to drive sustained activation of the MEK-ERK pathway, a major effector of oncogenic and endogenous KRAS. Going forward, it will interesting to test whether MEK inhibitors are able to prevent acinar cell reprogramming in the context of chronic pancreatitis and/or decrease the risk of chronic pancreatitis progressing to PDAC.

Given the dramatic effects of *Ptf1a* deletion on transformation and inflammation, it will be important to determine which genes in our RNA-seq data set are directly suppressed or activated by PTF1A. It has been previously established that PTF1A regulates a network of transcription factors controlling acinar-specific gene expression (Masui et al., 2008, 2010). Among these are *Bhlha15/Mist1* and *Nr5a2*, both downregulated in the *Ptf1a cKO* condition (Figure 5), and both previously shown to inhibit PanIN development (Shi et al., 2009b; Flandez et al., 2014; von Figura et al., 2014b). Of these three genes, only *Ptf1a* is indispensable for acinar cell differentiation (Krapp et al., 1998;

Pin et al., 2001; *Kawaguchi et al., 2002*; *Holmstrom et al., 2011*; *von Figura et al., 2014b*), and it will be of interest to determine the relative rank of these factors as suppressors of cancer initiation and progression, and their epistatic relationship. It will also be useful to understand how KRAS, together with inflammatory and other insults, is capable of downregulating the expression and/or function of PTF1-network components during tumor initiation. Of note, recent studies indicate that oncogenic KRAS induces specific pathways dedicated to silencing tumor suppressor genes (*Wajapeyee et al., 2013*; *Serra et al., 2014*); a similar process may drive downregulation of *Ptf1a* and its partners during acinar cell reprogramming.

Because loss of *Ptf1a* strongly potentiated KRAS-mediated transformation (*Figures 2, 3*), we hypothesized that PTF1A inhibits KRAS-signaling activity in some capacity. Here, we demonstrate that loss of *Ptf1a* leads to upregulation of genes associated with KRAS-dependency in human cancer cells (*Singh et al., 2009*; *Loboda et al., 2010*). Future investigations should therefore move forward to test if different subtypes of human PDAC exhibit different extents of *PTF1A* repression, and whether variation in *PTF1A* expression within human PDAC correlates with KRAS-dependency or disease prognosis. Recent studies have classified ~1/3 of pancreatic cancers as 'exocrine-like', and several genes that are under *Ptf1a* control contribute to this signature (*Figure 5A,B*) (*Collisson et al., 2011*). Unfortunately, human *PTF1A* was not present on the microarray used in that study; nonetheless, their data suggest that PTF1A and its transcriptional targets are retained at low levels in some, but not all, cases of PDAC. Consistent with these previous reports, we found that sparse epithelial cells in human PanIN lesions retain nuclear PTF1A (*Figure 1—figure supplement 1*). In addition to supporting the contention that human PanINs and PDAC arise from mature acinar cells, these findings suggest that low levels of persistent PTF1A, held in check by epigenetic rather than genetic mechanisms, may be available for therapeutically targeted restoration. The fact that removal of a single allele of *Ptf1a* accelerates mouse PDAC development (*Figure 7D*) suggests that even incomplete inhibition of human PTF1A could promote acinar transformation and subsequent tumorigenesis. Our results suggest that PTF1A restoration provides an indirect approach to target KRAS-dependency in pancreatic cancer, inhibiting this currently 'undruggable', although ubiquitous, cancer-driving mutation (*Pasca di Magliano and Logsdon, 2013*). Additionally, our data suggest that PTF1A restoration may reduce inflammatory pathways that feed forward to synergize with oncogenic KRAS (*Maniati et al., 2011*; *Daniluk et al., 2012*; *Maier et al., 2013*). Future studies will focus on genetic PTF1A gain-of-function approaches to determine if sustained PTF1A expression can prevent and/or reverse acinar-to-ductal reprogramming, PanIN initiation and PDAC progression.

In summary, we show that acinar cell differentiation, maintained through PTF1A, suppresses multiple oncogenic pathways associated with PDAC initiation and progression. Our data suggest that PTF1A functions as a nodal point in PDAC initiation by maintaining acinar-cell gene expression, suppressing KRAS function, and resisting inflammation. The antagonism between KRAS and the pro-acinar transcription factor network captures, at a genetic level, the tension between differentiation and malignant transformation that has long been hypothesized to exist in cancer (*Harris, 1990*). Loss of normal differentiation and reprogramming of cell fate appear to occur during initiation of a diverse array of tumor types (*Blanpain, 2013*). Our results, for the first time, demonstrate that this process is rate-limiting for cancer development, thus, constituting a novel mechanism of tumor suppression. The mouse PanIN-PDAC model provides a new experimental system to relate genetic changes in cancer, such as *KRAS* mutation, to epigenetic changes such as PTF1A downregulation. Furthermore, understanding how PTF1A function is subverted during pancreatic cancer initiation, and whether its reactivation could suppress or reverse tumor development, may yield novel approaches to prevention and treatment.

Materials and methods

Mice

Experimental mice of the following genotypes have been previously described: *Ptf1a*^{CreERT} (*Ptf1a*$^{tm2(cre/ESR1)Cvw}$ [*Kopinke et al., 2012*; *Pan et al., 2013*]), *Pdx1-Cre* (*Tg(Pdx1-cre)89.1Dam* [*Gu et al., 2002*]), *Kras*$^{LSL\text{-}G12D}$ (*Kras*^{tm4Tyj} [*Hingorani et al., 2003*]), *p53*^{flox} (*Trp53*^{tm1Brn} [*Marino et al., 2000*]), and *R26R*^{EYFP} (*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ [*Srinivas et al., 2001*]). The *Ptf1a*^{flox} allele (*Ptf1a*^{tm3Cvw}) was generated using homologous recombination in mouse ES cells at the Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource. The 5′ and 3′ loxP sites were placed 1.7 kb upstream and 2 kb downstream of the *Ptf1a* transcriptional start site, respectively. Full details will be provided elsewhere (Wright et al., in preparation). Mice with a germ line deletion allele of *Ptf1a*, *Ptf1a*† were generated by

crossing *Ptf1a*^{CN} to the ubiquitous early deletor line *Sox2-Cre* (*Tg(Sox2-cre)1Amc*[*Hayashi et al., 2003*]). To activate CreERT-mediated recombination, mice were administered tamoxifen (Sigma, St. Louis, MO) dissolved in corn oil, via oral gavage at doses indicated in the text. All mouse experiments were carried out according to institutional and NIH guidelines.

Human histological specimens

All human pathological specimens were de-identified before their use. The utilization of these human specimens is therefore not considered human subject research under the US Department of Human and Health Services regulations and related guidance (45 CDR Part 46). Paraffin embedded specimens were sectioned (6 μm) and IHC was performed for PTF1A, as described below. Samples were analyzed by NMK, MPB, and LCM.

Tissue processing and histology

After euthanasia, pancreata were dissected in ice-cold phosphate-buffered saline solution (PBS), separated into multiple fragments, and processed for both frozen and paraffin sections as previously described (*De La et al., 2008*; *Keefe et al., 2012*; *Kopinke et al., 2012*). Briefly, tissues were fixed for paraffin embedding in zinc-buffered formalin (Z-FIX; Anatech, Battle Creek, MI), room temperature overnight, or 4% paraformaldehyde/PBS, 4˚C 1–2 hr, followed by processing into Paraplast Plus (McCormick Scientific) or Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA). Paraffin and frozen sections were cut at thickness of 6 μm and 8 μm, respectively, and collected sequentially across multiples slides, with ~100-μm spacing between individual sections on a single slide.

IHC and immunofluorescence followed our established procedures (*De La et al., 2008*; *Keefe et al., 2012*; *Kopinke et al., 2012*), including high-temperature antigen retrieval (Vector Unmasking Solution; Vector Laboratories, Burlingame, CA) prior to staining paraffin sections. Primary antibodies utilized in this study are listed in *Table 2*. Secondary antibodies, raised in donkey (Jackson Immunoresearch, West Grove, PA), were used at 1:250 dilution. Vectastain reagents and diaminobenzidine (DAB) substrate (Vector Laboratories) were used for IHC. Slides stained by immunofluorescence were counterstained with DAPI and mounted in Fluoromount-G (Southern Biotech), and photographed on an Olympus IX71 microscope, using MicroSuite software (Olympus America, Waltham, MA). Images were processed in Adobe Photoshop, with exposure times and adjustments identical between genotypes and treatment groups.

For Alcian Blue staining, paraffin sections were washed 5 min in 3% acetic acid, followed by a 10–12 min incubation in staining solution (1% Alcian Blue in 3% acetic acid), and extensive washing in 3% acetic acid. Sirius Red staining was performed on frozen sections that were fixed for 1 hr in Bouin's fixative at 55˚C. Specimens were subsequently washed in dH_2O and stained for 1 hr in Picro-Sirius Red (American MasterTech, Lodi, CA). Following staining, specimens were rinsed in 0.5% acetic acid, dehydrated and equilibrated into xylene, and mounted with Permount.

PanIN scoring

The entire tissue area of Alcian Blue/eosin-stained slides was photographed, at 4× original magnification, followed by photomerging (Adobe Photoshop) and surface area measurement using ImageJ software (NIH). Alcian Blue+ PanIN lesions were counted manually under the microscope, and PanIN burden calculated as the total number of Alcian Blue+ lesions per cm^2 surface area. As described in the text, metaplastic lesions that did not stain with Alcian Blue were not counted in the quantification. To avoid double-counting of potentially large and tortuous lesions, no more than one lesion was scored within an anatomically distinct pancreatic lobule (*De La et al., 2008*).

3D pancreatic acinar cultures

Acinar cultures were established according to previous publications (*Kurup and Bhonde, 2002*; *Means et al., 2005*; *Ardito et al., 2012*). Briefly, dorsal pancreata were minced in Hank's buffered saline solution and digested sequentially in 0.02% trypsin (5 min, 37˚C) and 1 mg/ml collagenase P (Roche Applied Science, Mannheim, Germany; 15 min, 37˚C), following filtration to eliminate undigested material, and repeated washing to eliminate debris and dead cells, acinar cell clusters were embedded in rat tail collagen gels (Corning, Corning, NY), and cultured in Waymouth's medium (Life Technologies, Carlsbad, CA) supplemented with 1% fetal bovine serum, 0.4 mg/ml soybean trypsin inhibitor, and 1 μg/ml dexamethasone. Cultures were fixed and imaged after 5 days. To

Table 2. Primary antibodies used in this study

Antigen	Species	Source	Catalog #	Dilution
Amylase	Sheep	BioGenesis	0480-0104	1:1000
Cleaved-caspase-3	Rabbit	Abcam	AB2302	1:1000
Cd45	Rat	eBioScience	14-0451-82	1:2000
Claudin-18	Rabbit	Invitrogen	700178	1:2000
Cpa1	Goat	R&D Systems	AF2765	1:1000
Cytokeratin-19	Rat	Developmental Studies Hybridoma Bank	–	1:50
Cytokeratin-19	Rabbit	Abcam	AB133496	1:5000
GFP	Chicken	Aves Labs Inc.	GFP-1010	1:5000
Ki67	Mouse	BD Biosciences	550609	1:500
Muc5ac	Mouse	NeoMarkers	45M1	1:500
Ptf1a	Rabbit	Chris Wright, Vanderbilt University	–	1:5000
Ptf1a	Goat	Chris Wright, Vanderbilt University	–	1:5000
Phospho-ERK1/2 (T202/Y204)	Rabbit	Cell Signaling	9101	1:1000
Sox9	Rabbit	Millipore	AB5535	1:1000
α-SMA	Rabbit	Abcam	AB32575	1:2000

SMA, smooth muscle actin.

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quantify cyst size, we randomly selected >10 fields per mouse, imaged, and quantified the maximal diameter of each transformed cyst using ImageJ.

Quantification of immunofluorescence images

In order to quantify $R26R^{EYFP}$ labeling, 10–12 randomly selected 20× fields per specimen (taken across multiple sections) were photographed. Using ImageJ software (NIH), cells co-expressing EYFP with the acinar differentiation markers Amylase or CPA1 were detected by additive image overlay of their staining with DAPI and anti-GFP, and counted using the Analyze Particles function, as described previously (***Keefe et al., 2012***; ***Kopinke et al., 2012***). To ensure counting accuracy, random images were spot checked by manual counting using Adobe Photoshop. All calculations were performed in Microsoft Excel and the results are reported as the mean ± standard deviation (error bars). p-values were determined by two-tailed, unpaired t-tests performed in Excel or Graphpad Prism 6.

RNA-seq analysis

Total RNA was isolated from pancreata of 4- to 5-month-old *Ptf1a* cKO mice ($Ptf1a^{CreERT/lox}$) and their corollary controls ($Ptf1a^{CreERT/+}$), 2 weeks after TM treatment (3 days, 0.25 mg/g/day), using the guanidine thiocyanate protocol previously described with minor modifications (***MacDonald et al., 1987***). Individual RNA-Seq libraries were prepared from 5 μg of pancreatic RNA from three control and three *Ptf1a* cKO mice with the Illumina True-seq protocol by the UT Southwestern Genomic Core. The sequence data sets from an Illumina HISEQ2500 contained 50-nucleotide uniquely aligned single-end reads of 25.1, 26.4, and 25.8 million for the control samples and 29.7, 24.7, and 26.7 million for the *Ptf1a* cKO RNA samples (Tophat2) (***Kim et al., 2013***). Genes with differential expression were derived using edgeR (***Robinson et al., 2010***), with the default trimmed mean of M-values (TMM) trim settings of 30% for M_g and 5% for A_g and an FDR cut-off of <0.05. The volcano plot of differentially expressed genes was generated using R (http://www.r-project.org/) with the log2 fold change (FC) plotted against the FDR (−log10) (***Supplementary files 1, 2***).

Gene signatures of RAS dependency (***Singh et al., 2009***; ***Loboda et al., 2010***), classical and exocrine-like PDAC (***Collisson et al., 2011***), were mapped to orthologous mouse genes via HomoloGene ID. The RAS dependency signature combines gene lists from two separate studies (***Singh et al., 2009***; ***Loboda et al., 2010***), comprising 264 genes with only five in common. For GSEA

(*Subramanian et al., 2005*), we analyzed signature enrichment within the entire *Ptf1a* cKO RNA-seq data set, ordered by log2 FC relative to control, using the GSEA desktop application (http://www.broadinstitute.org/gsea/index.jsp).

To identify regulatory pathways altered upon *Ptf1a* deletion, significantly increased and decreased genes were analyzed by IPA (QIAGEN, Redwood City, CA, www.ingenuity.com) at expression thresholds of 1.5-, 2.0,- and 3.0-fold (*Supplementary files 3–5*). In order to obtain an accurate comparison between enriched and downregulated pathways, we used the Comparison Analysis function from expression data filtered at a gene expression threshold of ±2.0-fold. Heat maps were generated according to the −log p-values given by the IPA software using the comparison analysis function and were constructed in R (http://www.r-project.org/).

Caerulein treatment

We induced acute pancreatitis by i.p. injection of caerulein (Bachem, Torrance, CA), 0.1 μg/g, eight times daily over two consecutive days, as previously (*Jensen et al., 2005*; *Keefe et al., 2012*). Negative controls were injected with saline vehicle alone. Pancreata from all caerulein- or saline-treated mice were harvested 1 week following the final injection and processed as described above.

Kaplan–Meier analysis

KPC mice (of the genotype *Pdx1-Cre*; $Kras^{G12D}$; $p53^{lox/+}$) and KPC mice with *Ptf1a* heterozygosity (*Pdx1-Cre*; $Kras^{G12D}$; $p53^{lox/+}$; $Ptf1a^{+/-}$) were aged until they exhibited lethargy or distress as determined by the authors (NMK and LCM) and the in-house veterinary staff, or until the detection of a firm abdominal mass by palpation. The presence of PDAC was confirmed by histological analysis in consultation with a surgical pathologist (MB). At sacrifice, all mice were thoroughly inspected for liver metastases. Survival analysis was performed in GraphPad Prism (Version 6) and p-values were calculated using a Log-rank test.

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Author contributions

NMK, J-PDLO, GHS, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; CQH, GMC, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; SGW, FCP, Drafting or revising the article, Contributed unpublished essential data or reagents; MPB, Analysis and interpretation of data, Drafting or revising the article; CVEW, Conception and design, Drafting or revising the article, Contributed unpublished essential data or reagents; RJMD, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article, Contributed unpublished essential data or reagents; LCM, Conception and design, Analysis and interpretation of data, Drafting or revising the article

Ethics

Animal experimentation: This study was performed according to institutional and National Institutes of Health guidelines for animal research (Guide for the Care and Use of Laboratory Animals), and followed protocols approved by the Institutional Animal Care and Use Committees of the University of Utah (protocol #13-09009), University of Texas Southwestern Medical Center (protocol #2013-0008) and Vanderbilt University (protocol #M10/106).

Additional files

Supplementary files

- Supplementary file 1. R markdown for RNA-seq analysis. HTML annotation of R software package analysis performed to generate the data and analyses presented in ***Figure 5***.

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- Supplementary file 2. Files used for RNA-seq analysis. Excel spreadsheet containing (as tabs) files used in R analysis of RNA-seq data, including differentially expressed genes (false discovery rate < 0.05), gene signatures of RAS dependency, classical pancreatic ductal adenocarcinoma (PDAC) and exocrine-like PDAC, and 'palette' file used for color-coding volcano plot.

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- Supplementary file 3. Ingenuity Pathway Analysis (IPA) analysis output for genes changed >1.5-fold. Excel spreadsheet output from IPA (www.ingenuity.com), indicating predicted up- and down-regulated pathways and regulators from *Ptf1a* conditional knock-out (cKO) RNA-seq data, based on a differential expression threshold of 1.5-fold.

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- Supplementary file 4. IPA analysis output for genes changed >twofold. Excel spreadsheet output from IPA (www.ingenuity.com), indicating predicted up- and down-regulated pathways and regulators from *Ptf1a* cKO RNA-seq data, based on a differential expression threshold of twofold.

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- Supplementary file 5. IPA analysis output for genes changed >threefold. Excel spreadsheet output from IPA (www.ingenuity.com), indicating predicted up- and down-regulated pathways and regulators from *Ptf1a* cKO RNA-seq data, based on a differential expression threshold of threefold.

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Major dataset

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset ID and/or URL	Database, license, and accessibility information
Hoang Chinh, Galvin H Swift, Ana Azevedo-Pouly, Raymond J MacDonald	2015	Effects on the transcriptome of adult mouse pancreas (principally acinar cells) by the inactivation of the Ptf1a gene in vivo	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70542	Publicly available at NCBI Gene Expression Omnibus (Accession No: GSE70542).

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CHAPTER 4

PANCREATIC ACINAR CELL DEDIFFERENTIATION ABROGATES ANTI-INFLAMMATORY AND ANTI-TUMOR RESPONSES TO DEXAMETHASONE

Abstract

Pancreatitis-mediated injury stimulates *Kras*-mediated tumorigenesis in mouse models of pancreatic ductal adenocarcinoma (PDAC). We recently discovered that acinar cell dedifferentiation induced by *Ptf1a* deletion also promotes PDAC initiation. As we find that both injury and dedifferentiation are associated with inflammation, we hypothesized that tumorigenesis in these models could be alleviated by the anti-inflammatory drug dexamethasone. While dexamethasone subdued tumor initiation induces by the combination of injury and oncogenic *Kras*, it paradoxically accelerated tumorigenesis in the context of *Ptf1a* deletion. Our data indicate that tumor-promoting inflammation induced by dedifferentiation is distinct from that induced by injury, and suggest caution in targeting inflammation as a tumor driver in PDAC.

Introduction

Patients with pancreatic ductal adenocarcinoma (PDAC) have a dismal prognosis, partially due to limited treatment options. Activated *Kras* (*Kras*^{G12D}), the major driver oncogene of PDAC, is currently “undruggable” and targeting its immediate downstream signaling molecules has debilitating side-effects. Therefore, using preclinical models of PDAC to identify and modulate other targets that stall or reverse early disease is of the utmost importance (Ryan et al., 2014).

Expressing activated *Kras*^{G12D} exclusively in murine acinar cells is sufficient for preneoplastic pancreatic intraepithelial neoplasia (PanIN) formation, albeit at a slow pace (De La et al., 2008; Kopp et al., 2012). Several recent studies suggest that the transforming ability of *Kras* is greatly enhanced by extrinsic inflammatory signals (di

Magliano and Logsdon, 2013). This connection between acinar-intrinsic mutant *Kras* and acinar-extrinsic inflammation appears to be bidirectional; high levels of inflammatory signaling, as seen in caerulein-induced pancreatitis, activate the transforming ability of Kras, and high Kras activity is itself sufficient to induce rampant pancreatic inflammation (Daniluk et al., 2012; De La and Murtaugh, 2009; di Magliano and Logsdon, 2013; Huang et al., 2014; Ji et al., 2009). The positive feedback loop that emerges between inflammation and oncogenic Kras activity provides an attractive therapeutic target. Indeed, broadly inhibiting inflammation with the corticosteroid dexamethasone (DEX) preserves epithelial identity by preventing epithelial-to-mesenchymal transition and inhibits tumor cell dissemination in the well-characterized "KPC" (*Kras*^{G12D}, mutant *p53*, *Cre*) model of PDAC (Rhim et al., 2012). Understanding how inflammatory signals enhance the transforming ability of *Kras*^{G12D} will be essential to translating these findings into the clinic.

Results

While the microenvironment provides extrinsic inputs to amplifying *Kras*^{G12D} activity, cell-intrinsic factors, such as acinar differentiation determinants, act to prevent oncogenic transformation. When the acinar-specific transcription factor gene, *Ptf1a*, is deleted from exclusively acinar cells (*Ptf1a* cKO), spontaneous dedifferentiation, like that seen in pancreatitis, is rapidly induced (Hoang et al., 2016; Krah et al., 2015). *Ptf1a* cKO pancreata are also dramatically sensitized to *Kras*^{G12D}-mediated PanIN formation, with persistent leukocyte infiltration and fibrosis, similar to that seen when acinar-*Kras*^{G12D} mice are given caerulein-induced pancreatitis (Figure 4.1A-I) (De La and Murtaugh, 2009;

Kopp et al., 2012; Krah et al., 2015). Based on these phenotypic similarities, we hypothesized that broadly inhibiting inflammation with DEX would suppress the rapid transformation seen in both *Kras*^{G12D}/caerulein and *Kras*^{G12D}/*Ptf1a* cKO mouse models. Additionally, these experiments allowed us to test the epistatic relationship between inflammatory signals and acinar dedifferentiation during early PDAC initiation (Figure 4.1J, K).

To test these hypotheses, we developed two treatment regimens that generate a similar phenotype over an 18-day period. In this protocol, mice received either a daily injection of 10 mg/kg DEX or saline vehicle, beginning simultaneously with tamoxifen treatment (Figure 4.2). *Kras*/caerulein mice (genotype: *Ptf1a*$^{CreERT/+}$; *Kras*$^{LSL\text{-}G12D/+}$, Figure 4.2A) were administered tamoxifen (TM) on three consecutive days, before receiving caerulein treatment on days 7 and 8. Mice were sacrificed for histologic examination 10 days after the first caerulein administration (Figure 4.2B). In contrast, *Kras*/*Ptf1a* cKO mice were administered a single pulse of TM with no further intervention until sacrifice 18 days later (Figure 4.2B). The resulting histologic phenotypes exhibit widespread acinar cell transformation, fibrosis, and leukocyte infiltration, but minimal mature PanIN formation, which allowed us to evaluate the early stages of PDAC initiation and any acceleration/deceleration of disease.

Using this protocol, we found that DEX-treated *Kras*/caerulein pancreata exhibited reduced acinar cell transformation and a significant preservation of normal acinar tissue (Figure 4.3A-B, F), despite similar gross appearance and mass (Figure 4.3E). Overall, inflammation was reduced in DEX-treated pancreata, but was still present in transforming and fully transformed areas. Fibrosis, a hallmark of PanIN initiation and

progression, was highlighted with Sirius red staining and revealed a reduction in fibrotic tissue in DEX-treated *Kras*/caerulein mice (Figure 4.3C-D). These results corroborate those obtained in the KPC model, and confirm that the tumor-promoting effects of pancreatitis are mediated by DEX-sensitive inflammatory mechanism.

In contrast, *Kras/Ptf1a* cKO mice treated with DEX retained no histologically normal acinar tissue compared to saline treated controls (Figure 4.3 G-H) and exhibited a surprising number of high-grade PanINs following our 18-day protocol (Figure 4.3H). While pancreata from DEX-treated animals had a significant reduction in edema and organ mass (Figure 4.3M), they also exhibited a dramatically increased PanIN burden (Figure 4.3N), which was confirmed with Alcian blue staining and immunohistochemistry for Claudin18 (Figure 4.3I-L). Regardless of treatment, however, *Kras/Ptf1a* cKO pancreata exhibited a severe fibroinflammatory response, as indicated by Sirius red staining (data not shown), which is consistent with our previous findings (Krah et al., 2015).

Based on these results, we hypothesized that forced acinar dedifferentiation, via *Ptf1a* deletion, establishes a unique inflammatory milieu that alters the response to DEX. To begin to test this hypothesis, we performed immunofluorescence for different immune cell types to examine their abundance and localization. Although we found abundant CD45+ leukocytes (primarily F4/80^{+} macrophages) in both models, these cells were decreased by DEX only in *Kras*/caerulein mice (Figure 4.4A-H). Regions of the pancreas in which amylase+ acinar cells were preserved by DEX treatment contained scant CD45+ leukocytes. In contrast to macrophages, the infiltration of CD3+ T-cells that wasobserved in both models was uniformly abolished by DEX treatment (Figure 4.4I-L, M).

Interestingly, CD8+ T-cells, which have an inhibitory effect on PanIN initiation and progression (Zhang et al., 2014), were completely ablated by DEX treatment in *Kras/Ptf1a* cKO pancreata (Figure 4.4N-O).

Discussion

Our data suggest the existence of distinct inflammatory infiltrates, one that includes T-cells and is DEX-sensitive, and another that includes F4/80+ cells (macrophages and/or other monocytes), that is DEX-resistant at least in the context of *Ptf1a*-deficient epithelium (Figure 4.5). As both macrophages and T-cells are required for PanIN formation (Liou et al., 2015; McAllister et al., 2014; Zhang et al., 2014), we propose the possibility that an initial phase of DEX-sensitive inflammation is responsible for propagating acinar cell dedifferentiation, which in turn triggers a second, DEX-resistant phase that promotes *bona fide* PanIN development. Deletion of *Ptf1a* may effectively "skip" the first inflammatory phase, allowing the second phase inflammatory cells to infiltrate and rapidly induce PanINs.

Importantly, our results highlight the dynamics of the PDAC microenvironment during different stages of tumor initiation and suggest that these changes may influence the response to commonly used anti-inflammatory drugs. Consistent with previous studies, we found that DEX treatment preserves normal pancreatic architecture in differentiation-competent epithelium, which suggests that the inflammatory signals inhibited by DEX act upstream of acinar cell identity loss (Rhim et al., 2012). However, our results also suggest that other inflammatory signals, perhaps from macrophages/monocytes, act downstream of dedifferentiation to drive PanIN formation.

These signals are impervious to DEX treatment and act rapidly to further transform dedifferentiated cells. Identifying and inhibiting the signals that act upstream and downstream of cell identity changes will be crucial in treating PDAC that is associated with chronic inflammation (pancreatitis), and in patients with other risk factors that generate systemic inflammation, such as obesity and smoking (Krah and Murtaugh, 2016). As targeting the tumor microenvironment has emerged as a new anticancer approach, particularly in tumors whose cell-autonomous drivers are hard to directly target, our results merit further investigation.

We hypothesize that the first "wave" of inflammation is T-cell-dependent and mediates the initial steps in acinar cell transformation. To test this hypothesis, our lab will determine if IL-17a signaling from CD4+ T-cell is required for PanIN formation in the absence of *Ptf1a*. Ablation of CD4+ T-cells, which includes T-helper and T_H17 cells, has been shown to inhibit PanIN development in the *Kras*/caerulein model (McAllister et al., 2014; Zhang et al., 2014). The results described in this thesis, however, suggest that blocking T-cell infiltration with DEX does not prevent PanIN development in the Kras/*Ptf1a* cKO model; this implies that the major role for T-cells and IL-17 signaling during PanIN initiation is to promote acinar de-differentiation. This could be tested in two ways: first, CD4+ T-cells could be depleted in both the *Kras*/caerulein and *Kras*/*Ptf1a* cKO by weekly i.p. injection of purified GK1.5 anti-CD4 monoclonal antibody (BioXCell), as previously described (McAllister et al., 2014). Second, we could cross both *Kras*/caerulein and *Kras*/*Ptf1a* cKO mice onto an *Il17a*$^{-/-}$ background and determine whether this influences PanIN burden in both models (Nakae et al., 2002). What results would be expected if CD4+ T-cells act to cause dedifferentiation? If either

of these inputs (CD4+ T-cells or IL-17a) acts strictly at the dedifferentiation stage, then we expect that it should be dispensable for PanIN formation in a *Ptf1a* cKO background. However, if these cells/signals promote progression to the PanIN stage, we could expect to observe dedifferentiation lesions without hallmarks of progression (i.e., no Alcian blue^{+} lesions, or Claudin18^{-} lesions) (Krah et al., 2015).

It will also be necessary to test whether macrophage-derived IL-6 promotes PanIN development independent of differentiation state. Given the abundance of macrophages present in both our experimental models, we hypothesize that macrophage-derived IL-6 is required to fully activate Kras^{G12D} and drive dedifferentiated *Ptf1a*-null acini toward a PanIN fate. This represents the theoretical second "wave" of inflammation that is required to progress ADM into PanINs. To test whether macrophages themselves are required for ADM-to-PanIN progression, we will use gadolinium (III) chloride ($GdCl_3$, Sigma), which specifically kills macrophages due to their phagocytic activity (Hardonk et al., 1992). $GdCl_3$ will be administered in both Kras/caerulein and *Kras/Ptf1a* cKO models and we will quantify the PanIN formation and verify macrophage depletion. To determine whether *Ptf1a* loss abrogates the need for IL-6, we propose to cross *Kras* and *Kras/Ptf1a* cKO mice onto an Il6$^{-/-}$ background, which has previously been shown to dramatically curtail PanIN development (Kopf et al., 1994; Lesina et al., 2011). We hypothesize that macrophage derived IL-6 is required after dedifferentiation for Kras-induced PanIN formation; therefore, blocking either macrophages or IL-6 should halt tumor initiation at the ADM stage, even in Ptf1a cKO pancreata. Such a result would encourage further development of this approach for treatment of tumors that have progressed beyond the point of redifferentiation. However, if PanINs do form, despite

macrophage or IL-6 depletion, it would infer that macrophage and IL-6 signaling is require solely to drive acinar metaplasia, and may not be a viable target in advanced PDAC. As we anticipate that the future of PDAC therapies will involve not only chemotherapy, but manipulation of differentiation, inflammation, and antitumor immunity, these results have the potential to inform future treatment decisions.

Materials and Methods

Mice

All of the following mouse alleles utilized in this study have been previously described in the referenced publications: *Ptf1a*^{CreERT} (*Ptf1a*$^{tm2(cre/ESR1)Cvw}$),(Kopp et al., 2012) *Ptf1a*^{lox},(Hoang et al., 2016; Krah et al., 2015) and *Kras*$^{LSL\text{-}G12D}$ (*Kras*^{tm4Tyj})(Hingorani et al., 2003). To activate CreERT-mediated recombination, mice were administered 0.25 mg/g body weight tamoxifen (Sigma, St. Louis, MO) dissolved in corn oil by oral gavage (see Figure 4.2 for experimental schematics). All experiments were performed per institutional and NIH guidelines.

Dexamethasone treatment

Kras^{G12D} mice (genotype: *Ptf1a*$^{CreERT/+}$; *Kras*$^{LSL\text{-}G12D/+}$) and *Kras*^{G12D}; *Ptf1a* cKO mice (genotype: *Ptf1a*$^{CreERT/lox}$; *Kras*$^{LSL\text{-}G12D/+}$) were injected intraperitoneally (i.p.) with either dexamethasone (10 mg/kg/day) or saline daily for 17 days (Rhim et al., 2012). Mice were sacrificed on day 18 of our protocol, and pancreata were excised for histological analysis.

Tissue processing and histology

Following euthanasia, pancreata were dissected into chilled phosphate buffered saline (PBS) and weighed, prior to being separated into several fragments for paraffin and frozen histology. Tissues were fixed for paraffin embedding in zinc-buffered formalin (Z-FIX; Anatech, Battle Creek, MI) at room temperature overnight and were processed into Paraplast Plus (McCormick Scientific). Frozen specimens were fixed in 4% paraformaldehyde/PBS at 4°C for 1–2 hours, settled into 30% sucrose in 1x PBS, followed by processing into Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA). Both Paraffin and frozen sections were cut at thickness of 6 μm and collected sequentially across multiples slides with at least 100-μm spacing between individual sections on a single slide; this allowed us to gain a representative picture of the histology throughout the entire organ on a single slide.

H&E staining, immunohistochemistry, and immunofluorescence were performed following standard procedure from our previously published papers. All IHC slides were subjected to high-temperature antigen retrieval (Vector Unmasking Solution; Vector Laboratories, Burlingame, CA) prior to primary antibody application. Primary antibodies utilized in this study are listed in Table 4.1. Vectastain reagents and diaminobenzidine (DAB) substrate (Vector Laboratories) were used for IHC. Immunofluorescence slides were counterstained with DAPI and mounted in Fluoromount-G (Southern Biotech) and photographed on an Olympus IX71 microscope using MicroSuite software (Olympus America, Waltham, MA). Images were taken with matching exposure times and identical adjustments were performed in Adobe Photoshop between genotypes and treatment groups. Sirius red staining was performed on frozen sections, which were fixed for 1 hour

at 55C in Bouin's fixative. Specimens were washed in dH_2O and stained for 1 hour in Picro-Sirius Red at room temperature. 0.5% acetic acid was used to rinse specimens after staining; slides were then dehydrated and equilibrated into xylene and mounted with Permount. Alcian blue staining was performed on paraffin sections which were washed for 10 minutes in 3% acetic acid prior to a 15-minute incubation in staining solution (1% Alcian blue in 3% acetic acid). Slides were subsequently washed in 3% acetic acid, dehydrated, and equilibrated to xylene before being mounted with Permount. All bright field images were acquired on an Olympus CX41 microscope using MicroSuite software.

Caerulein treatment

Acute pancreatitis was induced in *Kras*^{G12D} mice (genotype: *Ptf1a*$^{CreERT/+}$; *Kras*$^{LSL\text{-}G12D/+}$) by administering hourly 0.1 μg/g i.p. injections of caerulein (Bachem, Torrance, CA) six times daily over two consecutive days. Negative controls were injected with saline vehicle alone (Jensen et al., 2005; Keefe et al., 2012; Kopp et al., 2012). In DEX treated animals, the daily DEX injection was administered following all 6 caerulein injections. Pancreata were harvested 10 days following the final injection and processed as described above.

Quantifying PanIN burden

To determine the surface area of Alcian blue stained pancreata, sections were photographed at 4x original magnification and photomerged in Adobe Photoshop. Area was determined using ImageJ software (NIH). The number of Alcian blue positive lesions was counted manually under the microscope and PanIN burden was calculated as

the total number of Alcian blue positive lesions per cm^2 surface area. As previously described, we did not count metaplastic lesions that do not stain clearly with Alcian blue. To avoid double counting large or tortuous lesions, we scored no more than one lesion within an anatomically distinct pancreatic lobule, as previously described (De La et al., 2008; Krah et al., 2015). All statistical analyses and graphs were made using Graphpad Prism 7.

Scoring normal vs abnormal area

H&E and Sirius red stained slides were photographed at 4x original magnification and photomerged in Adobe Photoshop. Using these composite images, we over-layed a grid (1mm x 1mm) and quantified the number of square containing metaplastic areas of the pancreas versus areas that retained normal acinar cells using Adobe Photoshop. The proportion of diseased area was then calculated as a percentage of the entire section area and graphed using Graphpad Prism 7.

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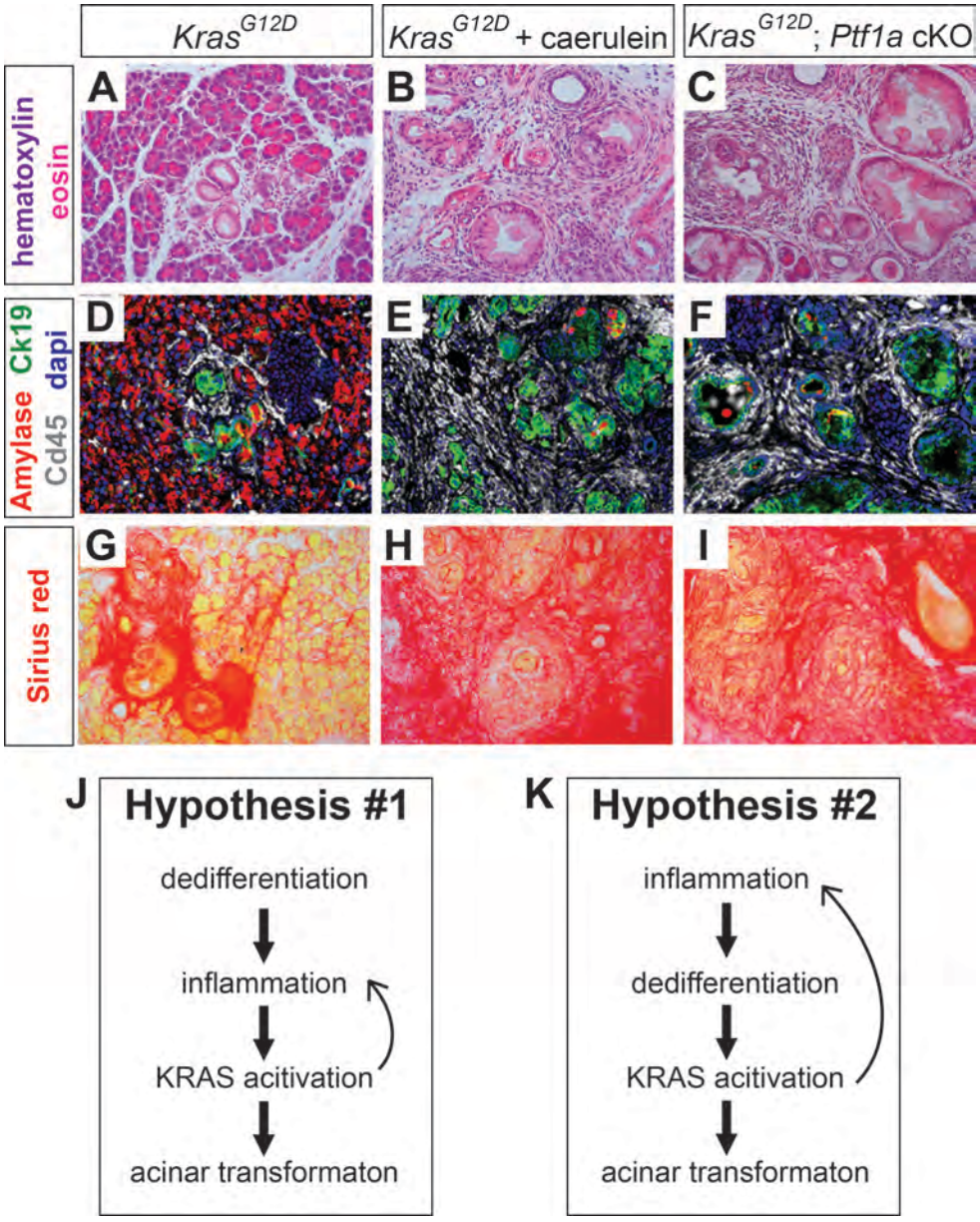

Figure 4.1. Phenotypic similarities in mouse models of pancreatic cancer initiation. H&E images from pancreata of mice expressing *Kras*^{G12D} in acinar cells 8 weeks after induction (A), mice expressing *Kras*^{G12D} in acinar cells three weeks after caerulein administration (B), and mice expressing *Kras*^{G12D} and with simultaneous *Ptf1a* deletion 6 weeks after induction (C). Immunofluorescence for amylase (acinar cells), CK19 (duct cells), and CD45 (leukocytes) (D-F), and Sirius red staining in mice of the same treatment groups (G-I). Immunofluorescent images are 20x and bright field images are 40x original magnification. (J-K) Contrasting naïve models of the relationship between dedifferentiation and inflammation during Kras^{G12D}-mediated acinar cell transformation.

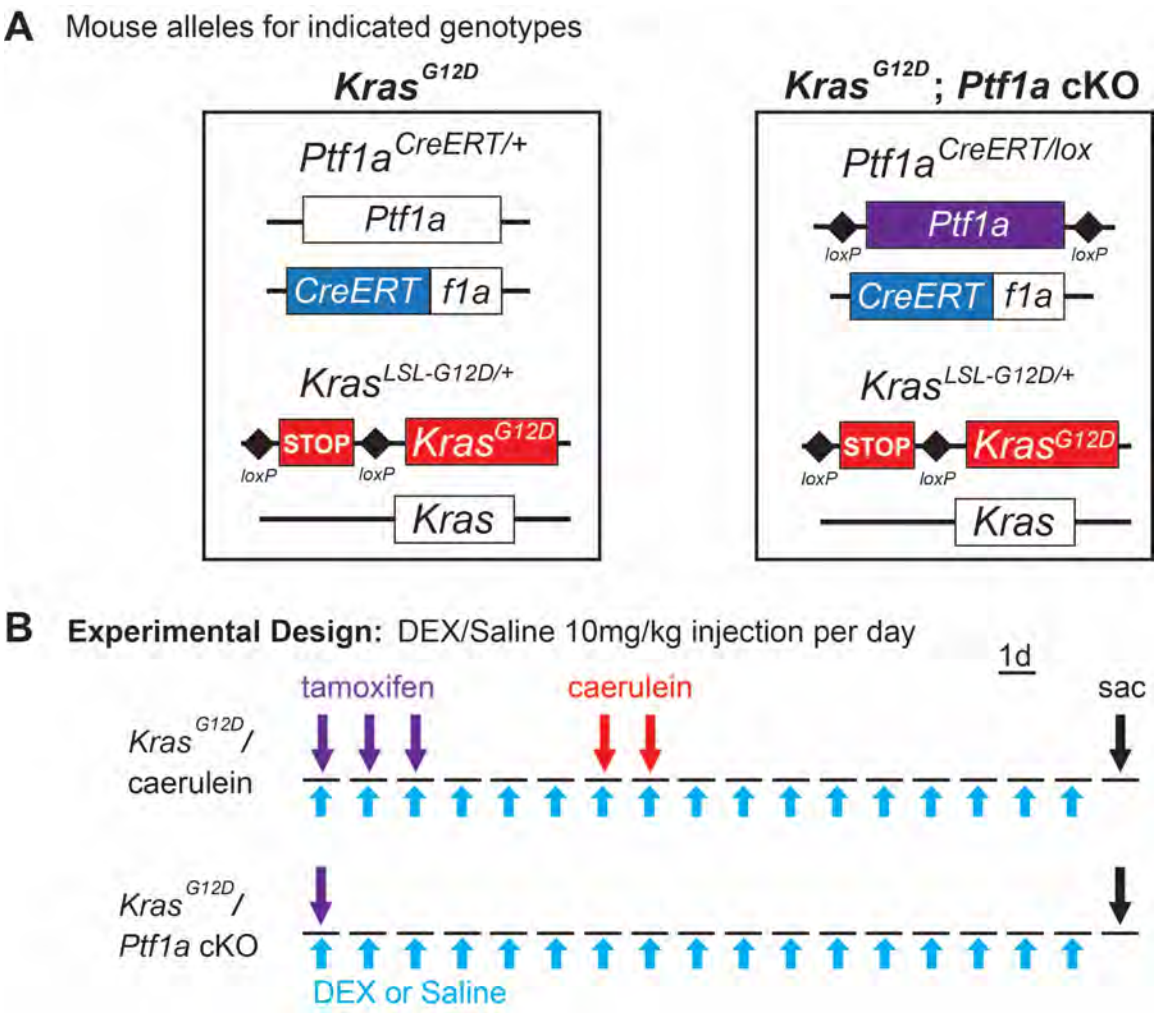

Figure 4.2. Mouse alleles and experimental schematics. (A) *Kras*^{G12D} mice contain an oncogenic *Kras*$^{LSL-G12D}$ allele which is can be activated by Cre mediated recombination, which removes the transcriptional stop cassette at the 5' end of the allele. CreERT is expressed exclusively in acinar cells by driving expression from the endogenous *Ptf1a* locus. *Kras*^{G12D}; *Ptf1a* cKO mice contain the same alleles and an additional Ptf1a "floxed" allele which can be excised by active Cre recombinase. (B) Experimental schematics for *Kras*^{G12D} mice administered Caerulein (top panel), and *Kras*^{G12D}; *Ptf1a* cKO mice (bottom panel).

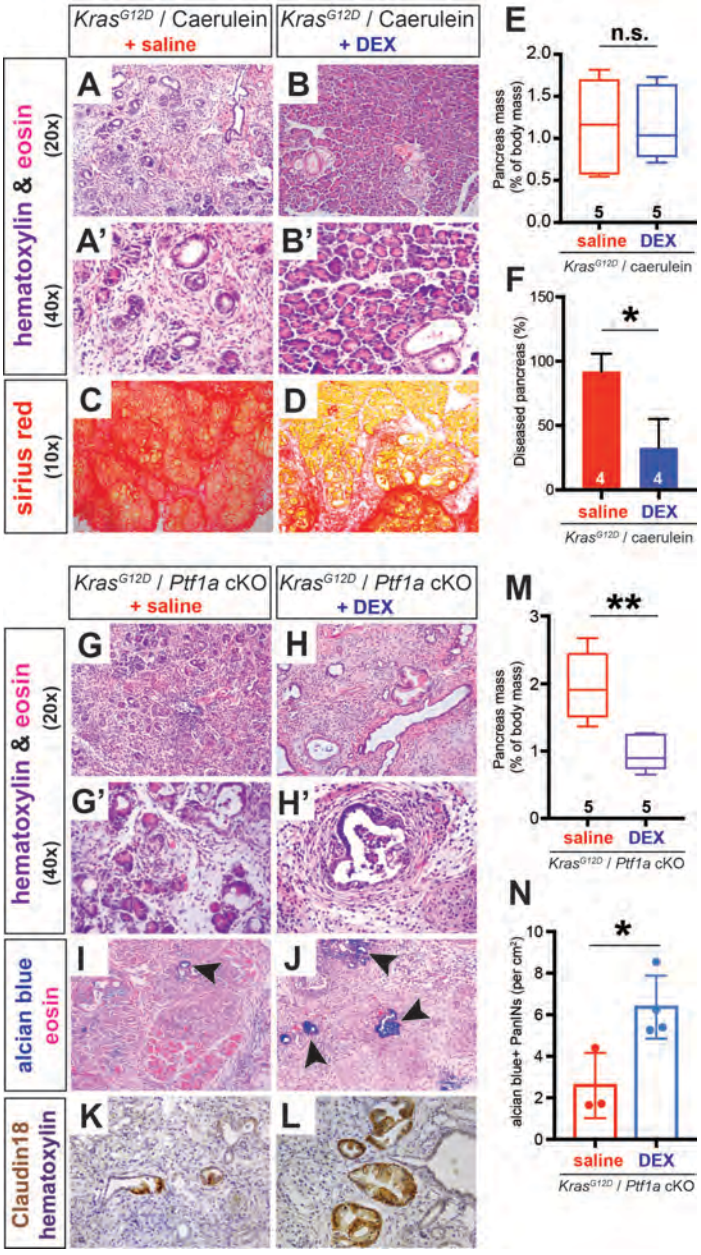

Figure 4.3. Differing responses to dexamethasone treatment in two models of PDAC initiation. Representative H&E and Sirius red images from *Kras*^{G12D} pancreata with indicated treatment **(A-D)**. Quantifications of pancreatic mass (E), and PanIN/fibrosis vs. normal histological area **(F)**. H&E, Alcian blue, and Claudin-18 staining from *Kras*^{G12D}; *Ptf1a* cKO pancreata with and without dexamethasone treatment **(H-M)**. Arrowheads indicate Alcian blue positive lesions (I-J). Quantification of pancreatic mass **(M)**, and PanINs per area **(N)**.

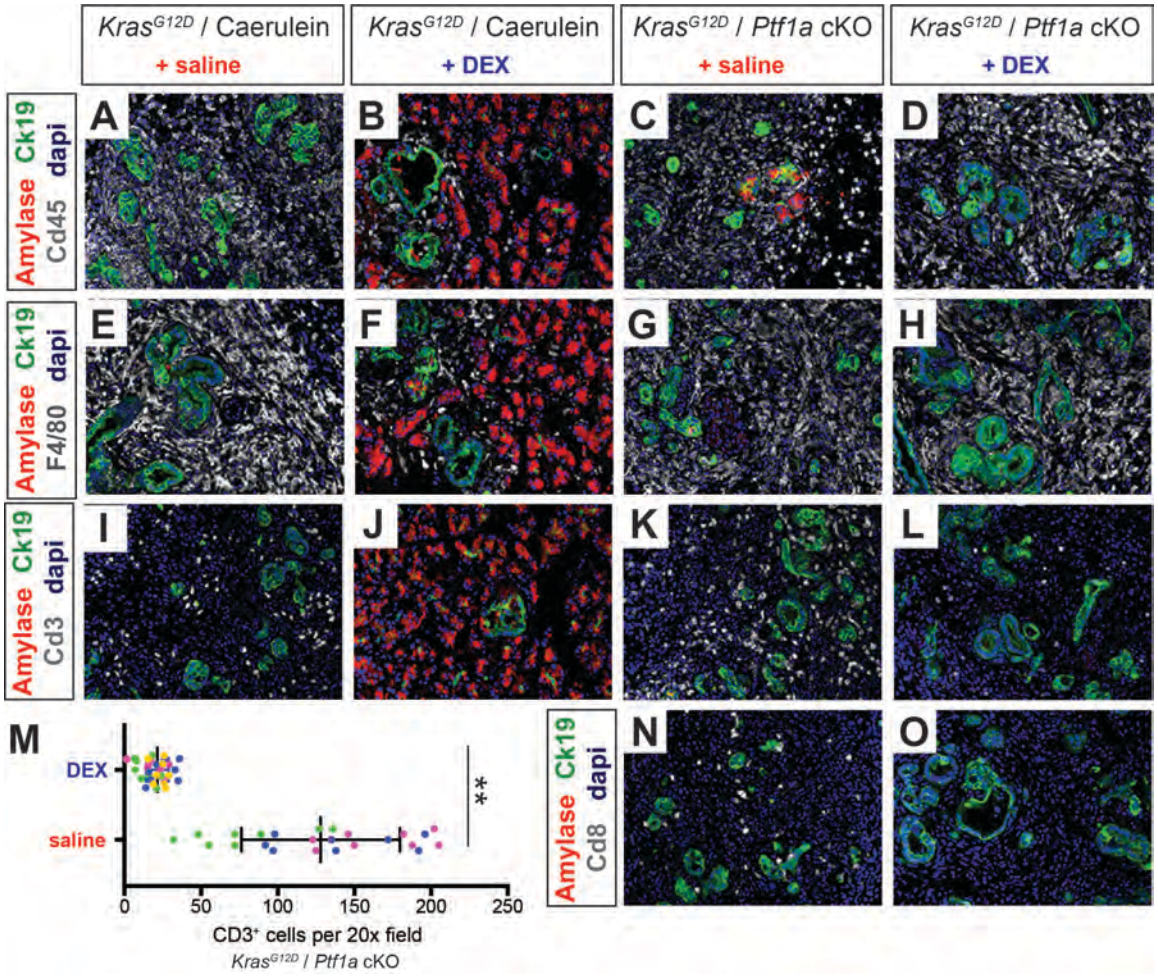

Figure 4.4. Dexamethasone treatment depletes T-cell infiltration, but not macrophage presence, in *Kras*^{G12D}; *Ptf1a* cKO pancreata. Immunofluorescence for acinar cell amylase, ductal CK19, DAPI, and either the pan-leukocyte marker CD45 (A-D) or lineage-specific markers: F4/80+ macrophages (E-H), CD3+ T-cells (I-L), or CD8+ cytotoxic T-cells (N-O). Quantification of T-cell infiltration in indicated treatment groups; each color represent an individual biological replicate, with each technical replicate shown (M). **P<0.01

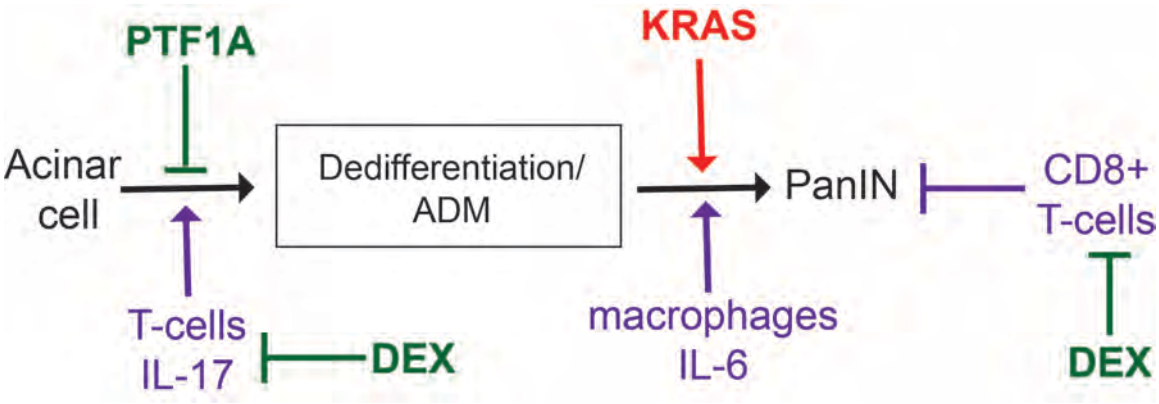

Figure 4.5. Model diagram of inflammatory infiltration during PDAC initiation. Our data suggest that acinar cell dedifferentiation plays a key role in the recruitment of tumor-promoting immune cells during KRAS-induced PDAC initiation, as well as in the response of KRAS-mutant epithelium to the signals produced by those cells. Our data suggest the existence of two distinct inflammatory responses involved in PDAC initiation, one that is DEX sensitive and PTF1A-regulated, and another that cannot be inhibited by DEX and is not directly related to PTF1A. We hypothesize that loss of PTF1A abrogates the requirement for CD4+ T-cells and IL-17. DEX-mediated inhibition of cytotoxic T-cells may also allow PanIN maturation, once acinar cells have undergone dedifferentiation.

Table 4.1. Primary antibodies utilized in this study

Antigen	Species	Source	Catalog #	Dilution used
Amylase	Sheep	BioGenesis	0480-0104	1:1000
Cd45	Rat	eBioScience	14-0451-82	1:2000
Cd3	Rat	AbD serotec	MCA500G	1:1000
Cd8a	Rat	eBioScience	14-0808-80	1:1000
Claudin-18	Rabbit	Invitrogen	700178	1:2000
Cytokeratin-19	Rabbit	Abcam	AB133496	1:5000
F4/80	Rat	eBioScience	14-4801-81	1:1000

CHAPTER 5

IN VIVO INHIBITION AND REVERSION OF PANCREATIC CANCER INITIATION THROUGH A DIFFERENTIATION-BASED MECHANISM

Abstract

Pancreatic cancer remains one of the worst prognoses in medicine, with a 5-year patient survival of only ~7%. Activating mutations in *Kras* are nearly ubiquitous in human pancreatic cancer and are sufficient to initiate precancerous pancreatic intraepithelial neoplasia (PanINs) when expressed in adult acinar cells of mice. PanINs normally take months to form, but can be rapidly induced by loss of acinar differentiation, suggesting that loss of mature cell identity is a rate-limiting step in tumor initiation. Here, we show that enforced acinar differentiation, mediated through the transcription factor *Ptf1a*, can inhibit and even reverse the early stages of pancreatic cancer. Using an established mouse model of this disease, we show that loss of *Ptf1a* allows for rapid progression to invasive and lethal adenocarcinoma, confirming that loss of acinar cell identity hastens tumor development independent of canonical tumor suppressor genes. Conversely, sustaining *Ptf1a* expression during pancreatic cancer initiation eliminates formation of *Kras*-driven PanINs. Additionally, reintroduction of *Ptf1a* into established PanINs reverts them to primitive acinar cells. To our knowledge, this is the first study establishing that a cell differentiation program can prevent and reverse oncogenic transformation *in vivo* and thus introduces a novel paradigm for solid tumor prevention and treatment.

Introduction

With a 5-year survival of only ~7%, pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid tumors and urgently requires novel interventions (Siegel et al., 2016). While the median survival of ~8 months suggests a rapid disease course,

mathematical modeling of human tumor mutation data suggests that PDAC has a gradual onset, requiring over 15 years to progress from initial genetic mutation to metastatic disease (Yachida et al., 2010). This window of time could allow for early detection, prevention and treatment before tumors metastasize and become incurable. Thus, understanding the way disease initiates and progresses is of the utmost importance.

While studying early PDAC in humans has been difficult due to the advanced presentation of disease, genetically engineered mouse models (GEMMs) have provided insight into the biology of PDAC initiation. The proto-oncogene *Kras* is mutated in over 90% of human PDAC cases (Bailey et al., 2016) and represents a driver mutation in GEMMs (Hingorani et al., 2003). Activated mutant *Kras* is required to initiate and maintain precancerous pancreatic intraepithelial neoplasia (PanINs) and drive progression to invasive PDAC; however, targeting mutant *Kras* directly, or RAS signaling, has been met with little clinical success (Collins et al., 2012a; Collins et al., 2012b; Collins and Pasca di Magliano, 2013; di Magliano and Logsdon, 2013; McCormick, 2015). While the majority of PanINs do not harbor mutations in genes other than *Kras* (Hosoda et al., 2017), progression to invasive cancer generally requires mutation in tumor suppressor genes, such as *TP53*, *DPC4/SMAD4*, *INK4A*, *CDKN2A* (Ryan et al., 2014). Accordingly, mutation or deletion of these tumor suppressors in conjunction with $Kras^{G12D}$ expression in pancreatic GEMMs recapitulates invasive PDAC with nearly 100% penetrance (Aguirre et al., 2003; Bardeesy et al., 2006; Hingorani et al., 2005; Whittle et al., 2015).

While the epithelial cells that comprise both PanINs and PDAC tumors resemble pancreatic duct cells, our lab and others have demonstrated that precancerous PanINs arise, almost exclusively, from mature acinar cells (Bailey et al., 2015; De La et al., 2008;

Habbe et al., 2008; Kopp et al., 2012). The transforming ability of *Kras*^{G12D}, though, is still relatively inefficient, even when it is expressed ubiquitously in acinar cells throughout the pancreas. However, caerulein-induced pancreatitis, which mimics a well-known human risk factor for PDAC development (Lowenfels et al., 1993), rapidly transforms *Kras*-mutant acinar cells into PanINs (De La and Murtaugh, 2009; Guerra et al., 2007; Kopp et al., 2012). Pancreatitis itself is sufficient to induce transient metaplasia of wild-type acinar cells, in which they assume a ductal morphology and downregulate key regulators of acinar identity in favor of a progenitor-like gene expression profile (Jensen et al., 2005; Karki et al., 2015). Of note, duct cells are largely resistant to pancreatic cancer initiation, as pancreatitis administered to mice with *Kras*^{G12D}-expressing duct cells generates minimal PanIN formation (Kopp et al., 2012).

Together, these observations lead to the hypothesis that loss of acinar differentiation determinants might inhibit PDAC initiation by restraining the transforming ability of oncogenic Kras (Murtaugh, 2014; Rooman and Real, 2012). Indeed, deletion of any of three key transcription factors that coordinate acinar cell target gene programs, in conjunction with activated Kras, is sufficient to initiate rapid and robust PanIN formation, similar to that seen in pancreatitis (Flandez et al., 2014; Krah et al., 2015; Shi et al., 2013; von Figura et al., 2014). Deletion of the master regulator of acinar cell identity, *Ptf1a*, alone, causes metaplasia and induces a PDAC-like, Kras-permissive gene expression profile (Krah et al., 2015). *In vitro*, E47, a partner transcription factor of *Ptf1a*, can return pancreatic cancer cell lines to a quiescent state and induce acinar-specific gene expression (Kim et al., 2015). We therefore hypothesized that maintaining *Ptf1a* expression *in vivo* would restrain PanIN formation and

progression. We additionally sought to determine the plasticity of precancerous cells in the pancreas: can *Ptf1a* reprogram established PanINs back to benign acinar cells?

In this study, we demonstrate that loss of *Pf1a* dramatically accelerates invasive PDAC formation by removing an early, rate-limiting step to PanIN formation that is independent of canonical tumor suppressor gene regulation. Consistent with these findings, we show that sustained *Ptf1a* expression abolishes PanIN formation *in vivo* and limits acinar transformation *in vitro*. Our study additionally demonstrates the plasticity of pre-cancerous cells, showing that they can be reprogrammed to a benign phenotype, despite harboring irreversible genetic lesions.

Results

Using a previously published RNA-seq dataset, we analyzed what cellular functions might be affected by *Ptf1a* deletion in adult acinar cells (Krah et al., 2015). The Ingenuity Pathway Analysis "Disease and Functions" tool revealed that deletion of *Ptf1a*, in the absence of oncogenic *Kras*, resulted in upregulation of gene expression modules relevant to cell migration, metastasis, cell invasion, and necrosis (Figure 5.1A). We therefore hypothesized that genetic deletion of *Ptf1a* may hasten development of invasive PDAC independently of canonical tumor suppressor genes such as *Tp53*. To test this hypothesis, we generated mice based on the well-characterized *KPC* (*Kras*, *p53*, *Cre*) pancreatic cancer model (Hingorani et al., 2005). In this system, an inducible, acinar specific Cre ($Ptf1a^{CreERT}$) activates $Kras^{G12D}$ (expressed from the endogenous *Kras* locus (Tuveson et al., 2004)) and deletes both endogenous floxed *p53* (*Tp53*) alleles following TM administration. Our experimental group contained these alleles, plus a "floxed" *Ptf1a*

allele, which we have utilized previously (Figure 5.2) (Krah et al., 2015). This allele combination allows us to determine if intact *Ptf1a* contributes to tumor suppression even when *p53* is gone, i.e., independent of *p53*. To test whether *Ptf1a* deletion and *p53* deletion had distinct effects on pancreatic cancer initiation, we administered a single pulse of TM (0.25 mg/g body weight) to 6-8-week-old *KPC* and *KPC*; *Ptf1a* cKO mice and harvested pancreata after a 3-week chase (Figure 5.1B). In both experimental genotypes, total pancreas mass was not elevated above controls, suggesting that *bona fide* tumors had not yet formed in either genotype (Figure 5.1O). Histological examination revealed that *KPC* pancreata were nearly indistinguishable from controls at this early time point, exhibiting only rare PanIN lesions (Figure 5.1C-D). In contrast, the pancreata from *KPC*; *Ptf1a* cKO mice were composed exclusively of PanINs, ductal structures and fibroinflammatory infiltrate with little or no normal acinar tissue remaining (Figure 5.1E). Alcian blue staining, which binds acidic mucins that are present in pancreatic PanINs but not normal ducts, confirmed that the PanIN burden in *KPC*; *Ptf1a* cKO was dramatically increased compared to *KPC* mice (Figure 5.1F-H, P). Given the severity of the pancreatic phenotype, however, surprisingly few of the abundant ductal lesions of *KPC*; *Ptf1a* cKO pancreata were organized into well-formed, Alcian blue-positive PanINs (Figure 5.1E, H). Instead, and in contrast to *KPC* pancreata, numerous disorganized clusters and sheets of CK19+ cells were found throughout *KPC*; *Ptf1a* cKO pancreata, resembling carcinoma *in situ* (Figure 5.1I-K). Sirius red staining revealed that the ubiquitous transformation in *KPC*; *Ptf1a* cKO pancreata was associated with a severe fibroinflammatory phenotype, suggesting that loss of acinar cell identity hastens the stromal reaction associated with PDAC (Figure 5.1L-N). These results demonstrate that

Ptf1a restrains PDAC initiation by limiting early transformation, independent of canonical tumor suppressor (p53, specifically) function.

To test whether deletion of *Ptf1a* promotes progression to *bona fide* PDAC, we aged *KPC* and *KPC*; *Ptf1a* cKO mice that received a single pulse of TM (0.25 mg/g body weight) until they exhibited weight loss (20% of peak body weight), ascites, or lethargy. All ailing mice were found to harbor localized or metastatic pancreatic cancer. *KPC*; *Ptf1a* cKO mice had a median survival of 96 days, succumbing to PDAC much more rapidly than age-matched littermate *KPC* mice, which had a median survival of 160 days (Figure 5.3A). Interestingly, *KPC*; *Ptf1a* cKO were significantly more likely to be underweight at time of sacrifice (Figure 5.3B), perhaps due to longstanding exocrine insufficiency. However, primary tumors were indistinguishable between genotypes at the histological level and exhibited the classical features of PDAC, such as necrotic areas and CK19+ ductules (Figure 5.3C-D, G-H). Long-lived mice of both experimental genotypes exhibited liver and diaphragm metastases and ascites (Figure 5.3E-F and data not shown). Molecular characterization of primary tumors using mRNA-seq analysis revealed that very few genes were significantly differentially expressed between tumors from *KPC* and *KPC*; *Ptf1a* cKO mice (Red data points, Figure 5.3I). Interestingly, among the 30 genes significantly downregulated in *KPC*: *Ptf1a* cKO tumors were acinar-specific genes, such as amylase (*Amy2b, Amy1*), carboxypeptidase (*Cpa2*), and Chymotrypsin-C (*Ctrc*) (Figure 5.3I). Together, these data suggest that loss of *Ptf1a* does not alter the phenotype or invasive potential of pancreatic tumors, but rather hastens tumor progression by removing an early, rate-limiting barrier to tumor initiation.

Given these results, we hypothesized that sustaining *Ptf1a* expression might be

able to inhibit PDAC initiation and progression. If correct, this hypothesis could lead to new therapeutic strategies for the disease, because unlike *p53* and *Kras, Ptf1a* and its partner transcription factors are almost never genetically mutated in pancreatic cancer (Figure 5.4) (Bailey et al., 2016; Cerami et al., 2012; Gao et al., 2013). To test this hypothesis, we developed an acinar cell-specific, tamoxifen- and doxycycline (DOX)-inducible mouse model that allows for independent control of $Kras^{G12D}$ and *Ptf1a* expression. In this model, TM administration activates $Kras^{G12D}$ and a reverse tetracycline transactivator protein (rtTA), which is constitutively expressed from the *Rosa26* locus (Belteki et al., 2005; Collins et al., 2012a). Importantly, an IRES-GFP element placed downstream of the rtTA coding region allowed us to monitor the expression of this transgene using immunofluorescence. Subsequent administration of DOX drives rtTA-dependent expression of a transgenic *tetO-Ptf1a* in as little as 24 hours, the expression of which can be monitored with a downstream IRES-LacZ element (Figure 5.5 and Figure 5.6) (Willet et al., 2014).

To test whether sustained *Ptf1a* expression could inhibit the earliest phases of *Kras*-mediated PDAC development, we subjected control, $Kras^{G12D}$ and $Kras^{G12D}$ + *tetO-Ptf1a* mice to high dose-TM and a subsequent 8-week chase during continuous administration of DOX (1 mg/mL in the drinking water) (Figure 5.7A). All mice harbored $Ptf1a^{CreERT}$ and $Rosa26^{LSL\text{-}rtTA}$, and we confirmed that a similar percentage of acinar cells expressed rtTA-IRES-GFP between groups, indicating a uniform frequency of inducible Cre recombination (Figure 5.8). After 8W, $Kras^{G12}$ pancreata exhibited areas of edema (data not shown) and large areas of PanINs with associated fibroinflammatory infiltrate (Figure 5.7C). By contrast, PanINs were nearly absent in $Kras^{G12D}$ + *tetO-Ptf1a*

mice (Figure 5.7D). Regions of oncogenic transformation were highlighted by immunohistochemistry for the ductal gene CK19 (Figure 5.7E-G) and Alcian blue staining (Figure 5.7H-J). Quantifying the genotype-dependent PanIN burden revealed a drastic reduction in oncogenic transformation in ***Kras***^{G12D} + ***tetO-Ptf1a*** pancreata compared to those that expressed ***Kras***^{G12D} alone (Figure 5.7N, $P<0.0001$).

To understand why a limited number of PanINs still formed in ***Kras***^{G12D} + ***tetO-Ptf1a*** pancreata, we characterized these lesions relative to PanINs found in ***Kras***^{G12D} pancreata. There was no difference in the amount of Ki67 staining in the PanIN epithelium between genotypes (Figure 5.7K-M); lesions from both genotypes had various degrees of proliferation, indicating that they did not differ relating to this hallmark of cancer (Figure 5.7O). Interestingly, nearly all PanIN cells observed were completely negative for Ptf1a protein regardless of genotype, in contrast to adjacent acinar cells (Figure 5.7P-Q, V). These data indicate that there is not a unique population of PanINs that expresses transgenic ***Ptf1a*** in the ***Kras***^{G12D} + ***tetO-Ptf1a*** genotype, and suggests instead that cells comprising PanINs in this genotype are "escapers" that recombined the oncogenic ***Kras*** locus, but not the ***Rosa26*** locus. Notably, a previous study found that the ***Rosa26***$^{LSL\text{-}rtTA}$ allele was relatively resistant to Cre-mediated recombination, relative to other floxed alleles tested (Liu et al., 2013).

To directly test whether PanINs arise from escaper cells in ***Kras***^{G12D} + ***tetO-Ptf1a*** mice, we quantified the number of PanINs that had individual cells coexpressing CK19 and GFP (from Cre activation of ***rtTA-IRES-GFP***). Most PanIN cells in ***Kras***^{G12D} pancreata co-expressed CK19 and GFP, indicating that they formed from acinar cells that had recombined both the ***Kras***^{G12D} and ***Rosa26*** loci (Figure 5.7R, W). In contrast, almost all

PanIN cells in *Kras*^{G12D} + *tetO-Ptf1a* pancreata were GFP-negative (Figure 5.7CS, W), indicating that they had not activated *Rosa26*$^{LSL\text{-}rtTA}$. Thus, unequal Cre-mediated recombination between loci in a small population of acinar cells explains the residual formation of PanINs in *Kras*^{G12D} + *tetO-Ptf1a* pancreata. We additionally confirmed that PanINs from *Kras*^{G12D} + *tetO-Ptf1a* pancreata did not express *tetO-Ptf1a* by staining for LacZ. Indeed, PanINs harbored only very rare LacZ-positive cells, indicating that cells expressing *Ptf1a-IRES-LacZ* transgene were excluded from these precancerous lesions (Figure 5.7T-U). Taken together, these results strongly suggest that downregulation of *Ptf1a* expression is necessary for initiation of pancreatic cancer.

While screening tests for PDAC remain limited, risk factors have been identified in human epidemiology studies including chronic pancreatitis, obesity, and type 2 diabetes (reviewed in Krah and Murtaugh, 2016). We therefore wanted to test whether sustaining *Ptf1a* expression in the context of a known pancreatic cancer risk factor would also inhibit cancer initiation. Since caerulein-induced pancreatitis is known to synergize with mutations in *RAS* to rapidly induce PanIN formation, we used this well-characterized model to test our hypothesis (De La and Murtaugh, 2009; di Magliano and Logsdon, 2013; Guerra et al., 2007; Morris et al., 2010). Control, *Kras*^{G12D} and *Kras*^{G12D} + *tetO-Ptf1a* mice were administered high dose TM five days prior to caerulein treatment, and pancreata were harvested at three weeks after the final caerulein injection (Figure 5.9A). In all groups, 1 mg/mL of DOX was present in the drinking water throughout the experiment. While control mice completely recovered from caerulein-induced pancreatitis, robust PanIN formation was seen in the *Kras*^{G12D} genotype, as expected (F Figure 5.9B-C). In contrast, *Kras*^{G12D} + *tetO-Ptf1a* pancreata exhibited little PanIN

formation and lacked the large fibro-inflammatory regions characteristic of caerulein-treated *Kras*^{G12D} mice (Figure 5.9D). To highlight PanIN burden, we performed immunohistochemistry for CK19 and stained with Alcian blue, which confirmed a dramatic reduction in PanINs in *Kras*^{G12D} + *tetO-Ptf1a* mice (Figure 5.9E-J, Q). As in our previous experiment, PanINs in *Kras*^{G12D} + *tetO-Ptf1a* were almost completely Ptf1a-negative (Figure 5.9K-M) and were comprised almost exclusively of escaper cells (Figure 5.9N-P, R). Thus, even in a robust model of PDAC initiation that mimics a well-characterized human risk factor (Lowenfels et al., 1993), sustained *Ptf1a* expression is sufficient to limit disease initiation.

As pancreatitis involves the complex interaction between the stromal microenvironment and epithelial acinar cells, we next wanted to determine whether *Ptf1a* could restrain transformation in the absence of extrinsic inputs. To do this, we utilized a 3-dimensional (3D) culture system in which clusters of acinar cells undergo metaplasia into ductal cysts in response to mutant *Kras* signaling, without stimuli from the stromal microenvironment (Ardito et al., 2012; Krah et al., 2015; Means et al., 2005). Acinar clusters were isolated from *Kras*^{G12D} and *Kras*^{G12D} + *tetO-Ptf1a* mice, not treated with DOX, 8-weeks after the final TM dose and embedded in a collagen matrix, as previously described (n=4 mice) (Ardito et al., 2012; Means et al., 2005). In initial experiments, we treated acinar cluster with vehicle alone or DOX from the time they were plated and measured the number of transformed clusters and the area of transformed cysts at 3 and 5 days after plating. At day 3, significantly fewer DOX-treated acinar clusters had transformed into ductal cysts (Figure 5.10A-C), and those that had transformed were significantly smaller, as measured by cyst area (Figure 5.10D). Five days after treatment,

however, most acinar clusters had undergone some degree of transformation regardless of treatment (Figure 5.10E-G). DOX-treated cysts remained significantly smaller (Figure 5.10H) measured by cyst area, suggesting that sustained *Ptf1a* expression delays acinar transformation and restrains cyst expansion in this *ex vivo* system.

The therapeutic potential of these findings would be most impactful if reintroduction of *Ptf1a* into established lesions could redirect their fate to healthy acinar tissue, induce cell death, or inhibit further progression toward invasiveness. To begin to test whether re-expression of *Ptf1a* could halt transformation, we allowed untreated cysts from *Kras*^{G12D} + *tetO-Ptf1a* pancreata to form in our 3D acinar culture system for three days and then treated with vehicle or DOX (Figure 5.10I). While cysts treated with vehicle increased in area from day three to five, those treated with DOX remained the same size, suggesting that reintroduction of *Ptf1a* can stop the progression of cysts in this system (Figure 5.10J-N). Taken together, these data suggest that the induction of *Ptf1a* inhibits initial metaplasia and the progression of established transformed cells.

We therefore sought to utilize our *Kras*^{G12D} + *tetO-Ptf1a* mouse model to examine whether reintroduction of *Ptf1a* into PanINs would have a similar effect *in vivo*. As PanINs in *Kras*^{G12D} + *tetO-Ptf1a on* DOX were comprised exclusively of rtTA-negative cells (Figure 5.7S), we first wanted to confirm that these mice form Rosa$^{rtTA\text{-}GFP}$-positive PanINs when administered TM, but not DOX. Indeed, 8-weeks after TM administration, DOX-untreated *Kras*^{G12D} + *tetO-Ptf1a* mice exhibited widespread CK19/GFP dual-positive PanINs, confirming that these lesions express the cellular machinery necessary to re-express transgenic *tetO-Ptf1a* (Figure 5.11A). To test whether transformed PanINs could express the *tetO-Ptf1a* transgene, we administered *Kras*^{G12D} + *tetO-Ptf1a* TM on three

consecutive days and allowed PanINs to form during an 8-week chase. These mice then either remained on normal drinking water or received DOX in their drinking water for 24 hours before we harvested pancreata. While mice that did not receive DOX (or did not have the *tetO-Ptf1a* transgene) had no LacZ-positive cells anywhere in their pancreata, we observed normal acinar cells and several PanINs that were robustly LacZ-positive in the 24h DOX treated group (Figure 5.11D and data not shown). These results confirm that *tetO-Ptf1a* can be expressed in PanINs using our TM and DOX inducible model.

To determine the effect of *Ptf1a* re-expression in PanINs, $Kras^{G12D}$ + *tetO-Ptf1a* received three pulses of TM on consecutive days before an 8-week chase *without* DOX. Once PanINs had formed at 8 weeks, we administered 1 mg/mL DOX in the drinking water for 3 or six 6 before sacrifice. Three weeks after induction with DOX, we observed misplaced acinar cells interspersed within PanIN lesions throughout $Kras^{G12D}$ + *tetO-Ptf1a* pancreata, which were noticeably absent from $Kras^{G12D}$ mice that underwent the same protocol (Figure 5.12A-B). Pancreata from $Kras^{G12D}$ + *tetO-Ptf1a* mice were heterogeneous, exhibiting what appear to be wide-spread resolving PanINs and ductules at this time-point, and consistent with this observation, Alcian blue staining revealed numerous small, dissolving PanINs, intermixed with misplaced acinar cells (Figure 5.12C-D). To determine whether these misplaced acinar cells were expressing *tetO-Ptf1a*, we performed immunohistochemistry for Ptf1a (Figure 5.12E-F) and immunofluorescence for rtTA-GFP, amylase, and Ck19 (Figure 5.11A-C). Indeed, 3 weeks after DOX treatment, there was a significant increase in the number of hybrid acinar-duct structures, which had the histological appearance of embryonic acinar cells emerging from CK19-positive PanINs (Figure 5.11B, H). Consistent with these being

cells that reexpressed Ptf1a (Figure 5.11B), these clusters were almost ubiquitously LacZ-positive, indicating that they express *tetO-Ptf1a* (Figure 5.11E).

To confirm that redifferentiating GFP+ cells were emerging out of PanINs (and not being recruited into them), we quantified the number of CK19/GFP dual positive lesions in the following groups: 8-weeks NO DOX treatment, 8-weeks OFF DOX + 3-weeks ON DOX, and 8-weeks OFF DOX + 6-weeks ON DOX. While *Kras*^{G12D} + *tetO-Ptf1a* mice that were not treated with DOX had ~70% CK19/GFP dual positive PanINs, *Kras*^{G12D} + *tetO-* treated subsequently with DOX for 3-weeks or 6-weeks had dramatically fewer (26% and 9%, respectively) GFP+ cells contributing to PanINs (Figure 5.11B-C, G), suggesting that these cells had lost their oncogenic potential. LacZ staining confirmed that there were few, if any, *tetO-Ptf1a*-expressing cells contributing to PanINs after 6 weeks with DOX treatment (Figure 5.11F). Taken together, these data suggest that *Ptf1a*-expressing cells cannot maintain a preoncogenic phenotype, and demonstrate that PDAC precursors can be reprogrammed to a benign phenotype, despite harboring irreversible cancer-driving mutations.

Discussion

Acinar-to-ductal reprogramming has become a widely-appreciated step in the genesis of pre-cancerous PanINs (De La et al., 2008; Habbe et al., 2008; Kopp et al., 2012; Merrell and Stanger, 2016). Several recent studies suggest that acinar differentiation determinants, such as *Ptf1a* and *Nr5a2*, are required to prevent acinar metaplasia, even under homeostatic conditions (Hoang et al., 2016; Holmstrom et al., 2011; Krah et al., 2015; von Figura et al., 2014). Indeed, genetic deletion of these

transcription factors allows for oncogenic *Kras* to rapidly reprogram acinar cells into PanINs, suggesting that alterations in cell fate are a rate-limiting step in PDAC initiation (Flandez et al., 2014; Krah et al., 2015; Shi et al., 2009; von Figura et al., 2014). Here, we show that this rate-limiting step toward PanIN development also accelerates the formation of carcinoma *in situ* and invasive cancer when *p53* is simultaneously deleted (Figures 5.1 and 5.3). Additionally, we demonstrate that loss of *Ptf1a* is necessary for the earliest steps of oncogenic reprogramming, as the maintenance or re-introduction of *Ptf1a* expression prevents acinar transformation and limits oncogenic potential.

The potent combination of $Kras^{G12D}$ activation with deletion of *Ptf1a* and *p53* was sufficient to generate carcinoma *in situ* within 3 weeks of Cre-mediated recombination (Figure 5.1). This rapid time-scale to potential invasiveness is consistent with the cellular functions that *Ptf1a* is projected to restrain, but surprising given that pancreata from *KPC* mice remain relatively normal, with only sparse, well-organized PanINs at this early time-point. The contrast between the indolence of *KPC* epithelial cells, 3 weeks after tamoxifen administration, and the invasive phenotype of KPC; *Ptf1a* cKO epithelial at this time point suggests that the presence of *Ptf1a* is able to inhibit tumor formation even after cells have already sustained genetic “hits” typical of advanced PanINs and PDAC (Hosoda et al., 2017). With *Ptf1a* removed, cells with $Kras^{G12D}$ and no *p53* are disinhibited and rapidly progress to carcinoma (Figure 5.3A). Our data therefore highlight the importance of cell differentiation, which can restrain transformation of cells with irreversible genetic lesions for months.

Of note, several recent studies demonstrate that other pancreatic differentiation determinants, such as *Nr5a2* and *Pdx1*, restrain acinar cell transformation in the presence

of oncogenic Kras. For example, the Crawford and Hebrok labs recently demonstrated that deletion of *Pdx1* dramatically hastens PanIN formation, when on a *Kras*^{G12D} background (Roy et al., 2016). Similarly, *Nr5a2*, which coordinates acinar-specific gene expression with *Ptf1a* (Holmstrom et al., 2011), greatly accelerates PanIN formation when even a single allele is deleted (Flandez et al., 2014; von Figura et al., 2014). Consistent with these findings in mouse, GWAS studies have shown that SNPs in *NR5A2* increase the risk of human PDAC development (Li et al., 2012; Petersen et al., 2010), highlighting the relevance of acinar differentiation to human tumors. Additionally, forced expression of the Ptf1a binding partner, E47, in PDAC cells *in vitro* prevents cell proliferation and induces the expression of acinar-specific genes, demonstrating the plasticity of invasive PDAC cells (Kim et al., 2015). Going forward it will be interesting to test whether simultaneous activation of Ptf1a, Nr5a2 and E47 in PanINs or PDAC cells leads to a more robust reprogramming effect.

To our knowledge, this is one of the first studies demonstrating plasticity *in vivo* of tumor precursor cells. Previous work from the Pasca di Magliano lab has shown that inhibiting *Kras*^{G12D} directly, using a similar DOX inducible system to what we utilize in this study, leads to the regression of PanINs and PDAC (Collins et al., 2012a; Collins et al., 2012b). However, targeting oncogenic RAS in solid tumors has proven to be a monumental challenge (Collins and Pasca di Magliano, 2013; McCormick, 2015). Previous studies from our lab suggest that loss of *Ptf1a* alone upregulates genes associated with RAS dependency in human cancer cells (Krah et al., 2015). Restoring *Ptf1a* expression may therefore provide inhibitory effect on RAS signaling in PanINs or tumor cells, bypassing the need to directly restrain mutant *Kras*. The fact that acinar

differentiation can restrain transformation of cells with oncogenic *Kras* and loss of *p53* for months in the KPC model, and reverse the phenotype of PanINs, speaks to the power of this transcriptional network in inhibiting tumor initiation. Future studies should aim to understand the mechanisms by which *Ptf1a* and other components of its regulatory networked are silenced in PanINs and PDAC to devise strategies for their reactivation. Similar concepts may apply to other insidious solid cancers, such as hepatocellular carcinoma and colorectal cancer, where cellular plasticity is well-documented and the transcriptional programs that define mature cell types are well characterized (Krah and Murtaugh, 2016).

Materials and Methods

Mice

Experimental mice of the following genotypes have been utilized in previous publications: $Ptf1a^{CreERT}$ ($Ptf1a^{tm2(CreER1)CVW}$) (Kopinke et al., 2012; Krah et al., 2015), $Kras^{LSL\text{-}G12D}$ ($Kras^{tm4tyj}$) (Hingorani et al., 2003), $p53^{lox}$ ($Trp53^{tm1Brn}$) (Marino et al., 2000), $Ptf1a^{lox}$ ($Ptf1a^{tm3Cvw}$) (Hoang et al., 2016; Krah et al., 2015), $Rosa^{rtTA}$ (Belteki et al., 2005; Collins et al., 2012a; Collins et al., 2012b), and *tetO-Ptf1a* (Willet et al., 2014). To induce Cre-mediated recombination, mice were administered tamoxifen (Sigma, St. Louis, MO) dissolved in corn oil, via oral gavage, at doses indicated within the text. All experiments involving mice were performed according to institutional and National Institutes of Health (NIH) guidelines.

Tissue processing and histology

Following euthanasia, pancreata were dissected into ice cold PBS and separated into multiple parts and processed for frozen and paraffin sections, as previously described by our lab (De La et al., 2008; Keefe et al., 2012). For paraffin sectioning, tissues were fixed in zinc-buffered formalin (Z-fix; Anatech, Battle Creek, MI) at room temperature overnight, followed by processing into Paraplast plus (McCormick Scientific). Frozen histological specimens were fixed for 1-2 hours in 4% paraformaldehyde in 1x PBS on ice, followed by processing into Tissue-Tek O.C.T. compound (Sakua Fineteck, Torrance, CA). Both paraffin and frozen sections were cut at a thickness of 6-8μm with over 100μm spacing between individual sections on a single slide.

Immunohistochemistry and immunofluorescence followed established protocols from our lab (De La et al., 2008; Keefe et al., 2012; Krah et al., 2015) and included high temperature antigen retrieval (Vector Unmasking Solution; Vector Laboratories, Burlingame, CA), prior to staining all paraffin sections. All primary antibodies have been previously utilized by our lab (see Table 3.2). Secondary antibodies, raised in donkey (Jackson Immunoresearch, West Grove, PA) were used at a dilution of 1:250 in blocking solution. Vectastain reagents and diaminobenzidine (DAB) substrate (Vector Laboratories) were used for all IHC experiments. Immunofluorescence were counterstained with DAPI and mounted in Fluoromount-G (Southern Biotech), and photographed on an Olympus IX71 microscope, using MicroSuite software (Olympus America, Waltham, MA). Images were processed in Adobe Photoshop, with exposure times and adjustments identical between genotypes and treatment groups.

Alcian blue staining was performed on paraffin sections following a 15-min wash

in 3% acetic acid, followed by a 10-min incubation in staining solution (1% Alcian blue in 3% acetic acid), followed by extensive washes in 3% acetic acid and 1x PBS. Sirius red staining was performed on frozen sections that were fixed in Bouin's fixative at 55˚C for 1 hour. Specimens were washed in dH_2O and stained for 1 hour in Picro-Sirius Red (American MasterTech, Lodi, CA). Following staining, all slides were washed in 0.5% acetic acid, dehydrated and equilibrated into xylene, and mounted with Permount.

PanIN scoring

To measure the number of PanINs in each pancreas, the entire tissue area of Alcian blue/eosin-stained specimen was photographed at 4x original magnification, followed by Photomerging (Adobe Photoshop). The surface area was measured using ImageJ software (NIH). Alcian blue+ PanINs were counted manually under the microscope and PanIN burden was calculated as the total number of Alcian blue+ lesions per cm^2 surface area. As previously described, metaplastic lesions that did not stain with Alcian blue were not counted in the quantification. To avoid double-counting tortuous lesions that could occupy multiple regions in three-dimensional space, no more than one lesion was scored within an anatomically distinct pancreatic lobule (De La et al., 2008; Krah et al., 2015).

RNA-sequencing analysis

Tumors from *KPC* and *KPC*; *Ptf1a* cKO mice were homogenized and total RNA was isolated using a modified guanidine thiocyanate-guanidine hydrochloride extraction (Hoang et al., 2016; Krah et al., 2015). After the first precipitation, the RNA was

dissolved in equal parts of Trizol and purified using the Direct-zol RNA (Zymo) according to the manufacturer's instructions. RNA quality was accessed by Agilent Bioanalyzer with KPC mice having an average RIN value of 8.5 and KPC+P an average value of 9. Poly-A selection and the Illumina TruSeq Stranded mRNA Library Preparation Kit were used to generate libraries. Fifty cycles of single-read sequencing were conducted using the Illumina HiSeq 2500 instrument. Reads were aligned to UCSC mm10 genome by Tophat (v2.1.1) and EdgeR default settings were used to determine differential expression of genes (FDR <0.05).

Caerulein treatment

Acute pancreatitis was induced by i.p. injection of caerulein (Bachem, Torrance, CA), 0.1 ug/g in saline, six times daily over two consecutive days, as previously described (Keefe et al., 2012; Kopp et al., 2012; Krah et al., 2015). Negative controls were injected with an equal volume of saline. Pancreata from all caerulein-treated mice were harvested three weeks following the final injection and processed as described above.

Kaplan-Meier analysis

KPC mice (genotype: $Ptf1a^{CreERT/+}; Kras^{G12D}; p53^{lox/lox}$) and *KPC*; *Ptf1a* cKO mice (genotype: $Ptf1a^{CreERT/lox}; Kras^{G12D}; p53^{lox/lox}$) were aged until they exhibited lethargy, distress, >20% weight loss of peak body weight, or ascites, as determined by NMK or the in house veterinary staff. The presence of PDAC and metastases was confirmed by histological analysis in consultation with a surgical pathologist. Survival analysis was performed in

GraphPad Prism 7 and p-values were calculated using a Log-rank test and Gehan-Breslow-Wilcoxon test.

3D pancreatic acinar cultures

Acinar cultures were established following protocols from previous publications (Ardito et al., 2012; Means et al., 2005). Briefly, the dorsal pancreas was minced in HBSS and digested in 0.02% trypsin (5 min, 37˚C) and 1 mg/mL collagenase P (Roche Applied Science, Mannheim, Germany; 15 min, 37˚C). the resulting digest was filtered to eliminate undigested material and repeated washing was performed to eliminate debris and dead cells. Acinar cell clusters were embedded in rat tail collagen gels (Corning, Corning, NY) and cultured in Weymouth's medium (Life Technologies, Carlsbad, CA) supplemented with 1% fetal bovine serum, 0.4 mg/mL soybean trypsin inhibitor, and 1 µg/mL dexamethasone. Cultures were fixed and imaged 3 or 5 days after plating. To quantify cyst size, we randomly selected >12 fields per mouse, imaged and quantified the area of each transformed cyst using imageJ (NIH).

Quantification of immunofluorescence images

To quantify the *Rosa26*^{rtTA} recombination frequency, we imaged 10-12 randomly selected 20x fields per specimen (taken across multiple sections). Using ImageJ (NIH), cell coexpressing GFP with the acinar differentiation marker, Amylase, were detected by additive image overlay of their staining with DAPI and anti-GFP, and counted using the Analyze Particles function as described previously (Keefe et al., 2012; Kopinke et al., 2012). To ensure counting accuracy, random images were manually spot-checked, using

Adobe Photoshop. All calculations were performed in Microsoft Excel and the results are graphed as individuals with error bars representing the standard deviation. P-values were determined by a two-tailed, unpaired t-test in Graphpad Prism 7.

To quantify the number of GFP-positive PanINs, 10 randomly selected field were imaged per mouse. Each PanIN was hand scored according to the number of GFP and CK19+ cells over-lay with one another. If more than two cells co-expressed CK19 and GFP, the PanIN was considered GFP-positive.

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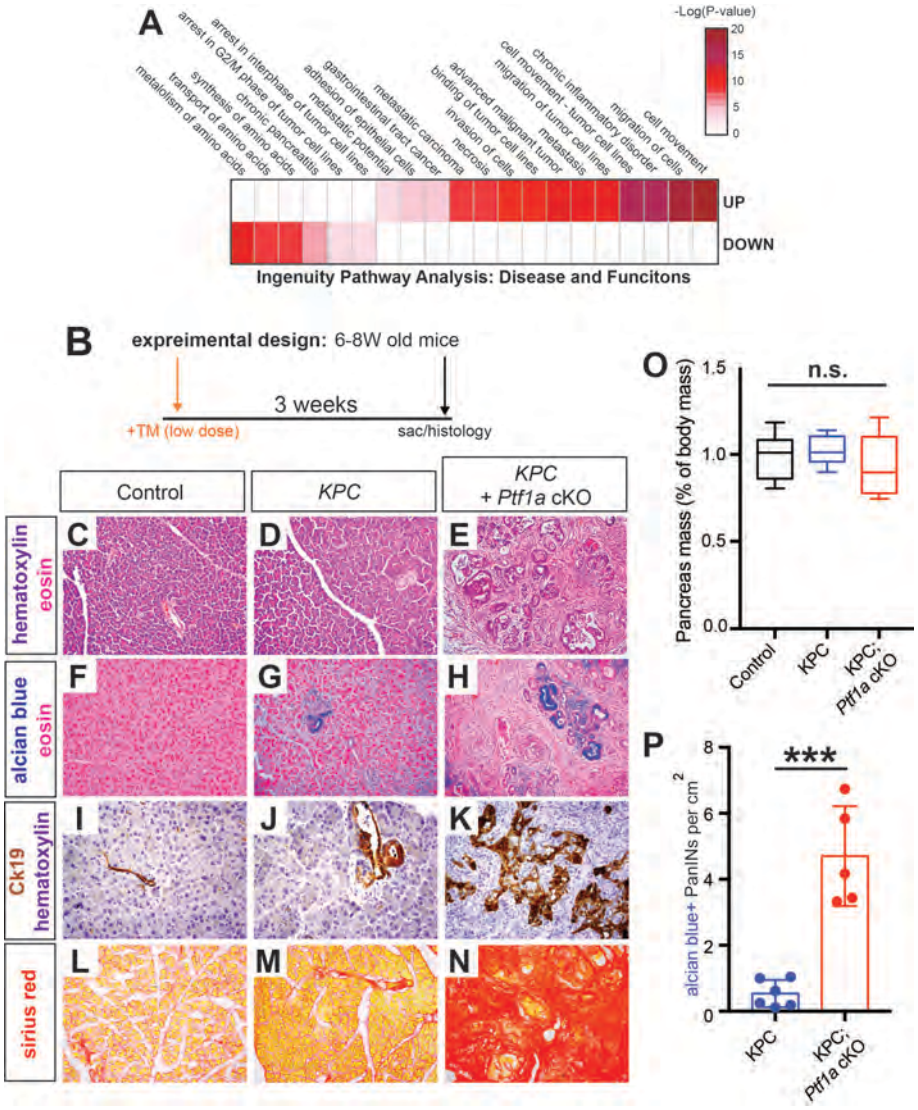

Figure 5.1. Loss of *Ptf1a* leads to rapid progression toward PDAC in *KPC* mice, independently of p53 loss. (A) Heat map of disease and functions from ingenuity pathway analysis predicted from the changes in gene expression in *Ptf1a* cKO pancreata. Analyses are based on a +/- expression threshold of 2.0. **(B)** Schematic of experimental design: mice of specified genotypes were administered a single pulse of 0.25 mg/g body weight TM to induce Cre-mediated recombination and were sacrificed three weeks later. **(C-E)** H&E staining of pancreata from mice of indicated genotypes three weeks after TM gavage. **(F-H)** Alcian blue staining, which highlights PanIN formation in KPC + *Ptf1a* cKO pancreata. **(I-K)** IHC for the ductal gene marker Cytokeratin-19 (CK19), highlighting normal ducts in control pancreata, well organized, oval PanINs in *KPC* mice, and sheets of disorganized epithelial cells in *KPC* + *Ptf1a* cKO pancreata. **(L-N)** Sirius Red staining, highlighting wide-spread fibrosis in *KPC* + *Ptf1a* cKO pancreata. **(O)** Pancreas mass, measured as a percent of body weight, was not significantly different between genotypes at 3W post TM administration. **(P)** Quantification of PanINs at 3-weeks post TM. *KPC* + *Ptf1a* cKO pancreata exhibited a 5-fold increase in Alcian Blue+ PanINs compared to *KPC* pancreata at this time-point (P<0.001).

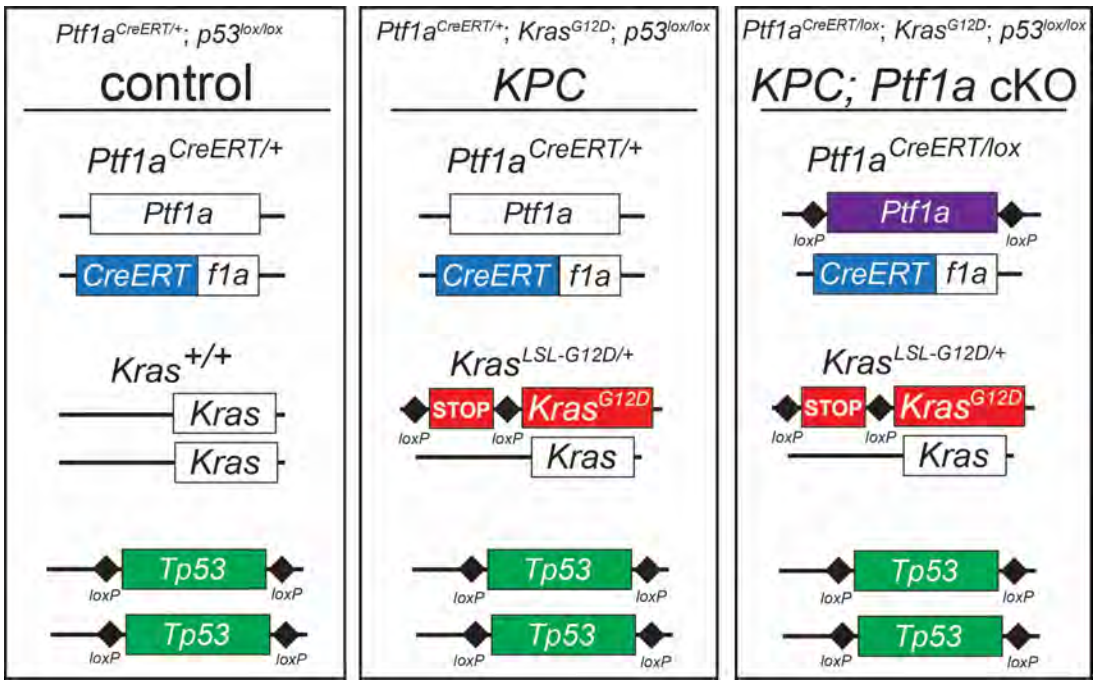

Figure 5.2. Schematic of mouse alleles used in Figures 5.1 and 5.3. Schematic representations of the alleles present in the genotypes referred to, in shorthand, as Control, *KPC*, and *KPC*; *Ptf1a* cKO.

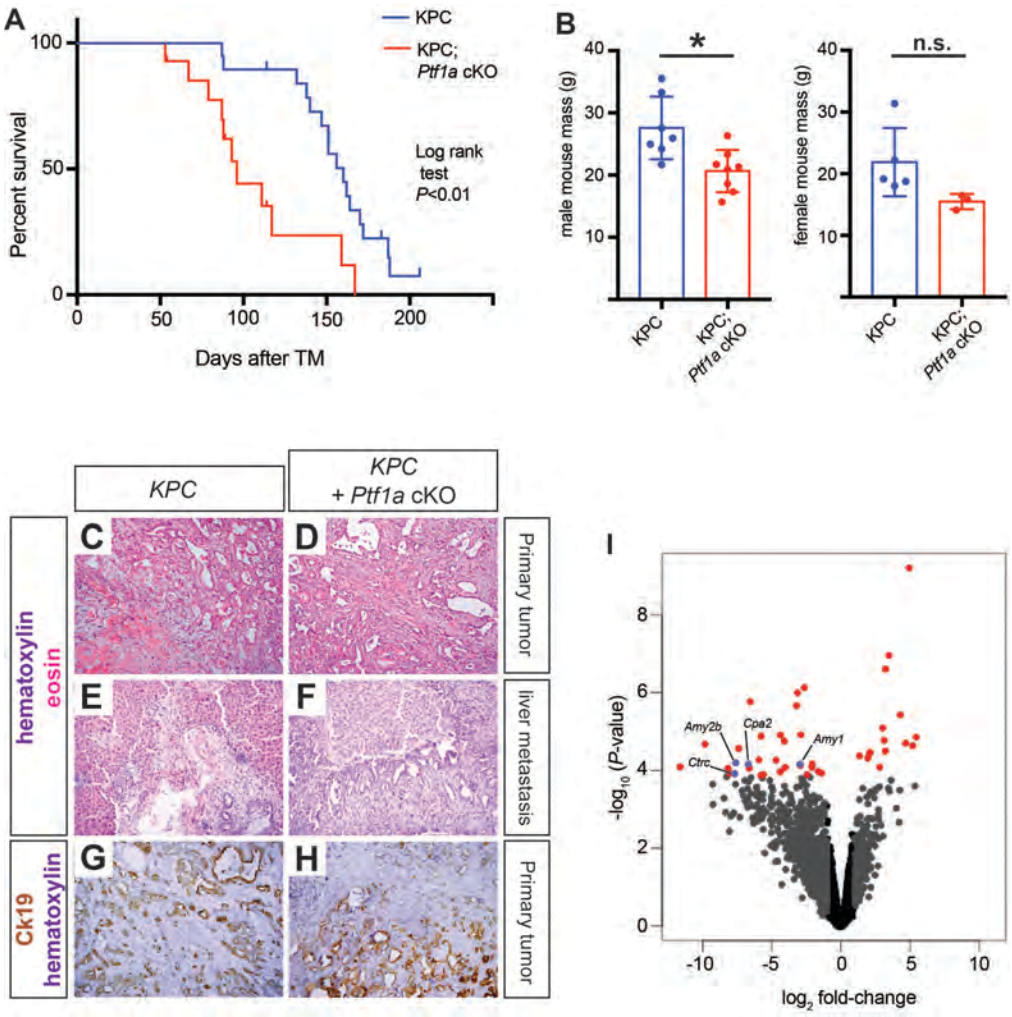

Figure 5.3. Loss of *Ptf1a* accelerates progression to PDAC, but does not drastically alter tumor phenotype. (A) Kaplan-Meier analysis from KPC mice (*Ptf1a*$^{CreERT/+}$; *Kras*^{G12D}; *p53*$^{lox/lox}$, blue line) and age-matched KPC; *Ptf1a* cKO (*Ptf1a*$^{CreERT/lox}$; *Kras*^{G12D}; *p53*$^{lox/lox}$, red line) mice that received TM at 6W of age (Log rank test: $P<0.01$). **(B)** Mass of male and female KPC and KPC; *Ptf1a* cKO mice at the time of sacrifice. Representative H&E stained histology from primary tumors **(C-D)** and liver metastases **(E-F)** from mice of indicated genotypes. **(G-H)** IHC for CK19, highlighting tumor epithelium in primary tumors from mice of indicated genotypes. **(I)** Volcano plot showing expressed genes in KPC and KPC; *Ptf1a* cKO tumors (Black dots). Grey data points represent genes that have a fold change >2.0 but do not reach statistical significance. Red dots are genes that have an FDR<0.05 and are differentially expressed. Downregulated acinar specific genes with FDR<0.05 are highlighted as blue dots and labeled with the gene name (Amy1, Amy2b, Cpa2, and Ctrc).

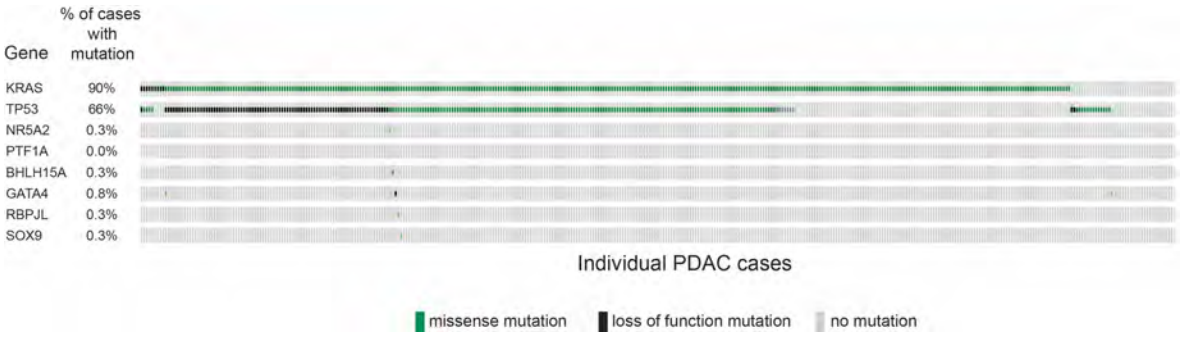

Figure 5.4. Common and uncommon mutations in human PDAC. Mutational profile of common driver mutations, *KRAS* and *TP53*, and uncommonly mutated genes such as, *NR5A2*, *PTF1A*, *RBPJL*, *GATA4*, and *SOX9*. Each gray bar represents an individual case. Green bars represent cases with a missense mutation in the indicated gene, while black bars represent loss of function mutations. Data obtained from cbioportal.org.

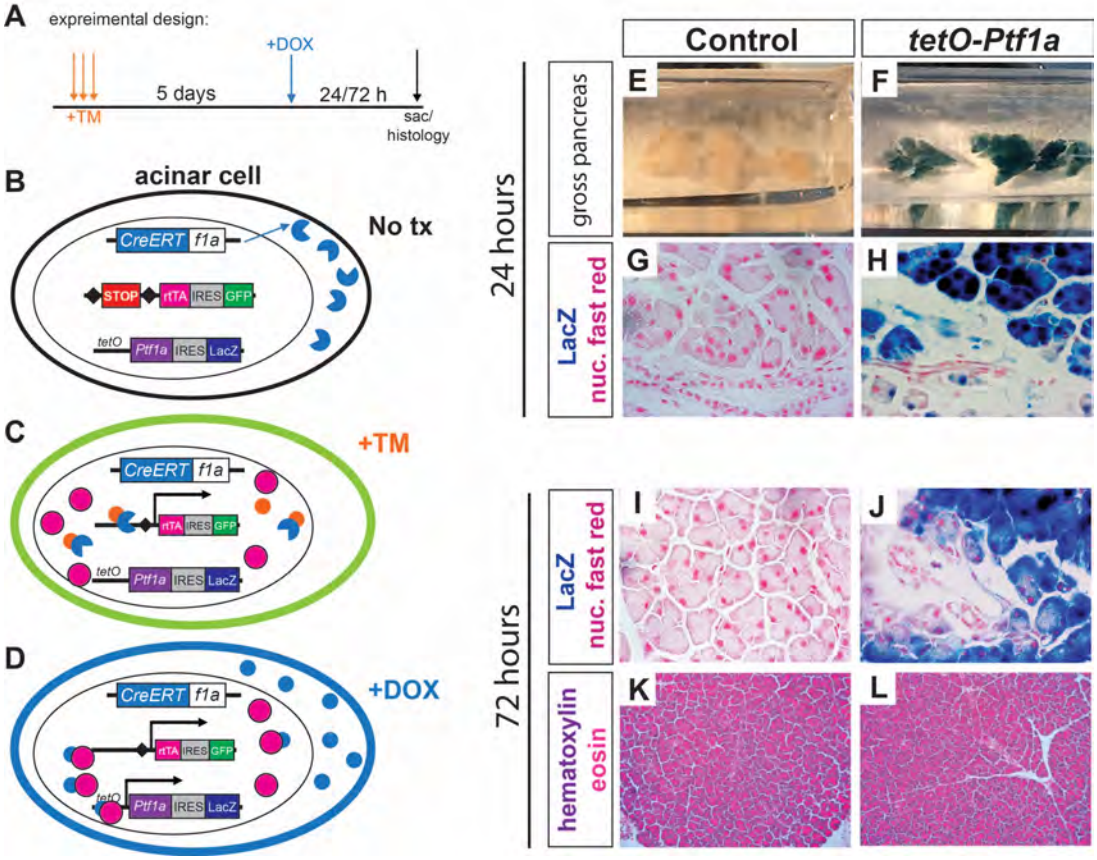

Figure 5.5. A mouse model to induce and sustain *Ptf1a* expression in acinar cells. (A) Experimental schematic to induce sustained *Ptf1a* expression using alleles described below **(B-D)**. Prior to tamoxifen (TM) administration, Cre is expressed from the endogenous *Ptf1a* locus, but is sequestered to the cytoplasm **(B)**. When TM is administered the *Rosa26* locus undergoes Cre-mediated recombination, which drives the constitutive expression of a reverse tetracycline transactivation element (rtTA) **(C)**. Since rtTA is conjugated to an IRES-GFP, the cells that recombine this locus permanently express GFP. **(D)** Upon administration of DOX, the rtTA can enter the nucleus and drive constitutive expression of the *tetO-Ptf1a* transgene; cells that express *tetO-Ptf1a* can be monitored with LacZ expression. **(E-F)** Gross pancreata of indicated genotypes 24h following DOX administration. **(G-H)** Histology of pancreata represented in E-F, which highlights the LacZ expression specific to acinar cells. **(I-J)** 72 hours following DOX administration, similar acinar specific LacZ expression, which does not alter the histological pancreatic phenotype **(K-L)**.

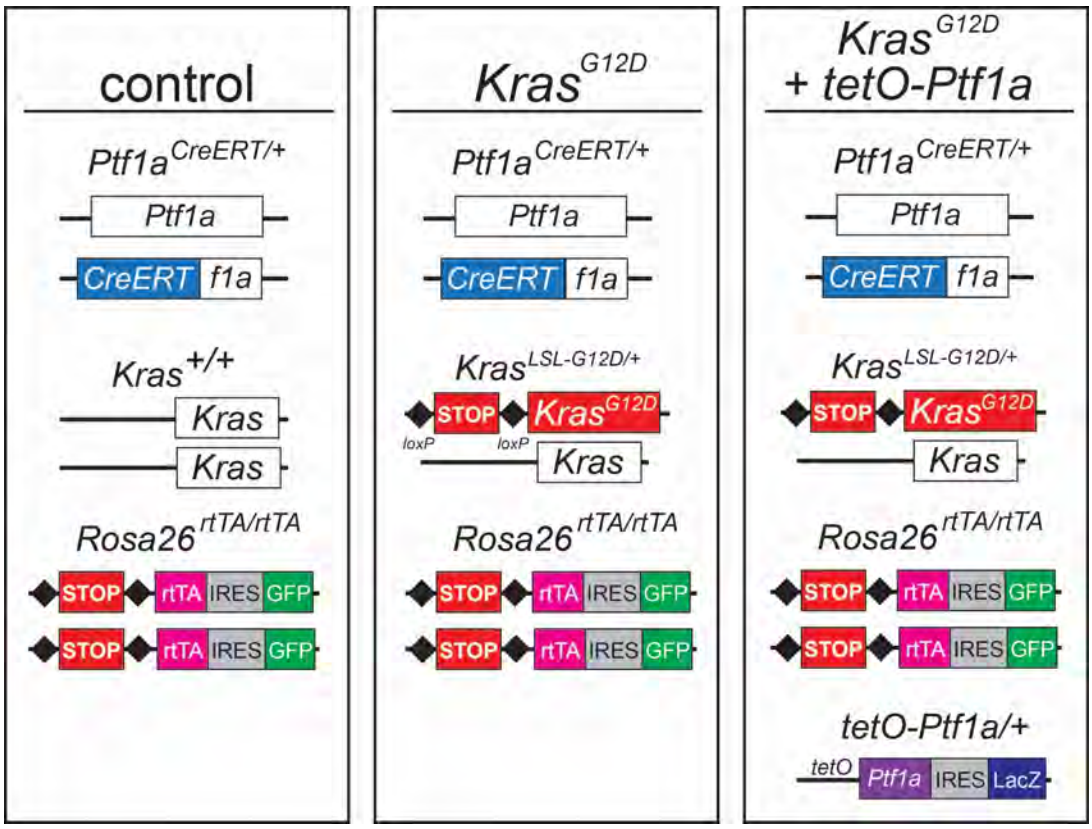

Figure 5.6. Mouse alleles utilized in Figures 5.7 through 5.12. Schematic representations of the alleles present in the genotypes referred to, in shorthand, as Control, *Kras*^{G12D}, and *Kras*^{G12D} + *tetO-Ptf1a*. Function of the system is described in **Figure 5.5**.

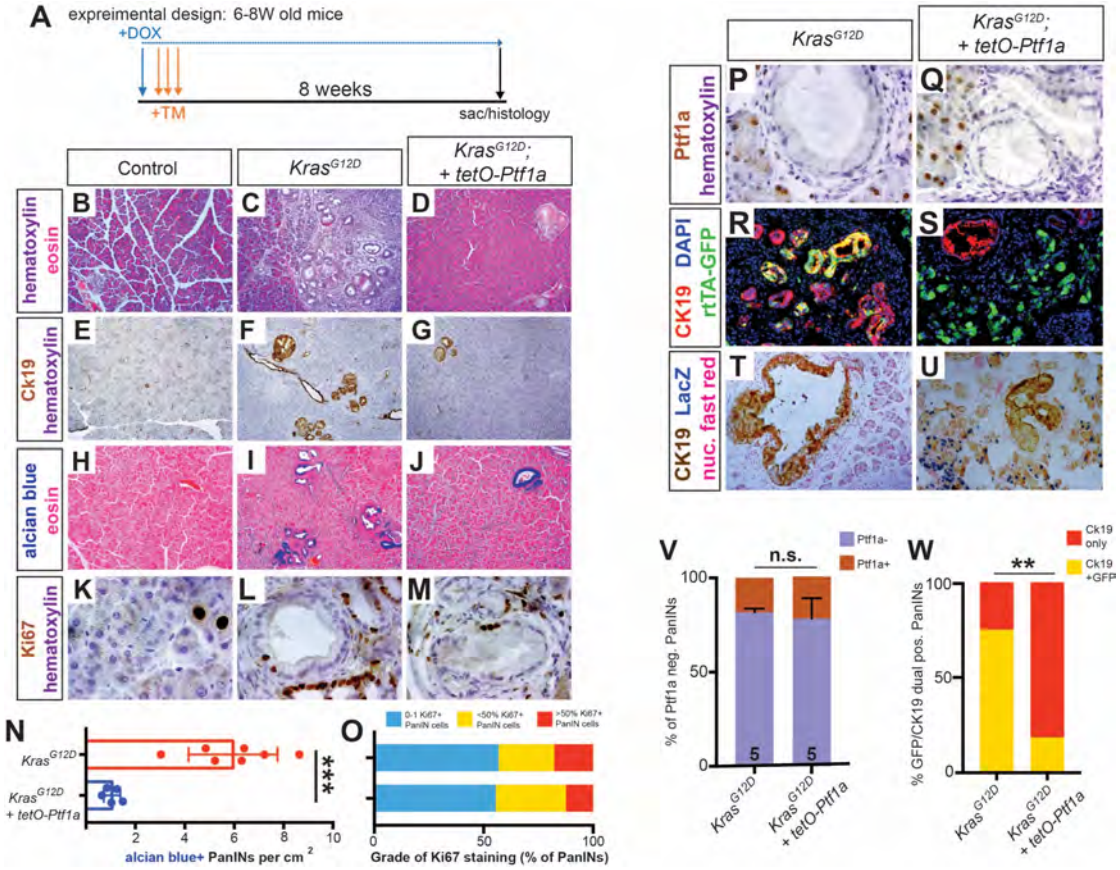

Figure 5.7 Sustained *Ptf1a* expression prevents *Kras*^{G12D}-driven PanIN formation. (A) Mice of indicated genotypes were administered 1 mg/mL DOX in their drinking water 24 hours before being gavaged with 0.25 mg/g body weight TM on three consecutive days to induce Cre-mediated recombination. 8W following the final TM gavage, mice were sacrificed for pancreatic histology. **(B-D)** H&E staining of pancreata from mice of indicated genotypes 8W after TM administration. IHC for CK19 **(E-G)** and alcian blue staining **(H-J)**, highlighting the reduced PanIN burden in in *Kras*^{G12D}*; tetO-Ptf1a* pancreata compared to those that express *Kras*^{G12D} only. **(K-M)** IHC for Ki67, specifically in PanINs of mice of indicated genotypes. **(N)** Quantification of the genotype dependent PanIN burden, showing a drastic reduction in the number of alcian blue+ lesions in *Kras*^{G12D}*; tetO-Ptf1a* pancreata compared to *Kras*^{G12D} pancreata (P<0.001). **(O)** Quantification of graded PanINs for proliferation based on Ki67 staining; there was no statistically significant difference between genotypes. **(P-Q)** IHC for Ptf1a, showing the lack of nuclear Ptf1a in PanINs, compared to adjacent acinar cells in both genotypes. **(R-S)** Immunofluorescence for the duct marker CK19 (red), the Rosa$^{rtTA\text{-}GFP}$ acinar lineage label (green), and dapi (blue). **(T-U)** Staining for CK19, the *tetO-Ptf1a-IRES-LacZ* and nuclear fast red. **(V)** Quantification of the number of PanINs that are completely Ptf1a negative vs. those that harbor 1 or more Ptf1a positive cells (n.s. = no significance between genotypes). **(W)** Quantification of the number of PanINs that are only CK19 positive vs. those that are CK19/ Rosa$^{rtTA\text{-}GFP}$ dual positive (Fisher exact test, P<0.01).

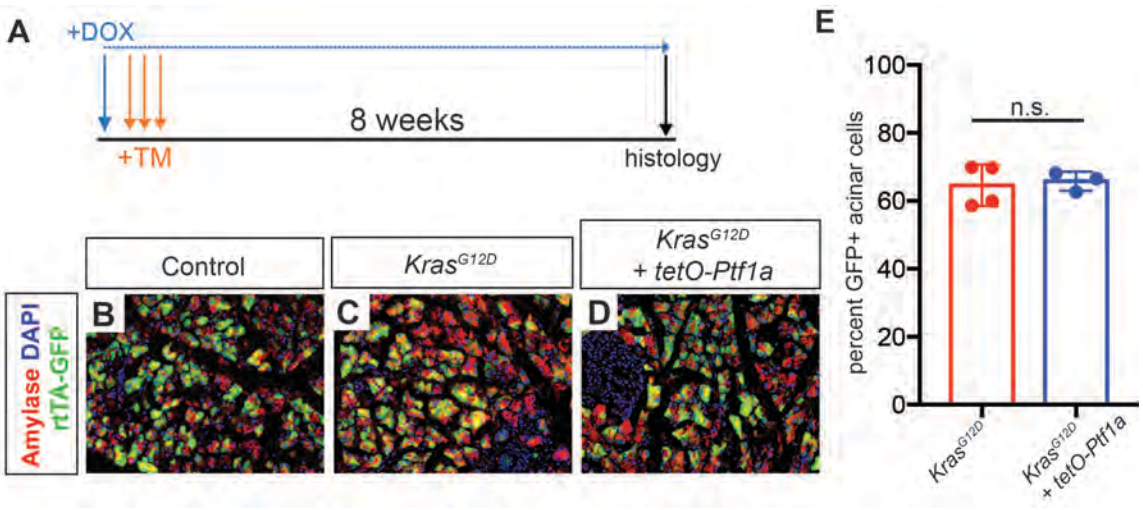

Figure 5.8*: Ptf1a*^{CreERT} recombination efficiency following tamoxifen treatment. (A) 6-8-week-old mice were administered tamoxifen on three consecutive days (3 x 0.25 mg/g mouse) while DOX (1 mg/mL) was present in the drinking water, and pancreata were harvested after an 8-week chase. **(B-D)** Immunofluorescence for amylase (red) and the Cre reporter *Rosa26*$^{rtTA\text{-}GFP}$ (green) on pancreata from mice of indicated genotypes. For all mice, only histologically normal areas were imaged in an effort to provide an accurate quantification of Cre-mediated recombination. **(E)** The proportion of GFP expression among amylase+ acinar cells were quantified for *Kras*^{G12D}, and *Kras*^{G12D} + *tetO-Ptf1a* genotypes. No significant difference was noted between any individuals or genotype groups.

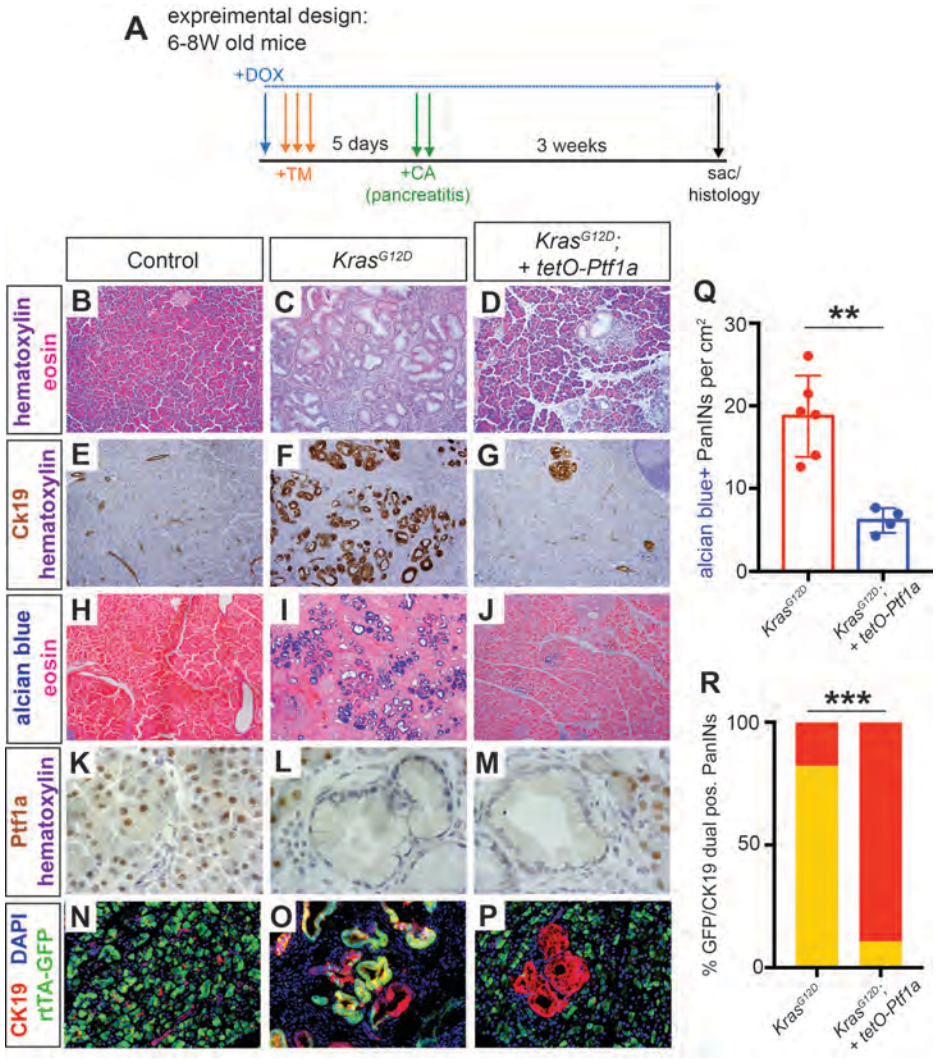

Figure 5.9. Maintenance of acinar identity inhibits Kras-driven PanIN formation during pancreatitis. (A) Experimental design: mice were first administered 1mg/mL DOX in their drinking water before receiving one dose of 0.25 mg/g Tamoxifen on three consecutive days. Five days following the final TM dose, mice were administered six hourly injections of 0.1µg/g caerulein on two consecutive days. Three weeks following the final caerulein injection, mice were sacrificed to analyze pancreatic histology. **(B-D)** H&E staining of pancreata from mice of indicated genotypes 3W after caerulein-induced pancreatitis. IHC for CK19 **(E-G)** and alcian blue staining **(H-J)** highlighting PanIN burden. **(K-M)** IHC for Ptf1a, showing the lack of nuclear Ptf1a in normal ducts in control mice **(K)**, and in PanINs **(L-M)**, compared to adjacent acinar cells. **(N-P)** Immunofluorescence for the duct marker CK19 (red), the $Rosa^{rtTA\text{-}GFP}$ acinar lineage label (green), and dapi (blue), highlighting CK19+ escaper PanIN cells in *$Kras^{G12D}$; tetO-Ptf1a* pancreata **(P)**. **(Q)** Quantification of the genotype dependent PanIN burden, showing a drastic reduction in the number of PanINs in *$Kras^{G12D}$; tetO-Ptf1a* pancreata ($P<0.01$). **(R)** Quantification of number of PanINs that are only CK19 positive vs. those that are CK19/ *$Rosa^{rtTA\text{-}GFP}$* dual positive (Fisher exact test, $P<0.001$).

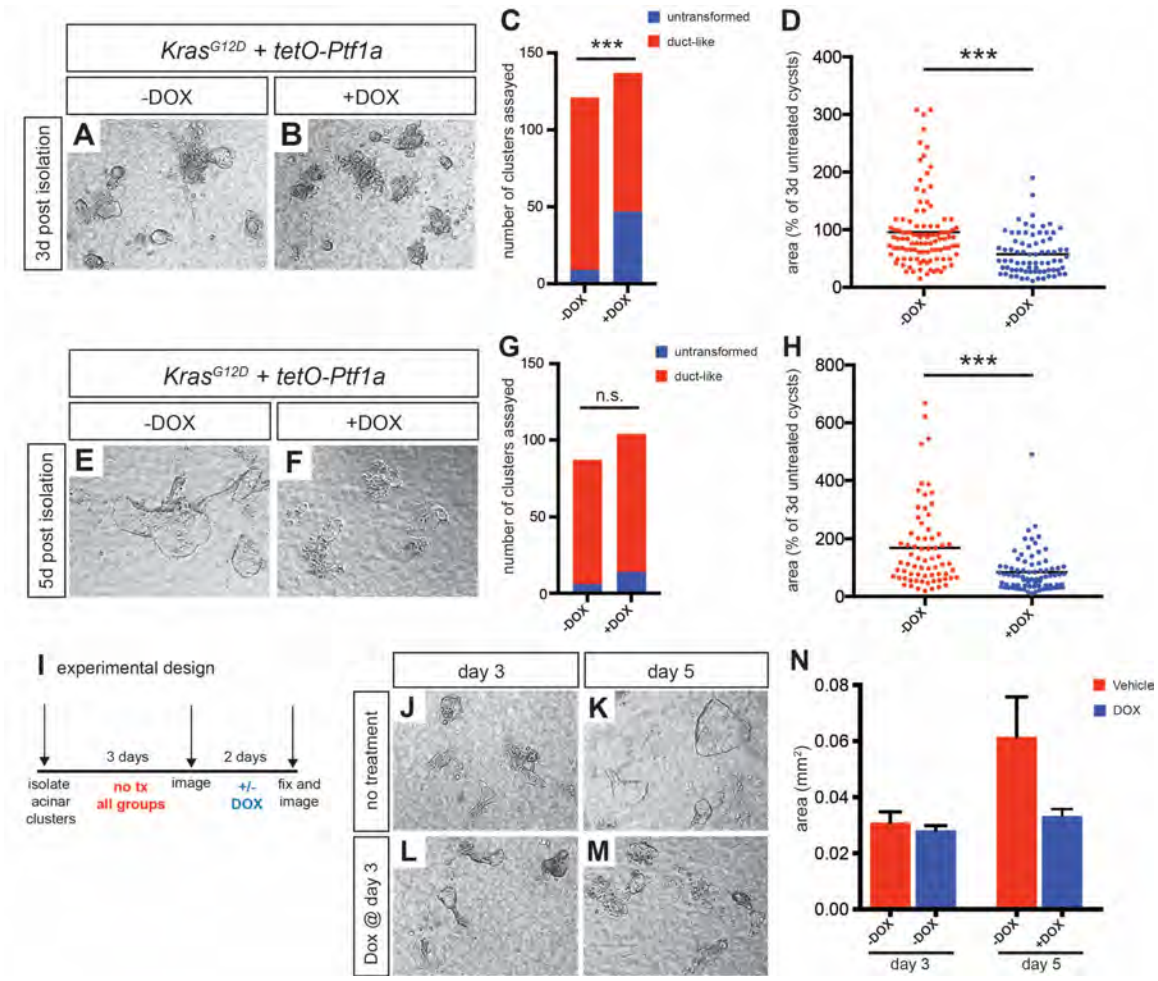

Figure 5.10. ***Ptf1a*** **limits acinar-to-ductal reprogramming and inhibits ductal expansion in 3D culture. (A-B)** Brightfield images of transforming acinar cluster from *Kras^{G12D}; tetO-Ptf1a* pancreata in the presence or absence of DOX three days after plating. **(C and G)** Quantification of the proportion of transformed clusters in the absence and presence of DOX either three **(C)** or five **(G)** days after plating (Fisher exact test, P<0.001). **(D, H)** Quantification of ductal area of transformed cysts 3 or 5 days after plating in the presence and absence of DOX. Each data point represents a single quantified sphere from one of four biological replicates (t-test, P<0.001). **(E-F)** Brightfield images acinar-derived ductal cysts from *Kras^{G12D}; tetO-Ptf1a* pancreata in the presence or absence of DOX five days after plating. Metaplastic ductules were allowed to form for three days before DOX or vehicle was added **(I)**. **(J,L)** Brightfield images of untreated transformed ducts at day 3, before the addition of DOX or vehicle. **(K,M)** Brightfield images of metaplastic ducts after 2 days of treatment with DOX or vehicle. **(N)** Quantification of duct area at day 3, prior to treatment, and day 5, after DOX/vehicle had been added to the media for 2 days.

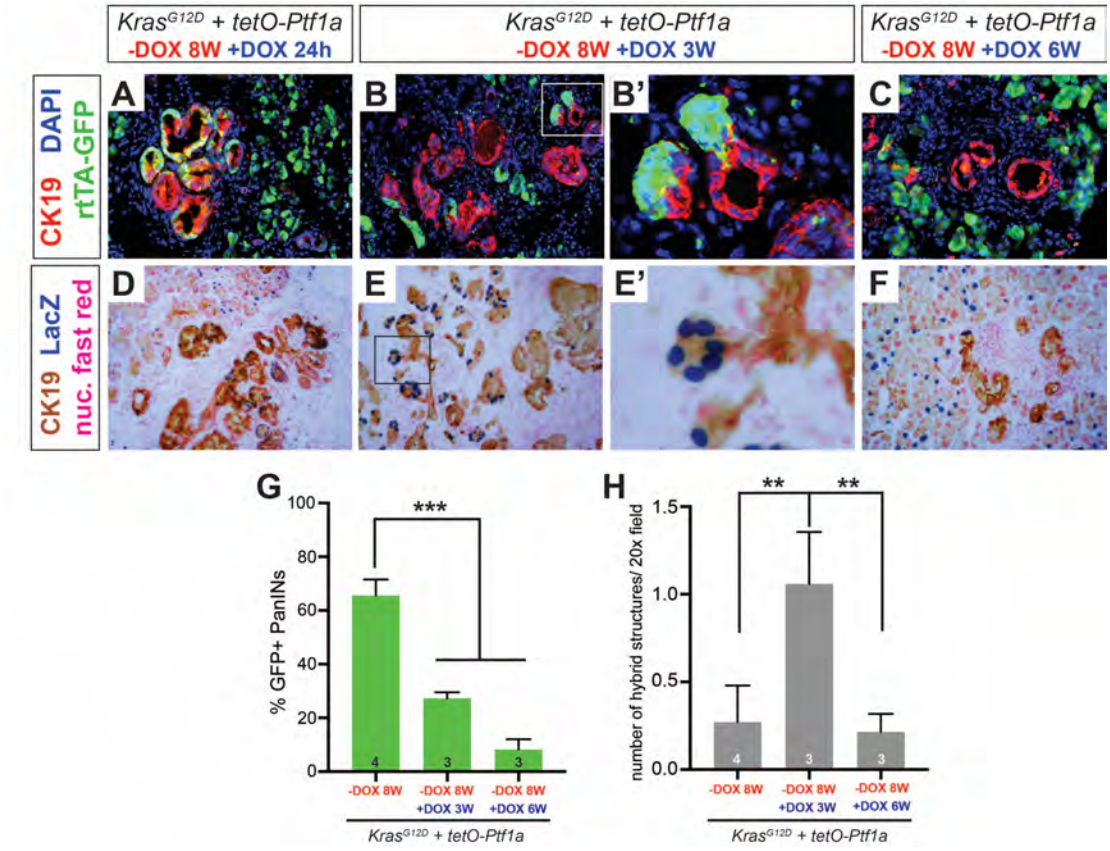

Figure 5.11. Re-expression of *Ptf1a* in PanINs leads to the emergence of primitive acinar cells. (A-C) Immunofluorescence for the duct marker CK19 (red), the Rosa$^{rtTA\text{-}GFP}$ acinar lineage label (green), and dapi (blue) demonstrating GFP+ PanINs in *Kras^{G12D}; tetO-Ptf1a* pancreata with only 24h DOX treatment **(A)** and highlighting largely GFP-negative PanINs in *Kras^{G12D}; tetO-Ptf1a* pancreata after 3 weeks or 6 weeks with DOX treatment **(B-C)**. **(B')** The blow-up image highlights GFP+ primitive acinar cells emerging from a CK19+ lesion. **(D-F)** Staining for CK19, the *tetO-Ptf1a-IRES-LacZ* and nuclear fast red in mice of indicated genotypes and treatments. 24 hours of DOX treatment in mice with pre-formed PanINs is sufficient to drive *tetO-Ptf1a* expression in PanINs **(D)**. **(E-F)** Staining for CK19, the *tetO-Ptf1a-IRES-LacZ* and nuclear fast red, highlighting clusters of LacZ-positive emerging from CK19+ lesions **(E')** and LacZ-negative PanINs 6 weeks after treatment **(F)**.

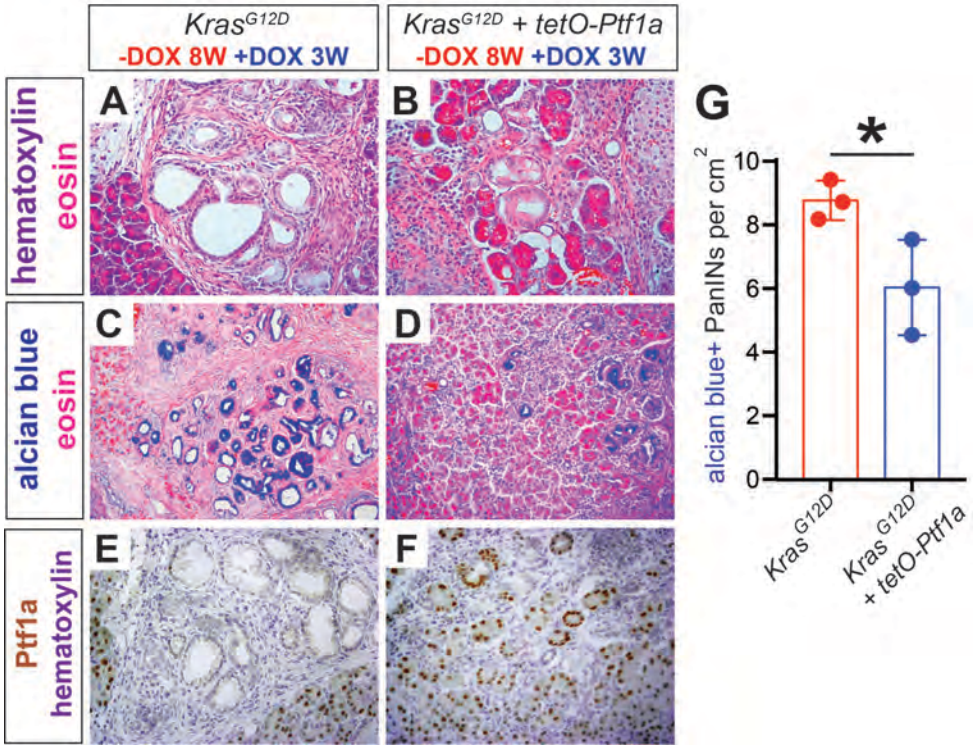

Figure 5.12. Re-expression of *Ptf1a* reduces PanIN burden in *Kras*^{G12D} + *tetO-Ptf1a* pancreata. H&E **(A-B)** and Alcian blue **(C-D)** staining of pancreata from mice of indicated genotypes and treatments. **(B)** Highlights primitive acinar cells trapped within PanIN lesions. **(E-F)** Immunohistochemistry for Ptf1a, showing absence of Ptf1a in PanINs from *Kras*^{G12D} pancreata and wide Ptf1a expression in duct-like structures in *Kras*^{G12D} + *tetO-Ptf1a* pancreata. **(G)** Quantification of genotype dependent PanIN burden following the treatment regimen 8 weeks without DOX, 3 weeks with DOX treatment. $P<0.05$.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

While pancreatic ductal adenocarcinoma (PDAC) remains one of the worst diseases in medicine, the last 10 years have seen remarkable developments in understanding the molecular origins of disease. Genetically engineered mouse models (GEMMs) have provided great insight into both the cell of origin and the pathways required to initiate PDAC. Mirroring what is seen in human disease, GEMMs of PDAC develop precancerous pancreatic intraepithelial neoplasia (PanINs), which can become invasive following tumor suppressor mutation or deletion (Aguirre et al., 2003; Bardeesy et al., 2006; Hingorani et al., 2003; Hingorani et al., 2005; Hruban et al., 2000). Notably, the classic progression model of PanIN-to-PDAC involves the increasing influence of non-cell-autonomous effects, such as desmoplasia and immune-epithelial cross-talk, which go on to make the fibrotic stroma associated with invasive tumors (di Magliano and Logsdon, 2013; Hruban et al., 2000; Morris et al., 2010b).

Although the duct-like phenotype of PanINs and the epithelial cells in PDAC tumors might suggest that the disease originates from duct cells, our lab and others have shown that acinar cells are much more susceptible to *Kras*-mediated oncogenic transformation than ducts (Bailey et al., 2015; De La et al., 2008; Habbe et al., 2008; Ji et al., 2009; Kopp et al., 2012; Ray et al., 2011). Evidence strongly suggests that PDAC is initiated by a metaplastic step in which acinar cells downregulate transcription factors that maintain cell identity, usually in response to $Kras^{G12D}$ mutation or pancreatic inflammation (De La et al., 2008; De La and Murtaugh, 2009; Guerra et al., 2007) (Figure 6.1). This intermediate, metaplastic cell type is uniquely sensitive to oncogenic transformation and PanIN formation. We therefore hypothesized that forced loss of acinar cell differentiation, via genetic *Ptf1a* deletion, could be a rate-limiting step for PDAC

initiation. In my dissertation, I demonstrate that *Ptf1a* maintains acinar cell homeostasis and limits metaplasia; in the presence of oncogenic *Kras*, *Ptf1a* limits the formation of precancerous pancreatic intraepithelial neoplasia (PanIN), and subsequent PDAC formation (Chapter 3). Acinar differentiation determinants, such as *Ptf1a* and *Nr5a2*, are also required for acinar cell regeneration following acute pancreatitis (Krah et al., 2015; von Figura et al., 2014). Perhaps most strikingly, my unpublished results indicate that maintaining acinar differentiation, via sustained or reintroduced *Ptf1a* expression, inhibits PanIN initiation (Chapter 5) (Figure 6.1).

The conventional PanIN-to-PDAC progression model depicts PanINs as intermediate stages in the progression to cancer, yet early-stage PanINs are almost found in more than half of randomly-selected healthy pancreata, despite a lifetime PDAC risk of only ~1% (Andea et al., 2003; Rebours et al., 2015). Consistent with an inherent low risk of progression, PanINs formed in the KC ($Kras^{G12D}$, *Cre*) mouse model, or our inducible $Kras^{G12D}$; *Ptf1a* cKO mouse (Chapter 3), rarely give rise to invasive carcinoma in the absence of engineered *p53* mutation/deletion, even when aged for 9-12 months (Hingorani et al., 2003; Hingorani et al., 2005; Krah et al., 2015). This observation is particularly striking in long-aged $Kras^{G12D}$; *Ptf1a* cKO mice where the entire pancreas is comprised of intermediate-grade PanINs for several months (Krah et al., 2015). These observations raise the possibility that PanINs could act in a host-protective manner, by isolating potentially oncogenic cells and denying them the ability to invade and metastasize (see Chapter 2). This idea is supported by the heterogeneity of these precancerous lesions; in humans, most cells comprising low-grade PanINs do not harbor *Kras* mutations, suggesting that PanINs emerge from multiple nonmutant cells and

remain polyclonal (Kanda et al., 2012). In contrast, in acinar-*Kras*^{G12D} mice, lineage tracing reveals that reprogrammed acinar ductules can be polyclonal, but PanIN-1 lesions are almost exclusively monoclonal (Maddipati and Stanger, 2015).

Due to a lack of appropriate molecular tools, however, it remains unclear whether wild-type acinar cells contribute to PanINs during PanIN formation in mice. To address this, our lab is currently engineering a *Kras*^{G12D}*-EGFP* allele, in which EGFP will be inserted upstream of endogenously expressed *Kras*^{G12D} allele and coexpressed via a viral T2A peptide (Murtaugh lab, unpublished). This allele will allow us to directly observe the phenotype of *Kras*-mutant and *Kras*-wild-type cells. With current methodology, Cre must recombine the *Kras* allele and the *Rosa26* allele, where reporter constructs are often placed for lineage tracing. This creates the opportunity for unequal Cre-mediated recombination, where one allele is activated, but the other is not (Liu et al., 2013). As we describe in Chapter 5, this unequal recombination leads to many Kras-mutant cells that do not express a lineage label (and vice-versa). However, the *Kras*^{G12D}*-EGFP* mouse will allow us to directly test whether PanINs contain a mix of *Kras*^{G12D}-positive and normal cells.

If wild-type acinar cells are reprogrammed to capture a sparse *Kras*-mutant acinar cell in this model, that would support this “encapsulation” hypothesis. It would also suggest that the initial waves of inflammation and fibrosis that promote acinar dedifferentiation, serve as a true wound-healing response to eradicate or isolate pathological cells that might be resistant to apoptosis. However, *Kras*-mutant cells may also signal to recruit bystander cells, which they require for their sustained proliferation and evolution. “Clusters” of ubiquitously *Kras*-mutant acinar cells may also be more

likely to form PanINs utilizing this bystander effect. A final and intermediate possibility is that *Kras*-mutant cells are initially restrained as PanINs until a catastrophic mitotic event, such a chromothripsis, causes immense mutational burden and imparts invasive potential on a *Kras*-mutant cell (Notta et al., 2016).

Regardless of whether PanIN cells are derived exclusively from *Kras*-mutant cells, our work demonstrates that they must downregulate *Ptf1a* to undergo transformation. We go on to show that loss of *Ptf1a* hastens the development of invasive PDAC, suggesting that loss of this transcription factor a rate limiting step for the entire acinar-to-PanIN-to-PDAC sequence. This makes *Ptf1a* unique among pancreatic master transcriptional regulators, as recent studies suggest that other differentiation determinants have dual roles in PDAC. For example, *Pdx1* is expressed at low levels in acinar cells and is required to suppress acinar cell reprogramming in the presence of oncogenic *Kras* (Roy et al., 2016). In stark contrast to *Ptf1a*, *Pdx1* remains highly expressed in PanINs but switches its transcriptional targets to facilitate RAS signaling and maintain tumorigenicity (Krah et al., 2015; Roy et al., 2016). Additionally, *Nr5a2*, a transcription factor that coordinates acinar-specific gene expression with *Ptf1a* during homeostasis (Holmstrom et al., 2011), also has a dual role in PDAC: it initially restrains acinar cell transformation, but is later re-expressed in PDAC cells to drive proliferation (Benod et al., 2011; Flandez et al., 2014; von Figura et al., 2014). The unique role for each transcription factor underscores the importance of understanding how differentiation programs may influence cellular phenotype at different cancer stages. Taken together, these studies also suggest that reactivation of *Ptf1a*, but not *Pdx1*, may hold promise as a therapeutic strategy in PDAC.

As PanINs and PDAC originate from acinar cells (Bailey et al., 2015; Habbe et al., 2008; Ji et al., 2009; Kopp et al., 2012), but do not express *Ptf1a* (De La et al., 2008; Krah et al., 2015), it will be important to determine the mechanism by which this gene is silenced. Since reactivation of *Ptf1a* and other components of the PTF1 transcriptional complex holds promise in preventing or re-differentiating PanINs/PDAC (Kim et al., 2015), understanding this mechanism could provide novel therapeutic approaches. Beginning in late embryonic development and continuing through homeostasis in adults, the PTF1 transcriptional complex (composed of Ptf1a, E proteins, and Rbpj-L) maintains *Ptf1a* expression through an autoregulatory transcriptional loop, which requires access to a highly conserved 5' autoregulatory enhancer (Masui et al., 2008). While mutations in the *Ptf1a* gene are not observed in human PDAC, mutations in this enhancer, which is located 13-15 kb upstream up the transcriptional start site, may not be picked up by whole exome sequencing. To test whether this intact enhancer element is required to prevent *Kras*-mediated transformation, the *Ptf1a* binding site could be mutated or deleted using CRISPR/CAS9. In collaboration with the MacDonald lab, Dr. Jane Johnson (University of Texas, Southwestern Medical Center) has recently engineered mice with such a deletion (unpublished correspondence). It will be interesting to cross these mice onto a $Kras^{G12D}$ background and determine whether deletion/mutation of this enhancer has the same effect as *Ptf1a* gene deletion (Krah et al., 2015). Additionally, oncogenic *Kras* signaling has been shown to promote the methylation and transcriptional repression of tumor suppressor genes, conferring a further growth advantage to colon cancer cells (Serra et al., 2014; Wajapeyee et al., 2013). If mutation of the autoregulatory is ruled out, could this mechanism of methylation be invoked in silencing the *Ptf1a* transcriptional

network during PDAC initiation?

The *Ptf1a* transcriptional network could also be silenced by changes in the transcriptional landscape of transforming acinar cells, promoted by stimulated oncogenic *Kras*. For example, oncogenic *Kras* may alter the balance of key transcription factors, such as *Ptf1a* and *Sox9*, to promote a duct-like gene expression profile. To determine whether *Kras*-mediated transformation alters chromatin accessibility, our lab is currently performing ATAC-seq on isolated $Kras^{G12D}$-expressing acinar cell clusters and metaplastic ductules derived from those same clusters in an acinar suspension system (Buenrostro et al., 2013). We hypothesize that the *Kras*-induced chromatin rearrangements make *Ptf1a* and other key network components inaccessible for transcription. Sox9, which is upregulated when *Ptf1a* is deleted (Chapter 3), could facilitate a transcriptional switch to ductal gene expression (Kopp et al., 2012; Krah et al., 2015). As we have previously demonstrated that decreased *Ptf1a* gene dosage sensitizes acinar cells to oncogenic transformation (Krah et al., 2015), it is easy to image that an attenuation of the autoregulatory loop described above would lead to rapid silencing of *Ptf1a* and its other acinar-specific targets.

While the above future direction focus on the cell-autonomous consequences of *Kras* mutation and *Ptf1a* loss, we provide substantial evidence that loss of *Ptf1a* alters the surrounding microenvironment. Loss of *Ptf1a* alone allows for the robust upregulation of several fibroinflammatory pathways, including stellate cell (pancreatic fibroblast) activation, dendritic cell maturation, and T-helper cell signaling – all of which have been implicated in PDAC development (Liou et al., 2015; Sherman et al., 2014; Zhang et al., 2014). Consistent with these findings, loss of *Ptf1a* during acute pancreatitis allows for

persistence of the severe fibroinflammatory reaction to caerulein and an absence of epithelial regeneration (Krah et al., 2015). Additionally, the robust PanIN formation seen in *Kras*^{G12D}; *Ptf1a* cKO pancreata is associated with leukocyte infiltration and fibroblast activation (Krah et al., 2015). Because this phenotype resembles that of *Kras*^{G12D} mice subjected to caerulein-induced pancreatitis, we reasoned that inflammation might be driving both phenotypes (De La and Murtaugh, 2009; Guerra et al., 2007; Morris et al., 2010a). Surprisingly, treatment with the corticosteroid, dexamethasone, produced opposite phenotypes in the *Kras*/caerulein and *Kras*/*Ptf1a* cKO mice.

There are several future directions pertaining to these findings (see Discussion, Chapter 4). It will be interesting to test how depletion of different immune cell types effects both *Kras*/caerulein and *Kras*/*Ptf1a* cKO mice. Particularly interesting will be depletion of macrophages and dendritic cells, as these cells are closely associated with PanINs in all models of PDAC that we have studied and have been implicated in PanIN initiation (Lesina et al., 2011; Liou et al., 2015; Liou et al., 2013; Seifert et al., 2016). Since myeloid cells and macrophages can have polarized phenotypes, by which they either promote or inhibit cancer initiation and progression, it will be interesting to identify which macrophage-derived signals are received by transforming acinar cells. Could some populations of macrophages promote acinar regeneration? Could others promote PanIN formation, perhaps through IL-6 signaling (Lesina et al., 2011)? Identifying these signals, and how they interact with *Kras* mutant acinar cells, may hold great potential for inhibiting pancreatic disease going forward.

Our work has greatly broadened the understanding PDAC initiation. While our understanding of disease initiation has greatly expanded over the past 10 years, clinical

outcomes have not significantly improved, despite advances in targeted therapy and immunotherapy. Further understanding of cell-autonomous changes in acini, and how those changes influence the surrounding microenvironment, should enhance our ability to form treatments that influence multiple aspects of PDAC earlier in the disease process. With a multitargeted approach, I believe that we will be able to impede disease progression and improve the quality of life of patients suffering from this terrible disease.

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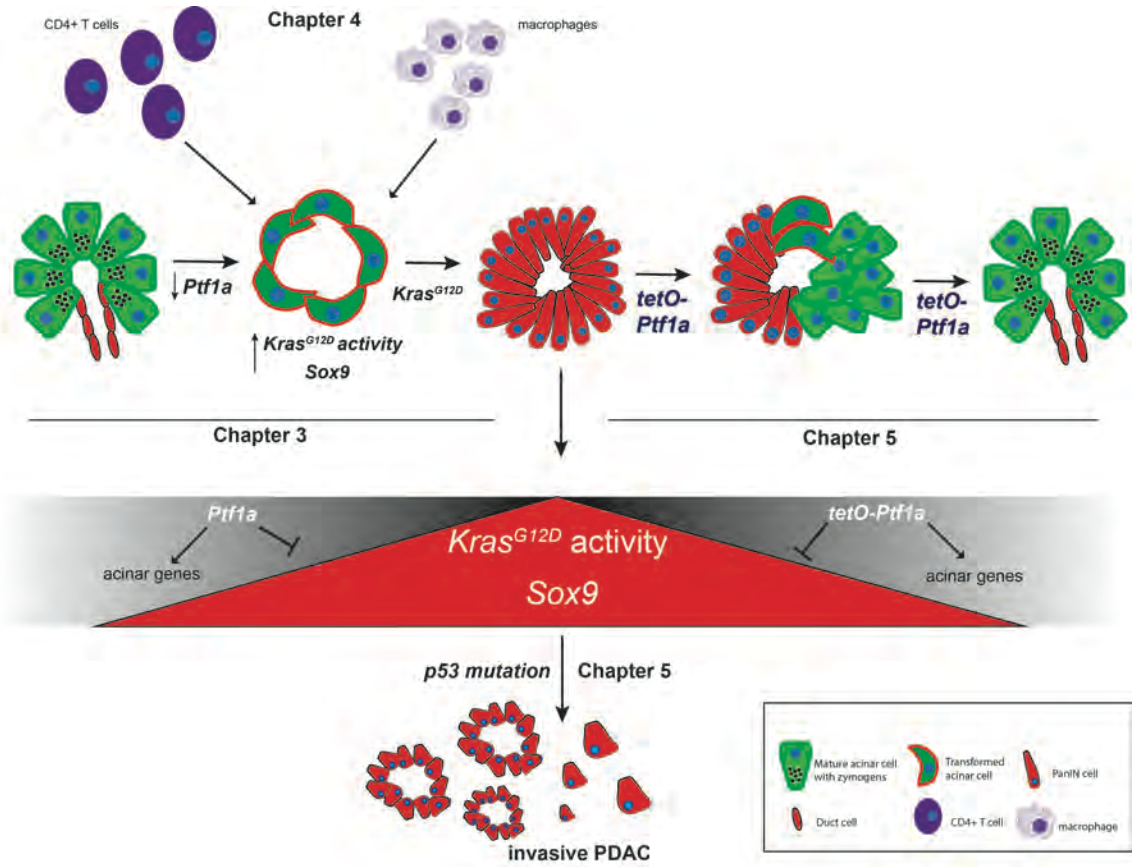

Figure 6.1. Overall model. In Chapter 3 (left side of diagram), I demonstrate that loss of *Ptf1a* is sufficient to induce acinar cell reprogramming, upregulate the ductal transcription factor *Sox9*, and produce a PDAC-like gene expression profile that sustains RAS signaling in human cancer cells. Consequently, *Ptf1a*-deficient cells are extremely sensitive to *Kras*^{G12D} –mediated oncogenic transformation. In Chapter 4 (top of diagram), I propose that there are two distinct inflammatory waves during PDAC initiation: one that is primarily predominantly T-cells and is responsible to acinar cell reprogramming, and another that is macrophage derived that promotes *bona fide* PanIN formation. In Chapter 5, I show that *Ptf1a* mediates a novel tumor suppressor mechanism, that is independent of *p53* loss. Consequently, *KPC* (Kras, p53, Cre) mice with *Ptf1a* deleted from acini, succumb much more rapidly to PDAC than littermate *KPC* mice. Finally, I show that reintroduction of Ptf1a into established PanINs is sufficient to revert them the amylase-positive acinar cells *in vivo*. This is among the first studies to demonstrate the plasticity of preoncogenic cells, and thus opens a new paradigm for solid tumor prevention and treatment.