ORIGINAL ARTICLE

Maintenance of acinar cell organization is critical to preventing Kras-induced acinar-ductal metaplasia

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers owing to a number of characteristics including difficulty in establishing early diagnosis and the absence of effective therapeutic regimens. A large number of genetic alterations have been ascribed to PDAC with mutations in the *KRAS2* proto-oncogene thought to be an early event in the progression of disease. Recent lineage-tracing studies have shown that acinar cells expressing mutant $Kras^{G12D}$ are induced to transdifferentiate, generating duct-like cells through a process known as acinar-ductal metaplasia (ADM). ADM lesions then convert to precancerous pancreatic intraepithelial neoplasia (PanIN) that progresses to PDAC over time. Thus, understanding the earliest events involved in ADM/PanIN formation would provide much needed information on the molecular pathways that are instrumental in initiating this disease. As studying the transition of acinar cells to metaplastic ductal cells *in vivo* is complicated by analysis of the entire organ, an *in vitro* three dimensional (3D) culture system was used to model ADM outside the animal. $Kras^{G12D}$-expressing acinar cells rapidly underwent ADM in 3D culture, forming ductal cysts that silenced acinar genes and activated duct genes, characteristics associated with *in vivo* ADM/PanIN lesions. Analysis of downstream KRAS signaling events established a critical importance for the Raf/MEK/ERK pathway in ADM induction. In addition, forced expression of the acinar-restricted transcription factor *Mist1*, which is critical to acinar cell organization, significantly attenuated $Kras^{G12D}$-induced ADM/PanIN formation. These results suggest that maintaining MIST1 activity in $Kras^{G12D}$-expressing acinar cells can partially mitigate the transformation activity of oncogenic KRAS. Future therapeutics that target both the MAPK pathway and *Mist1* transcriptional networks may show promising efficacy in combating this deadly disease.

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INTRODUCTION

With a 5-year survival rate of < 5% for patients with pancreatic ductal adenocarcinoma (PDAC), the disease remains one of the most lethal malignancies primarily due to late diagnosis and ineffective chemo and radiation therapies.[1] Recent identification of three distinct PDAC precursors—pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm and intraductal papillary mucinous neoplasm—provides new hope for early detection and a much better understanding of the underlying mechanisms that are instrumental in the progression of the disease.[2] Among these precursor lesions, PanINs are the best characterized.[3] Indeed, a number of genetic mutations associated with PDAC have been detected in PanINs at various stages including mutations in the *KRAS2*, *TP53* and *SMAD4* genes, which also are hallmarks of advanced PDAC, consistent with a PanIN→PDAC step-wise progression model.[2] These observations are further supported by genetically engineered mouse models where PanIN/PDAC progression is initiated by single-point mutations in the *Kras* gene and modified by additional genetic alterations in other oncogenes or tumor suppressors.[4–10] However, the molecular mechanisms that govern each step of PanIN/PDAC progression remain elusive.

More controversial is the cellular origin of PDAC. PDAC has long been considered a disease of pancreatic ducts. Early efforts to model the disease by expressing *Kras* in pancreatic duct cells did not yield discernable pathology,[11] although a recent study by Ray *et al.*[12] has shown that activation of endogenous $Kras^{G12D}$ in large pancreatic ducts leads to rare early PanIN lesions. Despite this example, increasing evidence supports the idea that PanIN/PDAC can originate from differentiated acinar cells, which represent the major cellular component of the pancreas parenchyma.[10,13–15] The development of duct-like PanIN lesions from acinar cells necessitates massive remodeling of these cells, both morphologically and with respect to gene expression profiles. The transition from acinar to ductal cell properties has been termed acinar-ductal metaplasia (ADM) and lineage-tracing studies have confirmed that this process results from direct transdifferentiation of adult acinar cells that convert to a duct cell phenotype upon $Kras^{G12D}$ expression.[10,13,16] The relevance of ADM to PDAC is also supported by the observation that ADM is frequently associated with human PanIN lesions from PDAC patients.[17,18] In addition, ADM development has been shown to precede PanIN formation in mouse $Kras^{G12D}$ models,[17] suggesting that ADM represents the initial stage of PDAC development.

Although it is clear that $Kras^{G12D}$ expression in adult acinar cells can generate ADM lesions that progress to PanINs and PDAC, the molecular signaling pathways that are instrumental in these conversion events remain poorly defined. Indeed, $Kras^{G12D}$

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expression alone does not guarantee ADM/PanIN development because in mouse PDAC models where ~95% of acinar cells express $Kras^{G12D}$ only a small cohort of cells generate detectable ADM/PanINs after a significant latent period (months), demonstrating that differentiated acinar cells are not universally responsive to KRAS signaling. This observation is compounded by the complexity of following ADM/PanIN development within the context of the intact organ. In an effort to develop an in vitro system to study the very earliest events of ADM formation, we focused on the initial transition of acinar cells converting to ductal cells upon $Kras^{G12D}$ expression in a three dimensional (3D) culture model. We found that $Kras^{G12D}$-expressing acinar cells in 3D culture rapidly converted to ductal cells that mimicked properties associated with in vivo ADM and PanIN lesions, including silencing of acinar genes and expression of duct genes. Analysis of RAS signaling components demonstrated that activation of the Raf/MEK/ERK pathway was essential for $Kras^{G12D}$-induced ADM. Furthermore, the ability of acinar cells to undergo a transdifferentiation process to ductal cells was significantly attenuated by forced expression of the acinar-restricted transcription factor Mist1, suggesting that maintaining acinar cell identity mitigates the strong oncogenic potential of $Kras^{G12D}$ expression. These results provide possible new therapeutic approaches to treat early ADM/PanIN cases by simultaneously targeting the MAPK pathway and Mist1 transcriptional networks.

RESULTS

$Kras^{G12D}$ expression converts pancreatic acini to ductal cysts in vitro

Kras mutations are virtually universal in human PDAC, although identification of the initial adult cell (duct, acinar and centroacinar) that acquires the mutation and initiates the transformation cascade remains unknown. Recent studies examining pancreatic cancer in mouse models have shown that Kras oncogene expression in adult acinar cells leads to PDAC that fully mimics the human disease.[10,13,15,16] Lineage-tracing of Elastase-CreER; LSL-$Kras^{G12D/+}$; $R26R^{mTmG}$ mice confirmed that activation of $Kras^{G12D}$ in adult acinar cells induces ADM, which can progress to PanIN and on to PDAC (Figure 1a).

Despite this fundamental process of initiating a transformation pathway, it has been difficult to study the intricacies of ADM→PanIN→PDAC in the intact animal. In an effort to dissect the initial $Kras^{G12D}$-dependent pathways in vitro, we asked if ADM could be modeled in tissue culture. Previous studies showed that wild-type acinar cells treated with TGFα and maintained in a 3D collagen matrix converted to duct-like cysts that activated epidermal growth factor receptor (EGFR) downstream signaling pathways, including Ras.[4,19] To determine whether constitutive KRAS activity could similarly lead to ductal cyst formation, $Mist1^{CreER/+}$; LSL-$Kras^{G12D/+}$; $R26R^{LacZ}$ or Elastase-CreER; LSL-$Kras^{G12D/+}$ mice were treated with corn oil (control) or tamoxifen (TM) to activate acinar-specific $Kras^{G12D}$ expression. Pancreata were then harvested 7 days post-treatment, digested with collagenase and individual acini clusters placed in a 3D collagen matrix. As expected, control and $Kras^{G12D}$-expressing acinar cells initially retained their normal apical–basal polarity with the rER accumulating at the basal aspect and zymogen granules clustered at the apical pole (Figure 1b). However, by 5 days in 3D culture there was a marked difference in cell viability between the $Kras^{G12D}$-expressing and non-expressing cell populations. Control acinar cells rapidly became disorganized and underwent massive cell death. In contrast, a high proportion (~70–90%) of $Kras^{G12D}$-expressing acinar cells survived and converted to ductal cyst structures that were comprised of a single layer of epithelial cells surrounding an empty luminal space, resembling the metaplastic ADM/PanIN lesions observed in vivo (Figures 1a and b). Interestingly, these metaplastic cysts were virtually identical to structures formed by primary pancreatic duct

cells.[20] Lineage tracing using the $R26R^{LacZ}$ reporter allele confirmed that the ductal cysts were derived via transdifferentiation of mature $Kras^{G12D}$-expressing acinar cells (Figure 1b). In agreement with a recent study by Heid et al.,[21] ductal cyst conversion was dependent on $Kras^{G12D}$ activity as the vast majority of control acinar cells (-TM) failed to convert to ductal cysts over the duration of the experiment (Figure 1c).

The profound morphological changes in the 3D cultures were reminiscent of the in vivo changes that take place in the whole organ upon $Kras^{G12D}$ activation. Indeed, a marked switch from acinar to ductal gene expression profiles occurred during the 5-day culture period. At day 0, individual acini expressed high levels of amylase, elastase, carboxypeptidase and lipase, with virtually no detectable expression of duct gene products including keratin 19 (K19), cystic fibrosis transmembrane conductance regulator and carbonic anhydrase II (Figures 2 and 3), confirming the purity of the starting acinar cell preparation. As expected, these cells also expressed high levels of the acinar-specific transcription factors Ptf1a and Mist1 (Figures 2 and 3), with Mist1 being essential to maintaining an adult acinar cell phenotype.[22] However, by day 5 $Kras^{G12D}$-induced ductal cysts exhibited a significant shift in their gene expression profiles that mirrored the expression pattern of PanIN lesions. Ductal cysts and PanINs uniformly silenced acinar genes and activated expression of duct-specific genes, including cystic fibrosis transmembrane conductance regulator, K19, carbonic anhydrase II and the duct-restricted transcription factors Sox9 and HNF6 (Figures 2 and 3; Supplementary figures S1 and 2). These cells also expressed Nestin (Figure 3; Supplementary figure S2), a marker of ductal cells in early PanINs.[23] As predicted, PanINs in $Mist1^{CreER/+}$; LSL-$Kras^{G12D/+}$ pancreata were highly proliferative and a similar proliferation index was observed with the $Kras^{G12D}$-induced ductal cysts (Figure 2; Supplementary figure S3). The parallel between ductal cyst formation in vitro and ADM/PanIN genesis in vivo suggests that the 3D acinar culture faithfully mimics the intracellular pathways that regulate ADM.

MAPK activity is elevated in PanINs and is essential for ductal cyst formation

It is well established that $Kras^{G12D}$ expression induces cellular transformation by activating downstream signaling events, including the Raf/MEK/ERK, PI3K/AKT and RalGDS/Ral pathways (Figure 4a). Although these pathways have been the subject of potential therapeutic benefit against pancreatic tumors,[24,25] little is known about the role they have in the earliest transformation events of ADM and PanIN formation. In an effort to dissect the importance of the PI3K/AKT and Raf/MEK/ERK pathways before tumor formation, we examined $Mist1^{CreER/+}$; LSL-$Kras^{G12D/+}$ (HET/Kras) pancreata 3 months post-TM to ascertain if ADM/PanIN regions exhibited elevated KRAS signaling. Interestingly, despite the fact that ≥90% of adult acinar cells express $Kras^{G12D}$, only regions of ADM/PanIN exhibited sufficiently high pAKT and pERK levels that could be detected by immunohistochemistry (Figure 4b). Thus, expression of $Kras^{G12D}$ in acinar cells (for up to 3 months) is not sufficient to uniformly activate the PI3K/AKT and Raf/MEK/ERK arms of the KRAS pathway in all cells.

To examine the importance of the pAKT and pERK pathways in converting acinar cells to ADM, we investigated these KRAS downstream pathways in the 3D culture model. As an initial test, we examined the importance of the upstream EGFR pathway with respect to $Kras^{G12D}$-induced ductal cyst formation. As previously reported,[4,19,21] wild-type acinar cells placed in 3D culture exhibited only background levels of ductal cysts. However, when cells were provided TGFα, rapid ductal cyst formation occurred (Figure 4c). As expected, inhibition of EGFR by the EGFR tyrosine kinase inhibitor PD153035 completely blocked ductal cyst formation of wild-type acinar cells but had no effect on
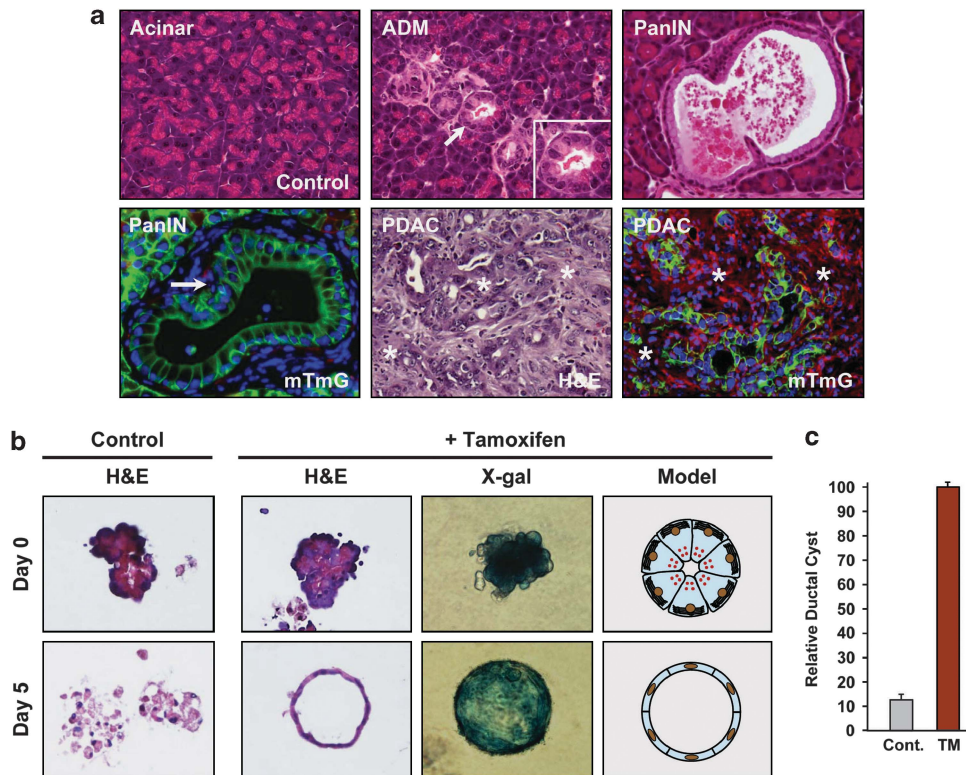
Figure 1. Acinar-specific Kras^{G12D} expression leads to ADM→PanIN→PDAC. (**a**) Adult Elastase-CreER; LSL-Kras$^{G12D/+}$; R26R^{mTmG} and Elastase-CreER; LSL-Kras$^{G12D/+}$; LSL-Trp53^{R172H}; R26R^{mTmG} mice were given corn oil (control) or TM and pancreas sections were processed for standard histology after 1–5 months. The earliest lesions to appear are ADM (inset) followed by PanINs and then PDAC. R26R^{mTmG} lineage tracing confirms that ADM, PanINs and PDAC are derived from Kras^{G12D}-expressing acinar cells (green). Red signal represents unrecombined R26R^{mTmG} stromal cells. Arrows, ADM and PanIN lesions; asterisks, adjacent stromal tissue. (**b**) Mist1$^{CreER/+}$; LSL-Kras$^{G12D/+}$; R26R^{LacZ} acinar cells were isolated from 7 day post-treatment mice and individual acini were cultured for 5 days in a 3D collagen matrix. The majority of control acinar cells undergo extensive cell death by 5 days, whereas Kras^{G12D}-expressing acinar cells rapidly transdifferentiate into ductal cysts (hematoxylin and eosin (H&E) sections) that retain β-gal expression (whole mount X-gal staining). Identical results were obtained with Elastase-CreER; LSL-Kras$^{G12D/+}$; R26R^{mTmG} mice. (**c**) Whereas corn oil-treated control Mist1$^{CreER/+}$; LSL-Kras$^{G12D/+}$; R26R^{LacZ} acinar cells generate very few ductal cysts, TM-treated acinar cells generate a large number of ductal cysts in 3D collagen matrix.

HET/Kras ductal cyst formation, confirming that KRAS functions downstream of EGFR (Figures 4a and c).

We next examined the importance of the PI3K/AKT and Raf/MEK/ERK pathways for ductal cyst formation in Het/Kras mice. As predicted, elevated pAKT was observed in Kras^{G12D}-expressing acinar cells when compared with control cells isolated from corn oil-treated littermates (Figure 4d). Treatment of the cultures with the PI3K inhibitor LY294002 led to a ~70% reduction in pAKT levels but had no significant effect on ductal cyst formation in the 3D culture model. Likewise, 90% reduction of pAKT levels by the PI3K inhibitor VIII produced only modest decreases in ADM conversion (Supplementary Figure S4). Together, these results suggest that the PI3K/AKT pathway is not required for, nor essential to, ADM.

A similar analysis was performed on the Raf/MEK/ERK pathway. Whereas control cells exhibited low levels of pERK, HET/Kras acinar cells had elevated pERK levels (Figure 4e). Treatment of cells with the MEK inhibitor PD98059 efficiently blocked accumulation of pERK at concentrations as low as 20 μM. Interestingly, inhibition of MEK activity perfectly correlated with decreased ductal cyst formation (Figure 4e). We propose that activation of the Raf/MEK/ERK pathway in a subset of Kras^{G12D}-expressing acinar cells is required to initiate an ADM response in mouse models of PDAC.

Sustained Mist1 expression suppresses ductal cyst formation in vitro and ADM/PanIN formation in vivo

Kras^{G12D}-expressing acinar cells rapidly lose their acinar characteristics as they acquire a duct-like phenotype.[13,17] An early event of

this switch is silencing the Mist1 locus, which encodes a basic helix-loop-helix transcription factor critical to acinar cell differentiation.[4,17] Mist1 is highly expressed in pancreatic acinar cells and its expression correlates with cellular differentiation and the establishment of the secretory machinery.[22,26] Previous studies have shown that human acinar cells lose Mist1 expression upon ADM/PanIN formation,[4,17] and in mouse models devoid of the Mist1 gene, Kras^{G12D}-expressing acinar cells rapidly undergo ADM and develop extensive PanIN lesions through a pathway that involves elevated pERK activity.[4,27] Therefore, we hypothesized that MIST1 promotes acinar cell differentiation and suppresses the oncogenic activity of Kras by keeping pERK levels low. Given that ADM is dependent on MAPK activity (Figure 4e), we examined if sustained Mist1 expression could restrict the conversion of Kras^{G12D}-expressing acinar cells to ductal cysts.

To test this concept, a transgenic mouse line (LSL-Mist1^{myc}) that produces constitutive Mist1 expression upon Cre-mediated recombination was generated (Figure 5a). Transgene expression of myc-tagged Mist1 was driven by a CMV early enhancer/chicken β-actin hybrid promoter, whose activity is independent of the differentiation status of cells.[28] When Mist1$^{CreER/CreER}$; LSL-Kras$^{G12D/+}$; LSL-Mist1^{myc} (KO/Kras/Mist1^{myc}) mice were treated with TM, Mist1^{myc} expression was observed in >90% acinar cells with no expression detected in duct cells or in corn oil-treated (-TM) control mice (Figure 5b). Next, we examined MAPK activity in pancreata +/− Kras^{G12D} and Mist1 expression. As shown in Figure 5c, loss of Mist1 (KO) led to elevated pERK levels, even in the absence of Kras^{G12D} expression. However, sustained Mist1^{myc}
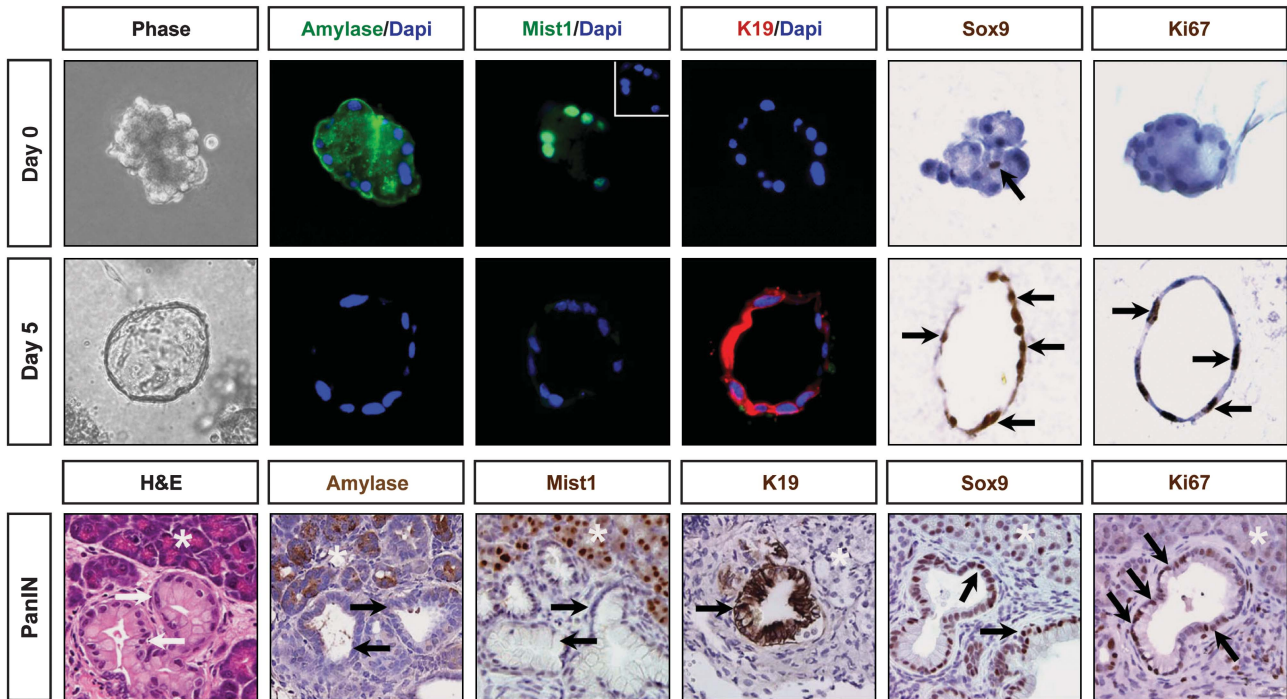
Figure 2. Kras^{G12D}-expressing acinar cells undergo conversion to a ductal phenotype when maintained in 3D collagen matrix. Fluorescence and light microscopy reveals that Kras^{G12D} acinar cells express acinar gene products at day 0 but rapidly convert to a ductal cell phenotype with activation of K19 and Sox9 expression. Inset for day 0 Mist1 panel shows the 4'6-diamidino-2-phenyl indole (Dapi)-stained nuclei for this acinus. Note that a single centroacinar cell (arrow) in an isolated acinus is SOX9 positive. Pancreas serial sections from experimental littermates confirm the absence of AMYLASE and MIST1 and the expression of SOX9 and K19 in acinar-derived PanINs (arrows). Both PanINs and ductal cysts are also Ki67 positive (arrows). Asterisks, adjacent acinar tissue.
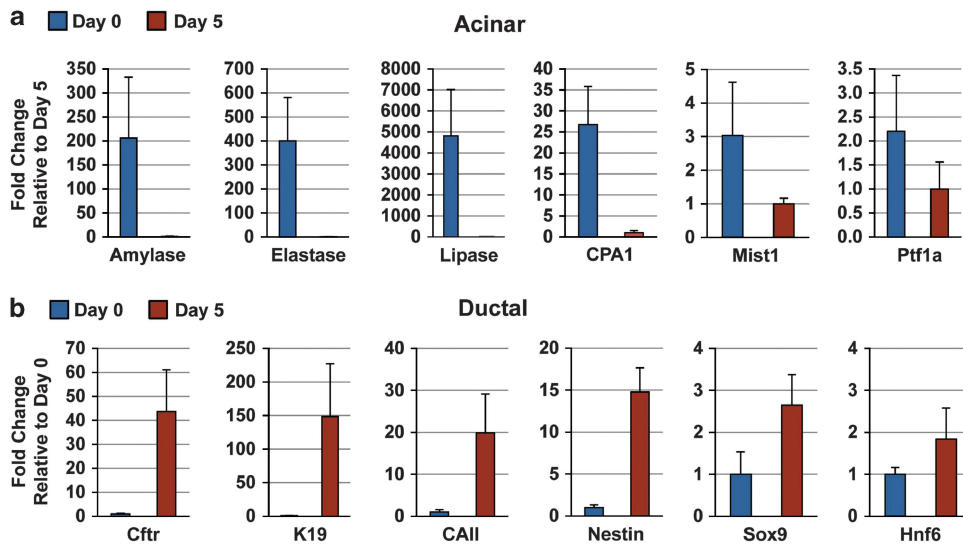

Figure 3. Kras^{G12D}-induced ductal cyst conversion leads to repression of the acinar gene program and activation of the ductal gene program. (**a**, **b**) Reverse transcriptase–qPCR of day 0 and day 5 cultures confirms that Kras^{G12D} acinar cells express high levels of acinar gene products at day 0 but rapidly downregulate acinar genes and upregulate duct genes by day 5, a time that corresponds to maximum ductal cyst formation.

expression in the KO/Kras/Mist1^{myc} pancreata generated lower pERK levels when compared with KO/Kras littermates (Figure 5c). We then examined how constitutive Mist1^{myc} expression influenced ductal cyst conversion in 3D cultures. As expected, Mist1$^{+/-}$ (HET) and Mist1$^{-/-}$ (KO) acini generated few ductal cysts when placed in collagen matrix for 5 days (Figure 5d). In contrast, TM-treated Mist1$^{CreER/+}$; LSL-Kras$^{G12D/+}$ (HET/Kras) acini rapidly

converted to ductal cysts and the percentage of conversion increased when Mist1$^{CreER/CreER}$; LSL-Kras^{G12D} (KO/Kras) acinar cells were tested (Figure 5d; Supplementary figure S5). Importantly, when KO/Kras/Mist1^{myc} acini were examined, ductal cyst formation was reduced to levels approaching those observed in Kras^{G12D}-negative (Mist1$^{+/-}$, Mist1$^{-/-}$) cells (Figure 5d; Supplementary figure S5). Similar results were obtained with the
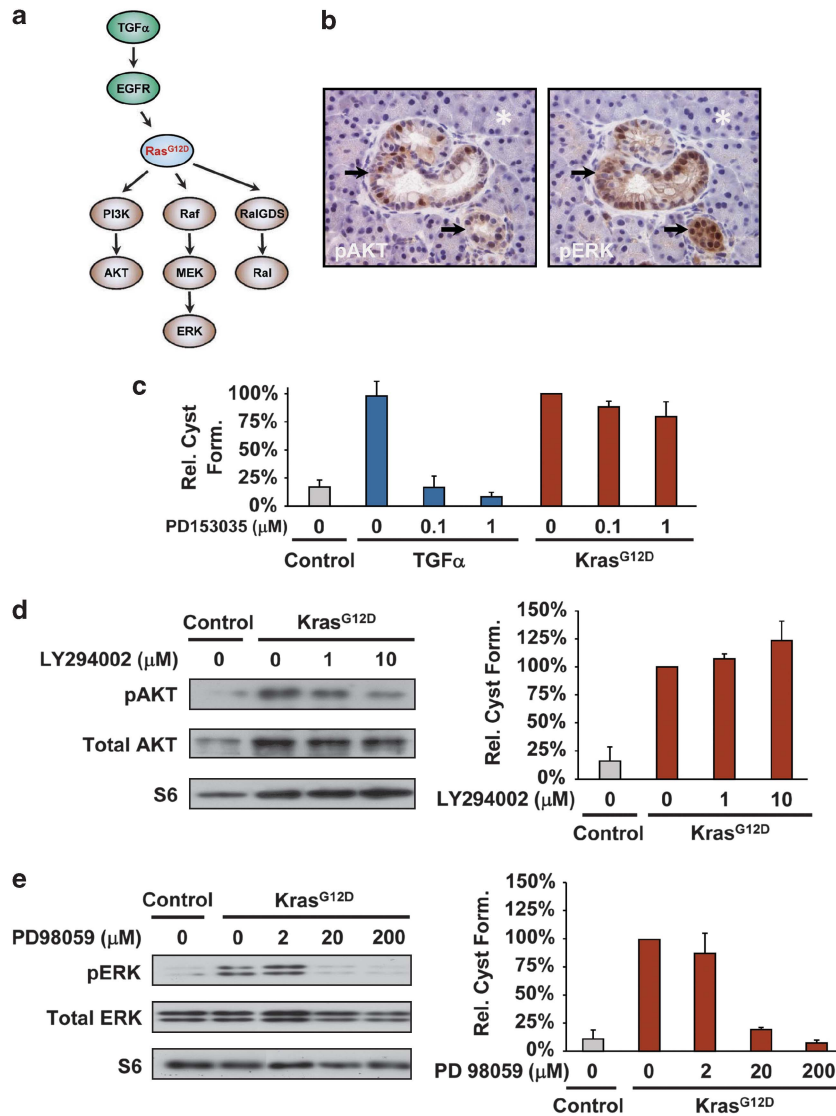
Figure 4. The Raf/MEK/ERK pathway is essential to early ADM/PanIN formation *in vitro* and *in vivo*. (**a**) Canonical upstream and downstream pathways associated with *Kras*. (**b**) ADM/PanIN lesions (arrows) in the HET/Kras pancreas exhibit elevated levels of pAKT and pERK. Note that the majority of acinar cells (asterisks) in HET/Kras pancreata are Kras[G12D] positive but pAKT/pERK negative. (**c**) TGFα treatment of control wild-type acinar cells leads to ductal cyst formation in 3D culture. As expected, inhibition of EGFR activity (PD153035 treatment) blocks TGFα-dependent ductal cyst formation but has no effect on the downstream Kras[G12D] pathway of HET/Kras acinar cells. (**d**) Inhibition of PI3K by LY294002 blocks pAKT activity but does not inhibit ductal cyst formation. (**e**) The MEK inhibitor PD98059 efficiently blocks pERK activity in HET/Kras acinar 3D cultures. Inhibition of MEK also leads to complete inhibition of Kras[G12D]-induced ductal cyst formation.

TGFα model where *Mist1[myc]* expression blocked TGFα-induced ductal cyst formation of *Mist1^{−/−}* acinar cells (Supplementary Figure S6). These results demonstrate that sustained *Mist1* expression lowers pERK levels and protects acinar cells from TGFα- or Kras[G12D]-induced ADM formation *in vitro*.

Finally, to determine whether sustained *Mist1* expression could similarly deter ADM/PanIN development *in vivo*, adult KO/Kras/Mist1[myc] mice and their littermate controls were treated with TM and pancreata were examined 3 months post-TM for the presence of ADM and PanIN lesions. HET/Kras mice developed rare focal areas of ADM and PanIN lesions at 3 months and in all cases the *Mist1* locus was transcriptionally silenced in acinar-derived PanINs and in advanced ADM lesions (Figures 6a and b). As expected, KO/Kras pancreata developed organ-wide extensive ADM/PanINs owing to the absence of *Mist1* before Kras[G12D] activation (Figures 6a and b). In contrast, sustained *Mist1[myc]* expression greatly attenuated ADM/PanIN development in the KO/Kras/Mist1[myc] mice as the majority of the organ consisted of normal

appearing acinar tissue with only isolated areas of ADM/PanINs (Figures 6a and b). Indeed, the number of PanINs that developed in KO/Kras/Mist1[myc] mice and reported in Figure 6b actually represent an upper limit because not all PanINs (∼18%) were MIST1[myc]-positive, presumably due to selected Cre-mediated recombination of the *LSL-Kras[G12D]* locus and not the *LSL-Mist1[myc]* transgene. Thus, a subset of Kras[G12D]-expressing acinar cells did not have an opportunity to respond to sustained MIST1 protein. However, despite the clear protective effect of *Mist1[myc]* expression, MIST1[myc] protein *per se* did not fully inhibit Kras[G12D]-induced PanINs as MIST1[myc] + PanINs were still generated, albeit at a greatly reduced level (Figures 6a and b). The MIST1[myc]-dependent reduction in ADM/PanIN formation was also reflected in the expression profiles of key acinar vs ductal genes where MIST1[myc] significantly reduced ductal gene activity while maintaining acinar gene expression to levels approaching, or exceeding, those obtained with HET/Kras mice (Figure 6c). These results are consistent with MIST1 exerting an anti-ADM/PanIN activity in the

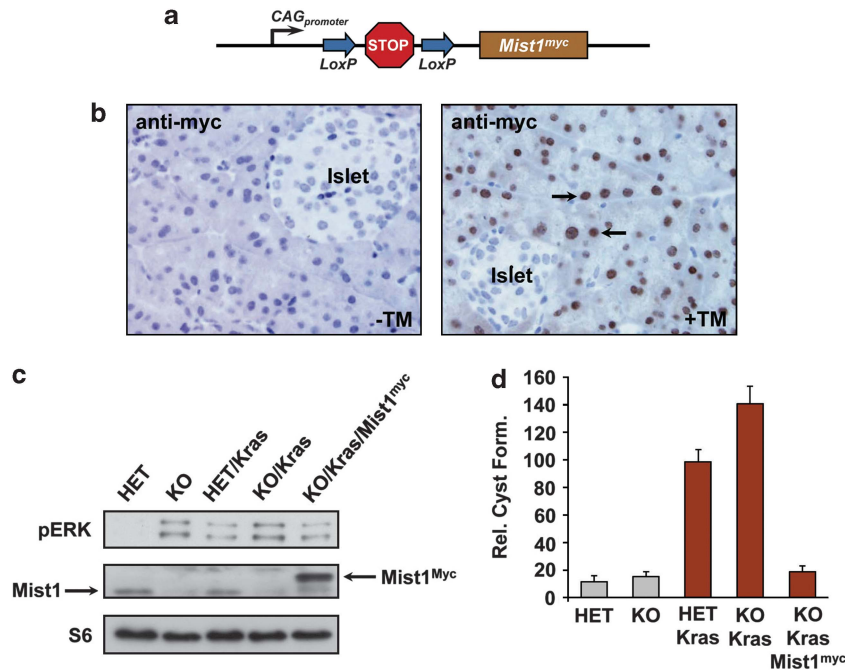**Figure 5.** Sustained Mist1 expression suppresses Kras[G12D]-induced ductal cysts. (**a**) Schematic diagram of the LSL-Mist1[myc] transgene. (**b**) Anti-myc immunohistochemistry of KO/Kras/Mist1[myc] pancreata reveals extensive acinar-specific expression of LSL-Mist1[myc] in TM-treated mice (arrows). (**c**) Immunoblot of pERK activity in HET vs KO and KO/Kras vs KO/Kras/Mist1[myc] pancreata. The absence of *Mist1* leads to increased pERK activity while sustained *Mist1[myc]* expression reduces pERK levels in KO/Kras/Mist1[myc] pancreata. (**d**) Quantification of ductal cyst formation in control and *Kras[G12D]*-expressing cells. Mice were treated with TM and acinar cells placed in 3D culture as described in Materials and methods. Sustained *Mist1[myc]* expression markedly inhibits *Kras[G12D]*-induced ductal cyst formation.

intact pancreas and support the concept that constitutive *Mist1* expression mitigates the activity of oncogenic *Kras[G12D]* in acinar cells, leading to a reduction in ADM/PanIN formation.

DISCUSSION

The role of acinar cells in pancreatic tumorigenesis has long been suspected, but definitive evidence had been lacking until lineage-tracing studies established that PanINs and PDAC can arise from adult acinar cells.[10,13,16] These studies also implicated the process of ADM as a bridge between normal acinar cells and duct-like PanINs. Consistent with this hypothesis, ADM occurrence precedes PanIN development in PDAC mouse models.[17] Despite the central role for *Kras[G12D]* in inducing ADM, *Kras* mutations are not a prerequisite. Mutant *Kras* is often detected in ADM and low-grade PanIN lesions in human diseased organs but these mutations are not universally present,[2,29] suggesting that additional underlying events can influence ADM formation. Indeed, exposure to EGFR growth factors leads to metaplastic lesions *in vitro* and *in vivo* in the absence of active *Kras*.[30,31] Similarly, EGFR activity is a hallmark of *Kras*-induced ADM/PanIN lesions,[17] implying a central role for this pathway in *Kras*-dependent and *Kras*-independent ADM. ADM is also commonly associated with acute and chronic pancreatitis in both humans and rodents,[18,32,33] suggesting that inflammation and/or cell damage can simultaneously generate an ADM response without *Kras* activity. Although a number of studies have shown that metaplastic cells in pancreatitis settings eventually resume an acinar cell phenotype,[33,34] the presence of *Kras* mutations efficiently redirects metaplastic cells toward PanIN development.[15,35] These results, together with the observation that chronic pancreatitis increases the risk for PDA,[36] underscore the relevance of ADM in pancreatic tumorigenesis and the need to elucidate the molecular mechanisms that regulate the fate of metaplastic cells.

In this study, we adopted a collagen matrix 3D culture assay to model the conversion of mature acinar cells to ductal metaplastic cells within a compressed timeframe (5 days vs months). As expected, expression of the *Kras[G12D]* oncogene promoted formation of duct-like cysts, with an efficacy similar to treatment of wild-type acinar cells with exogenous TGFα.[4,19,21] Further analysis confirmed that the starting cell preparation was highly enriched for amylase-expressing acinar cells, which converted to K19-expressing ductal cells in the presence of *Kras[G12D]*. Using inhibitors that specifically blocked individual signaling components, we also demonstrated that Raf/MEK/ERK represent a critical downstream effector pathway through which KRAS operates to induce ADM. Interestingly, *Braf[V599E]* and *Kras[G12D]*, the predominant *raf* and *ras* gene mutations, respectively, in pancreatic cancer operate in a mutually exclusive pattern in pancreatic tumors, suggesting that upstream (*Kras[G12D]*) or downstream (*Braf[V599E]*) effectors of the Raf/MEK/ERK pathway exhibit interchangeable functions in converting acinar cells to ADM lesions.[37] Recent lineage-tracing studies of primary human acinar cells also revealed that surviving acinar cells undergo an ADM response that is dependent on MAPK activity.[38] Likewise, we have found that TGFα-induced ADM is also dependent on the Raf/MEK/ERK pathway (unpublished results), revealing that these downstream effectors represent a common regulator of ADM in mouse and human Kras-dependent and Kras-independent settings. Future studies will test if individual dominant-negative mutants of the MAPK pathway similarly block ADM in models of tumorigenesis and pancreatitis.

While these studies were in progress, Heid et al.[21] reported that acinar cells isolated from *Ptf1a[Cre/+]; LSL-Kras[G12D]* mice were also capable of producing ductal cysts in 3D culture. In addition, conditional deletion of *Rac1* significantly blocked ductal cyst formation *in vitro* and PanIN development *in vivo*. This was a surprising finding as Rac1 is often thought to be activated by the PI3K pathway, which we showed by selective inhibition to be nonessential for ADM formation. However, a number of studies
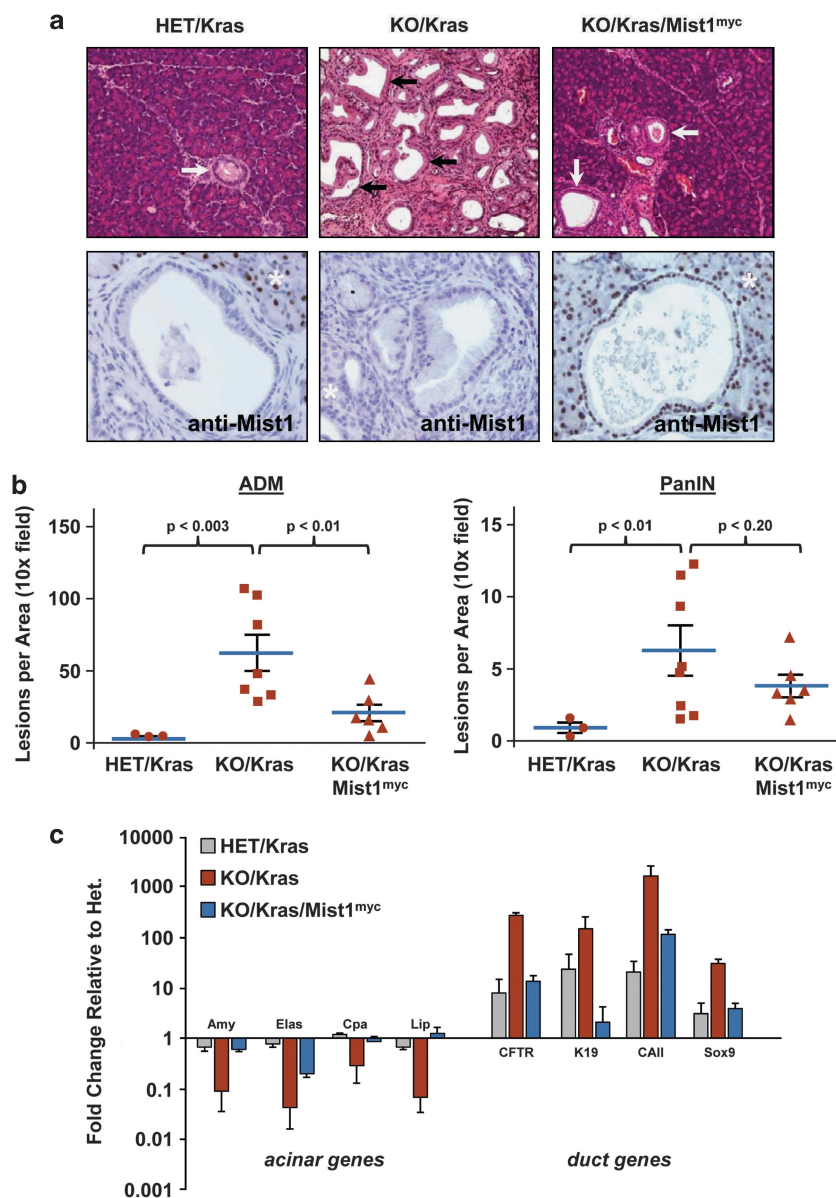
Figure 6. Sustained Mist1^myc^ expression inhibits ADM and PanIN formation *in vivo*. (**a**) PanIN formation (arrows) is greatly accelerated in KO/Kras pancreata but suppressed by sustained *Mist1^myc^* expression in KO/Kras/Mist1^myc^ mice. Acinar-derived PanINs are Mist1-negative in HET/Kras mice. Interestingly, induced *Mist1^myc^* expression alone is not sufficient to inhibit all PanIN formation as some MIST1^myc^+ PanINs still develop in KO/Kras/Mist1^myc^ pancreata. Asterisks, adjacent acinar tissue. (**b**) Quantification of ADM and PanIN lesions in HET/Kras, KO/Kras and KO/Kras/Mist1^myc^ samples. Note that constitutive *Mist1^myc^* leads to significant reductions in ADM/PanIN formation. (**c**) Analysis of gene expression profiles by reverse transcriptase–qPCR confirms the reduced ADM/PanIN formation in KO/Kras/Mist1^myc^ samples when compared with KO/Kras littermates. All values were normalized to the control HET samples, which were set to 1.0.

have shown that Rac can also be activated in a PI3K-independent manner via the Raf-specific guanine exchange factor Tiam1.[39,40] Importantly, *Tiam1* null mice are resistant to Ras-induced tumor formation,[41] confirming the importance of the Tiam1-Rac1 axis for the Ras signaling pathway. Thus, our study and the work by Heid *et al*.[21] strongly suggest that there are two key pathways (MAPK and Rac1) that are instrumental in *Kras*-induced ADM and that both pathways are essential to converting normal acinar cells into ADM and PanIN lesions. Future PDAC therapeutic strategies that target both pathways are likely to have higher efficacy than approaches targeting only a single effector.

Are there other ways to block ADM? We can find some leads in studies of pancreatitis, where most ADM spontaneously heals and re-differentiates in the absence of *Kras^G12D^* signaling. For example, β-catenin activation following acute pancreatitis is required for acinar cell regeneration, while stabilized β-catenin protects acinar cells against *Kras*-induced PanIN formation.[16] Similarly, *Mist1* exhibits a protective role not only in *Kras^G12D^*-induced ADM but also in ADM associated with acute pancreatitis, where *Mist1* null pancreata endure more severe damage following caerulein treatment.[42] At this time, it is unclear how *Mist1* influences ADM decisions. One possibility is that MIST1 protects acinar cells from metabolic stress associated with pancreatitis or *Kras^G12D^* expression by regulating aspects of the MAPK pathway. Indeed, *Mist1* is silenced in pancreatitis-associated acinar cells (unpublished results) and *Mist1^KO^* acinar cells exhibit increased MEK and ERK phosphorylation.[4] Given the central role of the MAPK pathway in ADM and the observation that pancreatitis enhances *Kras^G12D^*-induced tumorigenesis,[15,35,43] it is likely that *Mist1* inhibits ADM by maintaining basal levels of these MAPK

effectors. As *Mist1* is influential in converting embryonic stem cells to an acinar cell fate,[44] sustained *Mist1* expression also likely keeps key acinar characteristics intact in *Kras*[G12D] cells. Further investigation into the mechanisms of this regulatory network and identification of MIST1 target genes should lead to better therapeutic interventions and improved clinical outcomes for PDAC patients.

MATERIALS AND METHODS

Mouse strains and genotyping
Mist1[CreER/+], *LSL-Kras*[G12D/+], *R26R*[LacZ] and *R26R*[mTmG] reporter mouse lines have been described previously.[4,13,45] *Elastase*[pr]*-CreER* mice were generated according to the standard protocols using a previously reported Elastase[pr]-Mist1[MB] construct where the Mist1[MB] coding region was replaced by a CreER[T2] coding region to drive acinar-specific expression of CreER[T2].[46] *LSL-Mist1*[myc] transgenic mice were generated using the *pCAG-LoxP-CAT-LoxP-LacZ* construct in which the *LacZ* gene was replaced by a myc-tagged *Mist1* coding sequence.[47] Induction of CreER[T2] activity was accomplished by providing adult mice (6–9 weeks) TM (4 mg/mouse/day) for 2–3 consecutive days. Genotyping primer sets are listed in Supplemental Table I. All animal studies were conducted in compliance with NIH and the Purdue University IACUC guidelines.

3D acinar cell culture
Mice were treated with TM 7 days before harvesting the pancreas. Immediately after sacrificing, pancreata were rinsed in cold 1X Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) and cut into small pieces with scissors. Primary acini were released by collagenase P (Roche Applied Science, Mannheim, Germany) digestion (200 μg/ml in 1X HBSS) for 10–20 min at 37 °C. Isolated acini were washed three times in cold 5% fetal bovine serum, 1X HBSS and then filtered sequentially through 500- and 105-μm nylon meshes (Spectrum Laboratories, Rancho Dominguez, CA, USA). Cell suspensions were carefully layered on top of 30% fetal bovine serum, 1X HBSS and acini were collected by centrifugation (1000 r.p.m., 2 min at 4 °C) and then resuspended in 8 ml of 3D culture base medium (RPMI, 10% fetal bovine serum, 0.1 mg/ml soybean trypsin inhibitor, 1 μg/ml dexamethasone and antibiotics). 24-Well tissue culture plates were coated with a 250 μl/well collagen layer (100 μl 10X RPMI, 900 μl 3 mg/ml collagen, neutralized with either 4.2% NaHCO₃ or 0.34N NaOH) at least 1 h before acini isolation. The cell suspension was then mixed with collagen 1:1 and plated (0.5 ml/well). The cell–collagen mix was allowed to solidify for 1 h at 37 °C before adding 1 ml 3D culture media. Media was changed on days 1 and 3. On day 5, the percentage of acini that converted to ductal cysts was calculated by counting individual clusters in all wells. Collagen matrix was then digested for 10–15 min at 37 °C with 200 μg/ml collagenase P in 1X HBSS to release cells. RNA was prepared using the E.Z.N.A. total RNA kit (Omega Bio-Tek, Norcross, GA, USA). Alternatively, collagen discs were fixed in 4% formaldehyde, embedded in paraffin and 5-μm sections were prepared for standard immunohistochemistry as described below.

Histology and immunohistochemistry
Mouse pancreata were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned to 5 μm and stained using conventional hematoxylin and eosin. Sections were deparaffinized and retrieved using the 2100-Retriever (PickCell Laboratories, Amsterdam, The Netherlands) and antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA). Samples were blocked using the MOM blocking reagent (Vector Laboratories) and incubation of primary antibodies was conducted for 1 h at room temperature or overnight at 4 °C. Biotinylated secondary antibodies were applied for 10 min at 25 °C. Visualization was accomplished via 3,3'-diaminobenzidine peroxidase staining or tertiary avidin-conjugated fluorescent antibodies. Primary antibodies and conditions are provided in Supplemental Table II.

Protein immunoblots
In all, 20 μg of whole-cell protein extracts were separated on 12% acrylamide gels, transferred to polyvinylidene difluoride membranes and incubated with primary antibodies (antibody conditions are provided in Supplemental Table III). Immunoblots were developed using an ECL kit (Thermo Scientific, Waltham, MA, USA).

Quantitative reverse transcriptase–PCR gene expression analysis
Pancreas RNA or RNA from 3D cultures was isolated using the E.Z.N.A. total RNA kit and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Equal amounts of cDNA reactions were amplified with FastStart Universal SYBR Green Master (Roche Applied Science, Indianapolis, IN, USA) using the primer sets listed in Supplemental Table IV. Target sequences were amplified with 95 °C/30 s, 59 °C/60 s and 72 °C/30 s conditions. Fold changes in gene expression between TM-treated and control animals or between day 0 and day 5 3D cultures were calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Error bars represent the s.e.m.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)