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Loss of Activin Receptor Type 1B Accelerates Development of Intraductal Papillary Mucinous Neoplasms in Mice With Activated KRAS

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Abstract

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Background & Aims—Activin, a member of the transforming growth factor-β (TGFB) family, might be involved in pancreatic tumorigenesis, like other members of the TGFB family. Human pancreatic ductal adenocarcinomas contain somatic mutations in the activin A receptor type IB (*ACVR1B*) gene, indicating that ACVR1B could be a suppressor of pancreatic tumorigenesis.

Methods—We disrupted *Acvr1b* specifically in pancreata of mice (*Acvr1b*$^{flox/flox}$;*Pdx1-Cre* mice) and crossed them with LSL-KRAS^{G12D} mice, which express an activated form of KRAS and develop spontaneous pancreatic tumors. The resulting *Acvr1b*$^{flox/flox}$;*LSL-KRAS*^{G12D};*Pdx1-Cre* mice were monitored; pancreatic tissues were collected and analyzed by histology and immunohistochemical analyses. We also analyzed *p16*$^{flox/flox}$;*LSL-Kras*^{G12D};*Pdx1-Cre* mice and Cre-negative littermates (controls). Genomic DNA, total RNA, and protein were isolated from mouse tissues and primary pancreatic tumor cell lines and analyzed by reverse transcriptase PCR, sequencing, and immunoblot analyses. Human intraductal papillary mucinous neoplasm (IPMN) specimens were analyzed by immunohistochemistry.

Results—Loss of ACVR1B from pancreata of mice increased proliferation of pancreatic epithelial cells, led to formation of acinar to ductal metaplasia, and induced focal inflammatory changes, compared with control mice. Disruption of *Acvr1b* in *LSL-KRAS*^{G12D}; *Pdx1-Cre* mice accelerated growth of pancreatic IPMNs, compared with *LSL-KRAS*^{G12D};*Pdx1-Cre* mice, but did not alter growth of pancreatic intraepithelial neoplasias. We associated perinuclear localization of the activated NOTCH4 intracellular domain to the apical cytoplasm of neoplastic cells and with expansion of IPMN lesions in *Acvr1b*$^{flox/flox}$;*LSL-KRAS*^{G12D};*Pdx1-Cre* mice. Loss of the gene that encodes p16 (*Cdkn2a*) was required for progression of IPMNs to pancreatic ductal adenocarcinomas in *Acvr1b*$^{flox/flox}$;*LSL-Kras*^{G12D};*Pdx1-Cre* mice. We also observed progressive loss of p16 in human IPMNs of increasing grades.

Conclusion—Loss of ACVR1B accelerates growth of mutant KRAS-induced pancreatic IPMNs in mice; this process appears to involve NOTCH4 and loss of p16. ACVR1B suppresses early stages of pancreatic tumorigenesis; the activin signaling pathway might therefor be a therapeutic target for pancreatic cancer.

Keywords

tumor suppressor; TGF-β; superfamily; pancreas; PDA

INTRODUCTION

Pancreatic intraepithelial neoplasias (PanINs) and pancreatic intraductal papillary mucinous neoplasms (IPMNs) are the two most common precursor lesions that can progress to invasive pancreatic ductal adenocarcinoma (PDA). PanINs arise in small ducts and are graded in three categories based on the degree of dysplasia: low-grade PanIN-1A and 1B, intermediate-grade PanIN-2, and high-grade PanIN-3. Clinically IPMNs can be subtyped based on their location within the duct system into main duct, branch duct, and mixed[1]. Histologically, IPMNs can be further classified into three main subtypes: gastric foveolar type, intestinal type, and pancreatobiliary type. IPMNs typically are abundant mucin producing premalignant neoplasms that are grossly and radiographically identifiable[2]. In contrast to PanINs, which are difficult to identify and monitor clinically, IPMNs represent a

unique opportunity to block the progression of pancreatic tumor before an incurable invasive cancer develops.

Genetically engineered mouse models (GEMMs) have established a robust platform for exploring the molecular mechanisms underlying the progression of these precancerous lesions to invasive PDA[3, 4]. For example, using Pdx1-Cre to activate mutant *Kras*^{G12D} allele in the pancreatic progenitor cells induces full spectrum of premalignant mPanIN lesions that can eventually progress to invasive PDA[5]. Concomitant inactivation of the *tumor suppressors p16, p19*, *p53,* or TGF-β receptor type 2 (*TgfβR2*) can synergize with oncogenic *Kras*^{G12D} in promoting the progression of the non- invasive mPanINs to invasive cancer *in vivo*[6–10]. Interestingly, concomitant deletion of *Smad4*, a common downstream mediator of TGFB/activin/BMP signaling pathways, favors the development of mucin-producing precursor lesions (IPMNs or mucinous cystic neoplasms [MCNs]), and the progression of these mucin-producing precursors to invasive cancer was the dominant route of tumorigenesis in these mice[11–13]. These mouse modeling studies not only have demonstrated PanINs, IPMNs, and MCNs are precursors to invasive pancreatic cancer, but also support that disruption of a specific tumor suppressive pathway could favor the development of a particular precursor lesion and lead to distinct *KRAS*-driven tumorigenesis sequence in the pancreas. Specifically, the inactivation of *TgfβR2* signaling accelerated the PanIN to PDA sequence in the context of KRAS-induced tumorigenesis seemly through a SMAD4-independent manner, because deletion of its downstream gene *Smad4* promoted a distinct *Kras*-induced tumorigenesis route from IPMN/MCN to carcinoma. We therefore hypothesized that other ligands of the TGFB superfamily, such as activin, might be responsible for the reported SMAD4-dependent IPMN/MCN phenotype.

Increasing evidence indicate that activin signaling plays crucial roles in human tumorigenesis[14, 15]. We previously described somatic biallelic inactivation of the activin A receptor, type IB (*ACVR1B*) gene in human pancreatic cancer[16], suggesting that *ACVR1B* may play a tumor suppressive role in pancreatic carcinogenesis. To further demonstrate the role of activin signaling in pancreatic tumorigenesis *in vivo*, we have generated a conditional *Acvr1b* mouse line to circumvent the embryonic lethality resulted from conventional *Acvr1b* knockout[17]. Here, we report that pancreas-specific *Acvr1b* deletion alone (*Acvr1b*$^{flox/flox}$*;Pdx1-Cre*) resulted in focal inflammatory-like lesions and acinar to ductal metaplasia (ADM) in mice beyond 8 months-old. In combination with activated *Kras*^{G12D} expression, loss of ACVR1B preferentially promoted the development of pancreatic IPMNs but not PanINs. Additional mutations of tumor-suppressor genes such as *p16* appeared to be required for the progression of these IPMNs to PDA. This *in vivo* study further demonstrates that ACVR1B signaling plays a tumor suppressive role in the pancreatic tumorigenesis, which functions distinctively from TGFB receptors, specifically, in the development of IPMN-derived PDA.

Materials and Methods

Generating mouse lines

Acvr1b$^{flox/flox}$ mice (C57BL/6)[17] were mated with *Pdx1-Cre* or *LSL-Kras*^{G12D} (129/SvJae/C57BL/6) mice[5, 9] to generate *Acvr1b*$^{flox/flox}$*;Pdx1-Cre*, *Acvr1b*$^{flox/flox}$*;LSLKras*^{G12D}*;*

Pdx1-Cre (AKP), *LSL-Kras^{G12D};Pdx1-Cre* (KP), and Cre-negative littermate controls (*Acvr1b$^{flox/flox}$;LSL-Kras^{G12D}, LSL-Kras^{G12D}*, and *Acvr1b$^{flox/flox}$* mice) in multistep processes. The *p16$^{flox/flox}$;LSL-Kras^{G12D};Pdx1-Cre* mice (PKP) were generated as previously described[9]. All mice were housed in the Animal Care Facility at Columbia University Medical Center (CUMC). And the studies were conducted in compliance with the CUMC IACUC guidelines.

Molecular and cellular analyses

Mouse pancreatic tumors were resected and cultured to establish primary pancreatic tumor cell lines as previously described[9]. Genomic DNA, total RNA and protein of mouse tissues and primary pancreatic tumor cell lines were extracted for subsequent PCR, RT–PCR, sequencing, and western blotting as described in the same publication[9, 17]. RNA-Seq analyses were performed on AKP (n=3) and KP (n=3) tumor cell lines at the Columbia Genome Center[18–20]; 1130 genes were differentially expressed between the two genotypes (adjusted p-value <0.05). Details of the RNA-Seq analyses and the antibodies for western blot analyses are described in the Supplementary Methods.

Histology and tissue analyses

Murine issues were fixed either in 10% neutral buffered formalin overnight, and embedded in paraffin. Routine H&E staining was done by the Histology Service Core Facility at CUMC. IPMNs are defined as mucinous cystic papillary proliferations involving main or branch ducts with a diameter larger than 1mm. IPMNs in this model are grossly visible cystic lesions. Immunohistochemistry (IHC), BrdU incorporations, and TUNNEL assays were performed as previously described[17]. More details including antibodies are listed in the Supplementary Methods.

The levels of activin A in serum and unfixed pancreata were measured by ELISA assay from *Acvr1b* mutant and wild-type sibling at the age of two to three months (n=8 per group). The Quantikine ELISA Kit for mouse activin A was purchased from R&D systems.

The acquisition of the human IPMN tissue specimens was approved by the Institutional Review Board of CUMC and performed in accordance with Health Insurance Portability and Accountability Act. The details on these human IPMN specimens can be found in our previous publication[30]. The IHC were scored independently by two pathologists (H.E.R. and A.T.T.).

Statistical analysis

Data were expressed as mean ± SD. Paired two-tailed *t* tests were used to analyze differences in mouse lesion incidence, frequency, size, BrdU and Ki67 counts analysis.

RESULTS

Acvr1b deletion induces focal inflammatory and metaplastic changes in the pancreas

The pancreas-specific ACVR1B deficient mice (*Acvr1b$^{flox/flox}$;Pdx1-Cre;* referred to as AP mouse hereafter)[17] (Figure 1A) were live-born at the expected frequency. Their pancreata

appeared to be grossly normal at macroscopic and microscopic levels at early ages, exhibiting normal pancreatic architecture and appropriate mature lineage-specific markers (Figure S1). However, focal chronic inflammatory changes appeared in the pancreata of the AP mice beyond eight months of age, which were characterized by periductal stromal proliferation, infiltration of lymphocytes, ADM, ductal proliferation, and fibrosis (26 out of 36 mice, Figure 1B). None of these alterations were detected in the ACVR1B wild-type control animals (Cre-negative littermates) of comparable ages (n=36).

The IHC analyses showed that the vast majority of the infiltrating immune cells in the ACVR1B deficient pancreata were $CD3^+$ T cells and $Gr\text{-}1^+$ monocytes (Figure 1C). Few macrophages and $CD20^+$ B cells were also noted among the infiltrating immune cells. Significantly higher levels of activin A were detected in the pancreata and sera of the mutant mice by ELISA (Figure 1D).

The AP mice were monitored up to 23 months (n=5). Histological analysis of the aged AP mice revealed low-grade PanIN lesions associated with chronic inflammatory changes, but no high-grade PanIN lesions or carcinomas. These findings indicate that *Acvr1b* deletion alone is insufficient to cause pancreatic tumor formation.

Acvr1b deletion synergizes with oncogenic $Kras^{G12D}$ in shortening animal survival and the development of invasive cancer

To investigate the role of activin signaling in pancreatic tumorigenesis in the context of mutant KRAS, we generated $Acvr1b^{flox/flox}$;$LSL\text{-}Kras^{G12D}$;*Pdx1-Cre* mice (referred to as AKP mouse henceforth). AKP had a median survival of approximately seven months (218±94 days), significantly shorter than the control animals (Figure 2A) and $LSL\text{-}Kras^{G12D}$;*Pdx1-Cre* mice (> 365 days; referred to as KP mouse hereafter)[7, 13].

Macroscopically, the pancreata of AKP mice were typically firm and irregular in shape with multilobulated mucin producing cystic tumor masses involving the entire pancreas (Figure 2B). Tiny cysts were visible mostly in the tail or body of the pancreata as early as one month of age (Table S1). Large cysts (14 to 30 mm in diameter) containing transparent or hemorrhagic fluid were frequently seen in mice >3-month-old (Table S1). The cystic neoplastic lesions were directly connected to the main pancreatobiliary ducts (Figure 2D). About 13% (5/39) of the mutant mice with invasive PDA were also found with visible liver and/or lung metastasis (Figure 2E).

Histologically, ADM, ductular proliferation, and active fibrosis were present already in AKP mice by 2 months of age. Large (>1mm) cystic complexes, composed of papillary, noninvasive epithelial proliferations with mostly low grade dysplasia were detected in AKP mice at and beyond two months of age (Figure 2F). These cystic ductal lesions, resembling human IPMNs, gradually replaced the pancreatic acinar parenchyma as the mutant mice aged. The lining epithelial cells of the cysts showed abundant mucin production with positive alcian blue staining (Figure S2A) and ductal differentiation with cytokeratin 19 immunostaining (Figure S2B). The occurrence of ADM was evident by the expression of acinar cell marker amylase in duct-like lesions (Figure S2C); while scattered endocrine cells were also found associated with the mucinous cystic complexes highlighted with glucagon

and insulin immunostaining (Figure S2D and E). In the stromal areas, active proliferation of myofibroblasts associated with cystic papillary neoplasms was demonstrated by positivity for α-smooth muscle actin (α-SMA) and desmin immunolabeling (Figure S2F and G). These stromal cells were negative for estrogen receptor and progesterone receptor by IHC analyses.

ACVR1B loss selectively expands oncogenic KRAS-initiated IPMNs

To further understand the impact of *Acvr1b* deficiency on the $Kras^{G12D}$–driven pancreatic tumorigenesis, we compared the AKP to KP mice at time points 2, 5, and 9 months of age. KP mice have been reported to develop full spectrum of PanIN formation, with a predominance of mPanIN-1A and mPanIN-1B lesions at 2-months of age, with increased mPanIN-2 and mPanIN-3 lesions at five months of age, and mPanIN-3 lesions and invasive carcinoma in 9-month-old mice[5]. In comparing to the KP mice, the pancreata of the AKP mice were generally larger starting at two months of age (Figure 3A), with significantly increased pancreatic tumor burdens at five and nine month time points (Figure 3C). Corresponding histologic analyses showed that the AKP mice always harbored larger areas of ADM, mucinous cystic papillary complexes, and active fibrosis in the pancreata than the KP mice at every time point (Figure 3B, Tables S1, S2). The rapid growth of the pancreatic tumor burden could be due to the significantly increased proliferation index of the pancreatic ductal epithelium in the AKP mice (Figure 3D–E).

Loss of ACVR1B in the pancreas particularly promoted the expansion of IPMNs in the context of oncogenic KRAS, which could be quantitated by the dramatic increase of alcian blue–positivity in the pancreata of the AKP mice (Figure 4A and B). To further prove that Acv1b deficiency might have led to the preferential expansion of the IPMNs over mPanINs, the pancreata of AKP and KP mice at two months of age were evaluated by a comprehensive scoring system that involved microscopic examination of all terminal ducts in the lobular units of the pancreas[3, 21]. A median of approximately 20 lobular units were analyzed for each animal. Lobular units were scored as normal or the highest grade of mPanIN identified. No significant difference in the percentages of mPanIN to total lobular units was observed between the two genotypes (Figure 4C). Dilatation of the ducts was scored by measuring the maximum linear diameter of a dilated pancreatic duct on the H&E stained sections. The KP mice had normal caliber pancreatic ducts with normal ductal epithelium and normal average diameter of the ducts 0.2mm. In contrast, the AKP mice had cystically dilated main and/or branched ducts with associated mucinous epithelium, showing an average increase of 6.5-fold in the diameter of the ducts (average at 1.3mm) (Figure 4D). The data demonstrate that ACVR1B inactivation selectively accelerated the formation of IPMNs initiated by mutant KRAS.

Immunolabeling with antibody for ACVR1B confirmed that ACVR1B protein was absent from IPMN-like lesions, but could be detected in some of the mPanIN-like areas (Figure 4E). Consistently, phosphorylation of SMAD2, which is a downstream mediator of ACVR1B signaling, was absent from the ACVR1B-negative IPMN lesions but detectable in the mPanIN lesions with ACVR1B expression (Figure 4E). Laser capture microdissection confirmed that ACVR1B-negative lesions had undergone Cre-induced recombination at the *Acvr1b* alleles and lost the exon 3 of *Acvr1b* (Figure 4F). The residual expression of

ACVR1B in some of the ductal lesions in this AKP GEMM reflects the mosaic recombination pattern of this particular Pdx1-Cre transgenic mouse line described previously[5, 22]. Thus, these data confirm that ACVR1B deficiency selectively enhanced KRAS-induced IPMNs.

NOTCH4 signaling pathway is involved in the IPMN development

The differentiation of the pancreatic progenitor cells has been shown to be tightly modulated by NOTCH signaling[23]. Overexpression and activation of NOTCH signaling pathways have been reported from PanINs to PDA[24, 25]. We therefore assessed the expression of NOTCH1, 2, 3, and 4 in the pancreatic tumor cells derived from our GEMM. Previously we have reported that the *p16$^{flox/flox}$;LSL-Kras^{G12D};Pdx1-Cre* mice (referred to as PKP mice henceforth) developed pancreatic tumors mimicking the human PanIN/PDA histologic presentations[9]. We found that the expression of NOTCH4 was more pronounced in the pancreatic tumor cell lines derived from the AKP GEMM, but significantly less from the KP and PKP mice (Figure 5A). Using an antibody specific for the intracellular domain (ICD) of the NOTCH4 protein, which could detect the activated form of NOTCH4 in the cytoplasm as well as in the nucleus[26], we observed upregulated NOTCH4 expression in the early ADM and IPMN lesions with a specific pattern of perinuclear expression in the AKP mice. This perinuclear NOTCH4 expression was not found in the mPanIN lesions of the KP and PKP mice (Figure 5B). This observation was duplicated in cultured AKP tumor cells, where TGF-β1 treatment increased the perinuclear accumulation of NOTCH4 proteins, but did not facilitate their translocation into the nucleus (Figure 5C). This IPMN-specific NOTCH4 expression pattern was also noted in human IPMNs but not PanINs (Figure 5D). These data were supported by RNA-Seq analysis comparing cultured KP *vs.* AKP tumor cells. NOTCH4 expression was significantly upregulated in AKP than KP tumor cells (Figure 5E), but there was no statistical significant differences in the expressions of *Notch1*, *Notch2*, or *Notch3* genes. Furthermore, three *Notch4* target genes (*Zdhhc14*, *Wdfy1*, and *Gbp1*)[27] were also upregulated along with *Notch4* as expected (Figure 5E), while none of the canonical NOTCH target genes such as *Hes* and *Hey* family[28] were differentially expression between AKP and KP tumor cells. These results suggest that NOTCH4 activation plays a unique role in IPMN tumorigenesis.

Previously we have reported that members of the oncogenic PI3K signaling pathway are commonly altered at genetic or expression level in human IPMNs[29, 30]. Consistent with this finding, we found that the downstream gene PDK1 (3-phosphoinositide-dependent protein kinase-1) was up-regulated in the high-grade IPMN lesions of the AKP mice by IHC (Figure S3A and B). Overexpression of Cox-2 is associated with many epithelial tumors including pancreatic cancer[31, 32]. We detected dramatic upregulation of Cox-2 in the mucinous ductal cells of the IPMN lesions (Figure S3C and D). In contrast, the nuclear immunolabeling of phosphorylated Stat3 Tyrosine 705 was exclusively observed in the ADM (Figure S3E and F), indicating that the activation of Stat3 might be a very early event in the IPMN development.

Additional p16 inactivation is required for the IPMN progression to PDA and metastasis

A serial histologic analysis conducted on the AKP mice from one to nine months of age revealed that invasive carcinoma could be detected as early as 82 days of age (Table S1). Among the 3–9 month-old age group, 72% (39/54) of the AKP mice developed invasive pancreatic carcinoma at the time of sacrifice (Table S1). In addition, 9% (5/54) of this age group showed signs of distant metastasis, such as bloody ascites and liver or lung metastasis (Figure S4). In contrast, only 17% (4/24) of the KP mice developed microscopically invasive disease during the same age range, and none was macroscopically observed to have ascites, liver or lung metastasis (Table S2) ($p<0.01$). Both the gross pathologic findings and histologic analyses ascertained that ACVR1B loss significantly promoted the IPMN-associated invasive diseases.

Using the primary pancreatic tumor cell lines established from the AKP GEMM, we confirmed that exons 2 and 3 of *Acvr1b* were completely deleted from tumor cells, but remained intact in the stromal fibroblast cells. And the mutant *Kras* allele was activated in all these cell lines (Figure 6A). Loss of the *Kras* wild-type allele, which is a frequent occurrence previously reported in the PDA and metastases of the PKP GEMM[9], was not observed in the AKP GEMM. As anticipated, the inactivation of the *Acvr1b* gene led to disrupted activin signaling pathway in the AKP pancreatic tumor cells, but not in the control cells with wild-type *Acvr1b* (from the PKP GEMM); and the TGFB/SMAD2 signaling axis was not affected by *Acvr1b* inactivation (Figure 6B).

No direct crosstalk could be inferred between activin and KRAS/RAF/MEK signaling by western blot analysis of KRAS downstream mediators Raf and MEK1/2 in AKP tumor cells with or without activin treatment (Figure 6B). By IHC, upregulated nuclear labeling of p53 and p19 were observed mainly in the early precursor lesions such as ADM and inflammatory nodules (Figure S5A–D). But no increased cell apoptosis was noted in these lesions (Figure S5E and F), indicating that the concurrent ACVR1B inactivation likely enabled the pancreatic epithelial cells to escape from apoptotic stress triggered by KRAS-induced p53/p19 upregulation in the initiating stage. The tumor-suppressor SMAD4, which is deleted in about 50% of human PDA, was retained in this GEMM (Figure 6A and B, S5G and H).

In contrast, the loss of p16 protein expression was universally observed in the primary pancreatic tumor cell lines from the AKP mice (Figure 6B). PCR analysis of genomic DNA revealed that the *p16* gene was deleted in these AKP tumor cells (Figure 6A). The loss of p16 is a late-event in tumorigenesis because p16 expression was detected at the low- to moderate-grade IPMN lesions in the AKP mice (Figure 6C). Progressive loss of p16 expression was also found to be associated with increasing grades in human IPMNs by IHC analysis (p=0.011) (Figure 6D, S6I and J). Together these data show that inactivation of *p16* is essential for the progression of IPMN to invasive and metastatic carcinomas.

DISCUSSION

Dysregulated activin signaling contributed to inflammation and metaplasia in the pancreas

We have demonstrated that when activin signaling was interrupted starting at E8.5–9 in the pancreas, all three pancreatic cell lineages developed fully, indicating that activin signaling

is only critical to pancreatic lineage development in early progenitor cells prior to Pdx1 expression. However, the ACVR1B-deficient pancreata exhibited increased spontaneous inflammation such as ADM formation, immune cells infiltrating into mesenchyme, stromal fibroblast proliferation, and formation of inflammatory cysts in aged mice. Activin A has been shown to be an important component of inflammatory response[33], and may induce the migration of those inflammatory cells[34]. While the precise molecular mechanisms that underlined the inflammatory changes in the AP mice remain to be elucidated, the significantly elevated levels of activin A, perhaps via a negative feedback triggered by the disruption of ACVR1B signaling, likely have contributed to these changes (Figure 1).

Loss of *Acvr1b* preferentially enhanced the expansion of pancreatic IPMN and the inactivation of *p16* is required for the progression of IPMN to invasive cancer

We previously reported *ACVR1B* as a bona-fide tumor-suppressor gene in human pancreatic cancer[16]. In the current study, we have shown that *Acvr1b* deletion alone was sufficient to induce the formation of focal ADM lesions (Figure 1) and the loss of *Acvr1b* synergized with oncogenic KRAS in promoting pancreatic tumorigenesis, dramatically boosting tumor burden and reducing survival (Figures 2, 3). These *in vivo* findings further support that *Acvr1b* functions as a tumor-suppressor gene in pancreatic tumorigenesis.

In the context of mutant *Kras*, loss of ACVR1B signaling selectively enhanced the reprogramming of KRAS-initiated IPMNs, but not mPanIN (Figures 3, 4). *In vitro* cultures of primary acinar cells from WT, AP, KP, and AKP mice revealed that comparable events of KRAS-induced ADM transdifferentiation occurred in the KP and AKP cultures, and both cultures consisted of significantly higher duct-like clusters (>90%) than the AP cells (13%) (Figure S6). The WT acinar cultures started to die by Day 6, and contained less than 5% of duct-like structures by Day 7 (data not shown). The low count of duct-like clusters in the AP cultures indicated that cell-intrinsic ADM could occur but at lower frequency, which is consistent to the *in vivo* data. Although similar numbers of duct-like units were counted in the KP and AKP cultures, their morphologies differed (Figure S7). Each AKP duct-like cluster was an aggregate of multiple small ducts, while most of the KP clusters consisted of one single duct (Figures S7, S8). This morphological difference might represent early ADM to PanIN *vs.* ADM to IPMN route in these two genotypes. However, further examination is needed to determine the cell origin question of IPMN in future studies. A recent Brg1-null IPMN GEMM reported that IPMN can come from duct cells[35]. There are some notable differences between our AKP GEMM and the Brg1-null IPMN GEMM- Unlike the Brg1-null IPMN GEMM, we observed an expansion of the IPMN lesions due to increased proliferation in these lesions, not diminishment of the PanIN lesions. It's possible that the two models may promote IPMN development through vastly different mechanisms.

In defining oncogenic signaling pathways uniquely related to the development of pancreatic IPMNs and commonly shared in both our GEMM and human IPMNs, we found that NOTCH4 overexpression was preferentially associated with pancreatic IPMN development (Figure 5). The unique pattern of apical perinuclear NOTCH4 localization suggests that NOTCH4 may be critical to the formation of IPMN papillary structures, since activated NOTCH components can co-localize with microtubules to maintain cellular polarity[36]. An

unbiased RNA-Seq analysis comparing cultured AKP and KP tumor cells revealed that *Notch4* along with three of its target genes[27] were preferentially upregulated in AKP cells at statistical significant levels (Figure 5E). The lack of significant change in the canonical NOTCH signaling target genes[28] suggests a compensatory regulation by the *Notch1, Notch2, Notch3* genes. The RNA-Seq data support the observation that NOTCH4 may play a unique role in IPMN development.

While activated KRAS alone is sufficient to initiate pancreatic tumorigenesis[5], additional inactivation of *p53* or *p16* is often necessary to overcome the oncogene-associated senescence for precancerous lesions to be fully transformed to malignant tumor cells[13, 37]. In this IPMN to invasive cancer model, p16 expression was observed in the early IPMNs but lost in the invasive cancer and metastases (Figure 6), suggesting that *Acvr1b* plays a tumor suppressive role in early tumorigenesis and the inactivation of *p16* is required to overcome KRAS-induced senescence for tumor progression to invasive disease in this GEMM.

TGF-β superfamily plays a decisive role in the progression route of pancreatic cancer: PanIN/PDA versus IPMN/PDA

Inactivating mutations of *SMAD4* have been reported in more than half of human PDAs[38]. It is commonly believed that *SMAD4* is targeted for inactivation to disrupt TGFB signaling during pancreatic tumorigenesis, while the role of activin signaling in the initiation, progression, and metastasis of pancreatic cancer is unexplored. A previous study by Ijichi *et al* has showed that mice with the combination of *Tgfbr2* deletion and mutant $Kras^{G12D}$ activation rapidly developed invasive PDA through the mPanIN path without the development of IPMNs[10]. In contrast, in the current study, the disruption of ACVR1B signaling concomitant with *Kras* activation favored the development of IPMNs. Similarly, loss of *Smad4* in the presence of oncogenic $Kras^{G12D}$ also has resulted in the development of pancreatic IPMN/MCN[11–13]. The overlapped phenotypes of our AKP and $Smad4^{flox/flox}$;*LSL-*$Kras^{G12D}$;*Pdx1-Cre* GEMMs are in stark contrast to the mPanIN/PDA observed in the $Tgfbr2^{flox/flox}$;*LSL-*$Kras^{G12D}$;*Pdx1-Cre* GEMM[10]. These findings support a model in which activin signaling inactivation selectively promotes the development of pancreatic IPMNs potentially in a SMAD4-dependent manner; while the inactivation of TGFB signaling would favor the PanIN/PDA path (Figure S8). These findings demonstrate that activin and TGFB signaling play crucial and distinct roles in the regulation of pancreatic tumorigenesis.

In summary, we provide *in vivo* evidence supporting that *Acvr1b* functions as a tumor-suppressor gene in pancreatic tumorigenesis and that the activin signaling pathway preferentially modulates the expansion and progression of pancreatic IPMNs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACVR1B	activin A receptor, type IB
ADM	acinar to ductal metaplasia
AKP	*Acvr1b*$^{flox/flox}$; *LSL-Kras*^{G12D}; *Pdx1-Cre* mice
BrdU	5-bromo-2-deoxyuridine
ELISA	enzyme-linked immunosorbent assay
GEMM	Genetically engineered animal model
ICD	intracellular domain
IHC	immunohistochemistry
IPMN	intraductal papillary mucinous neoplasms
KP	*LSL-Kras*^{G12D}*; Pdx1-Cre* mice
mPanIN	mouse pancreatic intraepithelial neoplasia
PanIN	pancreatic intraepithelial neoplasia
PDA	pancreatic ductal adenocarcinoma
PKP	*p16*$^{flox/flox}$*;LSL-Kras*^{G12D}*;Pdx1-Cre* mice
TGFB	transforming growth factor-β
TgfβR2	TGFβ receptor type II

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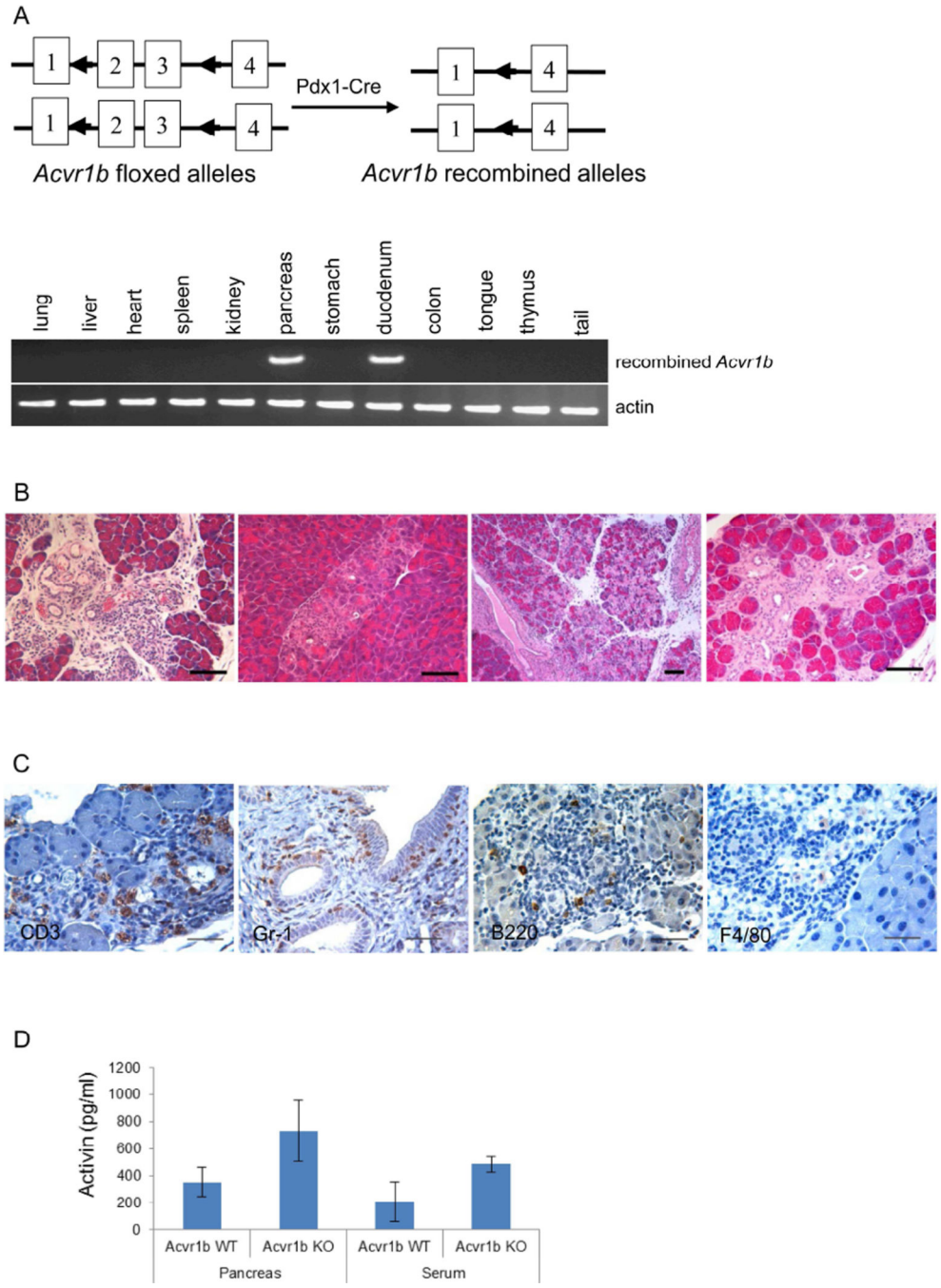


Figure 1. Conditional inactivation of ACVR1B in the pancreas induces chronic pancreatitis in the *Acvr1b*$^{flox/flox}$;*Pdx1-Cre* mutant mice

(A) The conditional floxed *Acvr1b* allele working scheme; and genomic PCR confirming Cre-induced recombination was restricted to the pancreas and duodenum of the mutant mice; (B) Representative inflammatory changes in AP mice- ductal proliferation and AMD in a mouse <9-month-old; focal ADM in a 12-month-old mouse; inflammatory lesions with marked inflammatory cell infiltration and ADM in a13-months-old mouse; and ductal proliferation and fibrosis in a 16-month-old mouse; (C) The inflammatory infiltrate was composed predominantly of CD3 positive T-cells and Gr-1 positive monocytes, with

scattered B220 positive B-cells and F4/80 macrophages in the pancreata of the AP mice. (D) Activin A level was significantly higher in the pancreata and sera of the mutant mice comparing to the control mice by ELISA assay (731.9±227.2 vs 353.4 ±106.7 pg/ml, n=8, p=0.0448; 484.8±59.2 vs 208.5±147.3pg/ml, n=8, p=0.0238, respectively). The scale bars in (B) represent 50 μm; scale bars in (C) represent 100 μm.

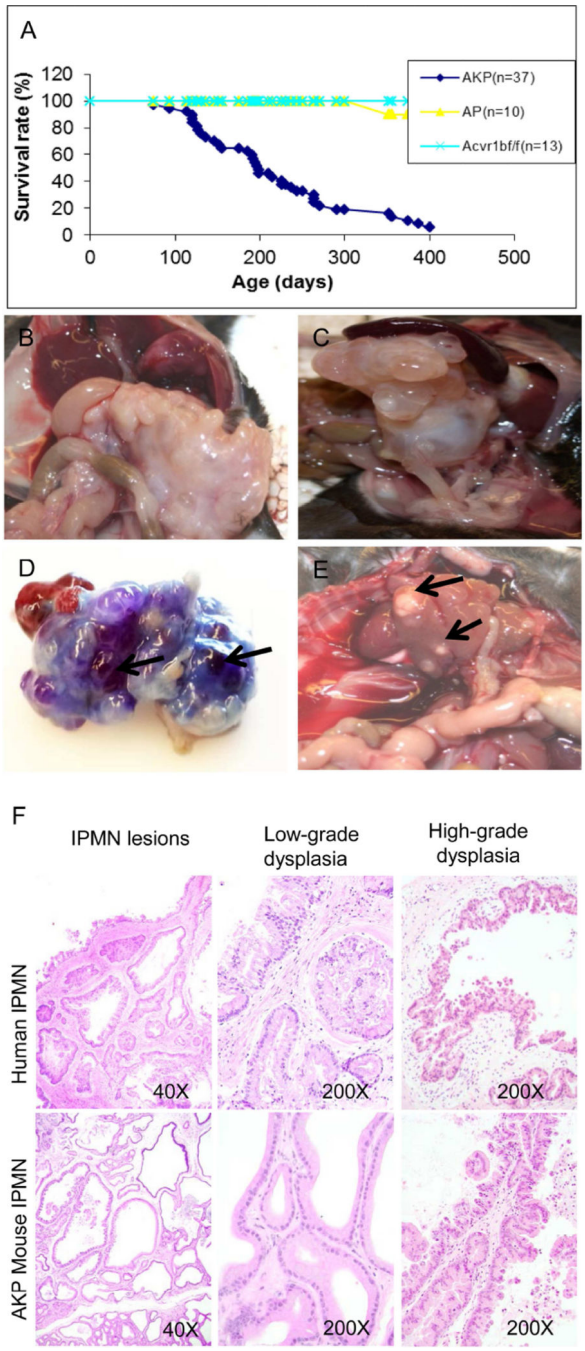


Figure 2. Inactivation of *Acvr1b* in the context of oncogenic *Kras*^{G12D} resulted in the development of IPMN-like lesions, invasive pancreatic tumors, and decreased survival

(A) Survival curve (Kaplan-Meier) of the AKP mice in comparison to AP and *Acvr1b*$^{flox/flox}$ mice; (B–C) are representative gross images of pancreatic IPMN tumors in the AKP mice; (D) 0.1% bromophenol blue dye was injected into the common bile duct and allowed to fill the duct system (common bile duct and pancreatic duct) in a retrograde manner. Blue dye was identified in the majority of the multilocular pancreatic cystic lesions in this 6 month old AKP mouse indicating a direct connection of the cystic neoplastic lesions to the main pancreatobiliary ducts; (E) some IPMN tumors were associated with invasive cancer and

with liver metastasis; (F) AKP mouse IPMN, multifocal branch duct and main duct with areas of low-grade dysplasia and high grade dysplasia, mimicking human IPMN, branch duct and main duct with areas of low-grade dysplasia and focal high-grade dysplasia.

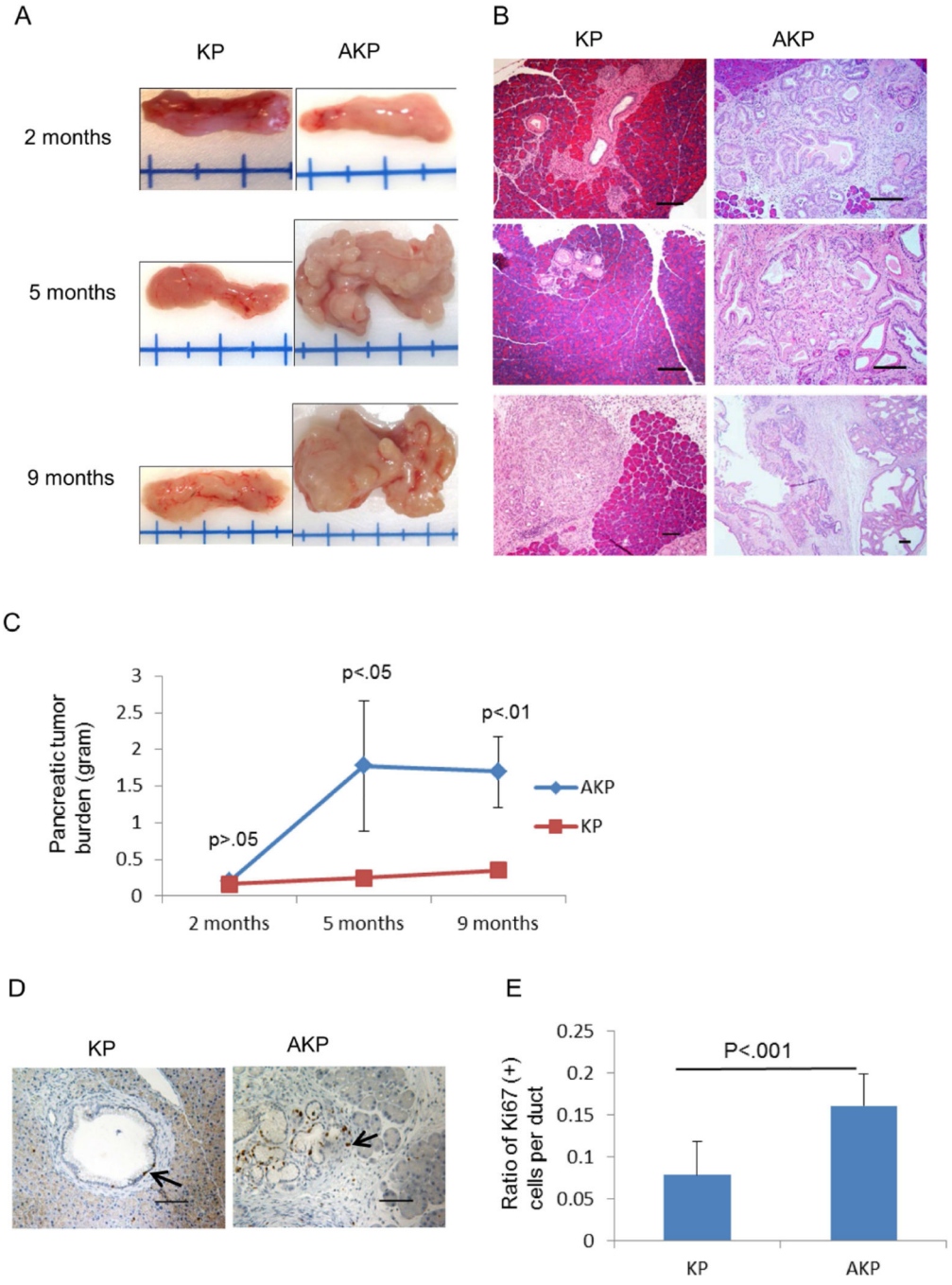


Figure 3. ACVR1B inactivation synergized with oncogenic KRAS in promoting pancreatic IPMN development and proliferation

(A) Gross analyses showed dramatic expansion of pancreatic tumor masses with age in the AKP mice compared to KP mice; (B) Histologic analyses revealed increased numbers of IPMN lesions in the AKP mice, which were rarely detected in the KP genotype; (C) increased pancreatic tumor burden in the AKP mice compared to the KP mice at 5 months (n=5, 1.77±0.89 *vs.* 0.25±0.05g, p=0.02) and at 9 months of age (n=4, 1.68±0.48 *vs.* 0.31±0.08, p=0.01); (D) The ratio of Ki67-positive cells to the total number of ductal cells in each low-grade dysplasia was used as a proliferative index of pancreatic ductal epithelial

cells. AKP mice exhibited a significantly higher proliferative index than KP mice at two months of age (n=4, 0.16±0.04 *vs.* 0.08±0.04, p=0.0001); (E) A representative Ki67 staining from each mouse model. Arrows indicate Ki67 positive epithelial cells. Scale bars indicate 100 μm.

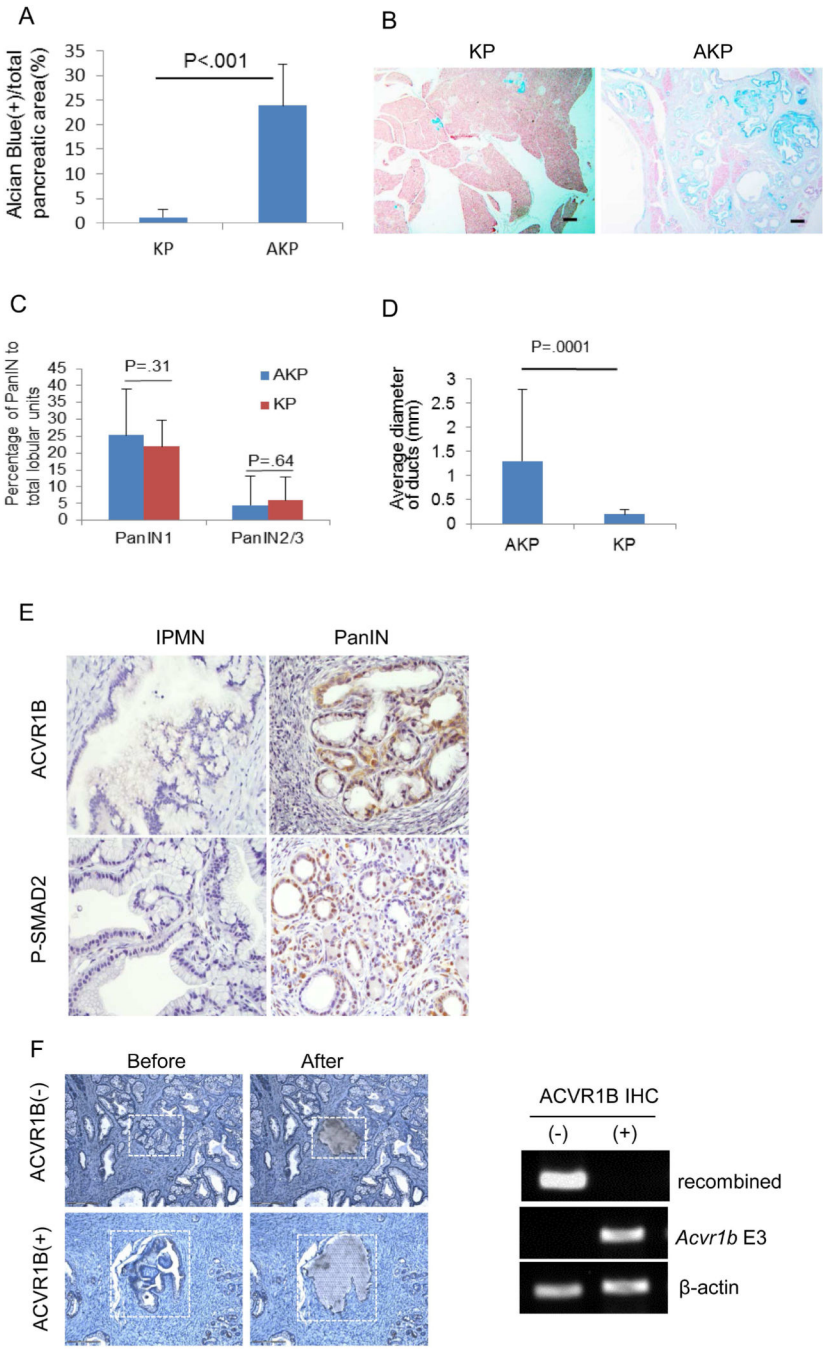
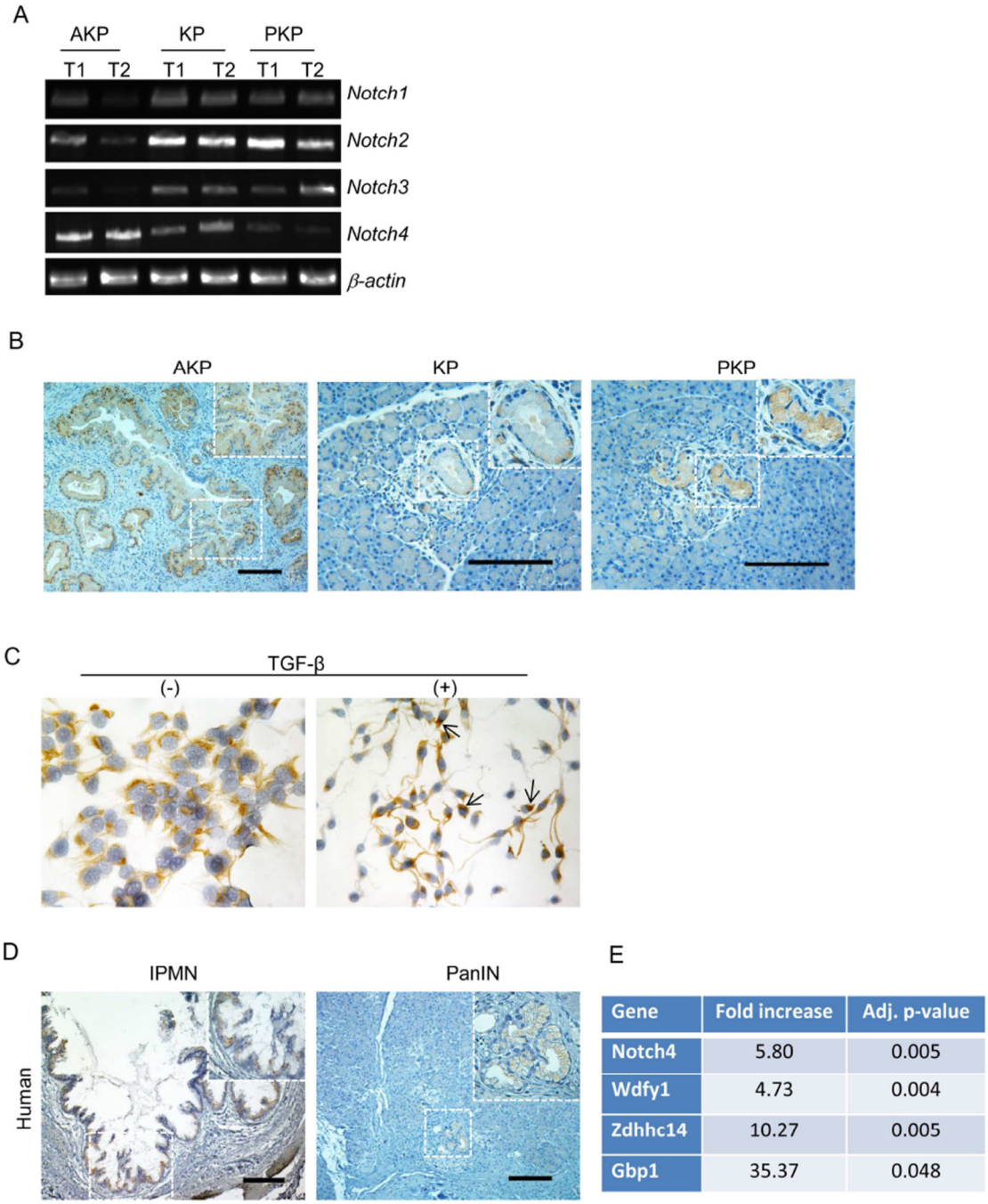


Figure 4. Disruption of activin signaling resulted in selective expansion of KRAS-induced IPMNs

(A) Quantification of Alcian blue-positive pancreatic area in 5-month-old of KP (n=6,1.2±1.6%) and AKP mice (n=6, 23.9±8.3%)($p<0.001$); (B) Representatives of Alcian blue staining in KP and AKP mice; (C) No significant impact observed on KRAS-driven mPanIN development comparing AKP *vs.* KP mice (mPanIN1: 25.35±13.51% *vs.* 22.02±7.78%, p=0.31; PanIN2/3: 4.4±8.8% *vs.* 6.04±6.9%, p=0.64); (D) Loss of ACVR1B significantly increased the dilation of the ducts in the AKP mice by 6.5-folds compared to in the KP mice (1.3±1.4 *vs.* 0.2±0.1, p=0.0001); (E) ACVR1B expression was absent from

IPMN-like lesions, but retained in some PanIN-like structures. Consistently, phosphorylation of Smad2 was not observed in the ACVR1B-negative IPMN lesions, but detectable in ACVR1B-expressing mPanIN in the AKP mice; (F) Laser capture microdissection confirmed that ACVR1B-negative lesions had undergone DNA recombination at the *Acvr1b* alleles and lost the exon3 of *Acvr1b*. Conversely, ACVR1B-expressing lesions retained exon3 without *Acvr1b* recombination. Scale bars indicate 100 μm.



E

Gene	Fold increase	Adj. p-value
Notch4	5.80	0.005
Wdfy1	4.73	0.004
Zdhhc14	10.27	0.005
Gbp1	35.37	0.048

Figure 5. NOTCH4 signaling is involved in the pancreatic IPMN development in mice and humans

(A) High expression of *Notch4* was detected in pancreatic tumor cells derived from the AKP mice while *Notch2* was preferentially upregulated in the PKP tumor cells using RT-PCR analysis; (B) A perinuclear granulation of cytoplasmic NOTCH4 expression was specifically identified in the pancreatic IPMN lesions in the AKP mice, but not in the PanINs predominantly found in the KP and PKP mice; (C) the perinuclear localization of NOTCH4 was duplicated *in vitro* in the AKP tumor cells. TGF-β1 treatment augmented the NOTCH4 expression but did not impact its localization; (D) This perinuclear granulation pattern of

NOTCH4 expression was also observed in the human IPMNs but not PanINs. Scale bars indicate 100 μm. (E) RNA-Seq analyses comparing gene expressions between AKP and KP tumor cell cultures identified statistical significant preferential upregulation of *Notch4* and *Notch4* target genes in AKP tumor cells.

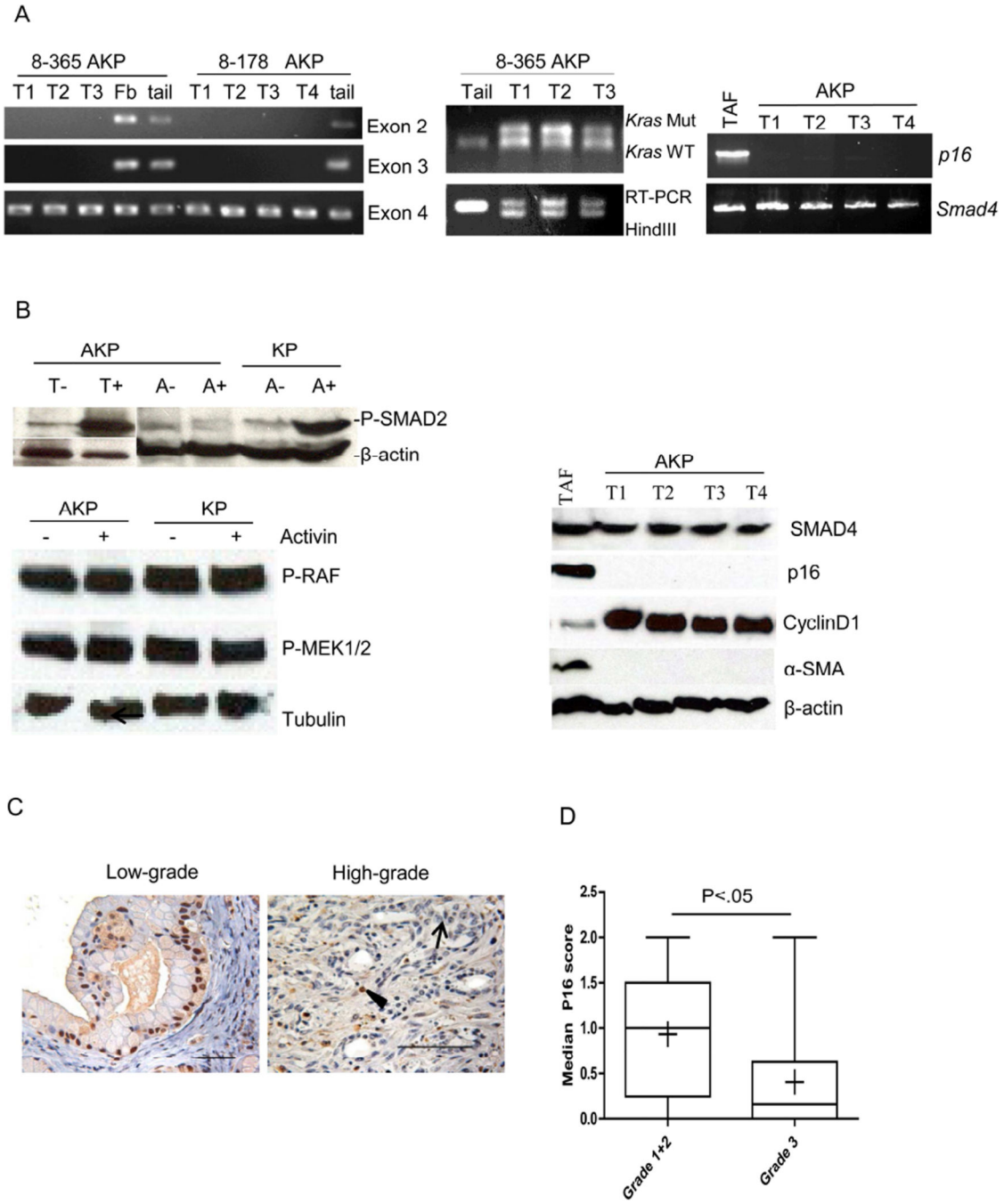


Figure 6. The loss of p16 was crucial for the progression of IPMN to PDA in both mice and humans

Molecular alternations were examined in pancreatic tumor cells derived from the AKP mice. (A) PCR analyses: In these tumor cells, exons 2 and 3 of the *Acvr1b* gene were completely excised from the genomic DNAs, but not in the tail DNAs of the AKP mice; the recombination of the $Kras^{G12D}$ mutant allele was detected at both the genomic DNA and RNA levels by PCR and RT-PCR analysis respectively; and the loss of p16 and retention of SMAD4 were detected at the genomic DNA level. (B) Western blot analyses: The phosphorylation of SMAD2 in response to activin A stimulation was abrogated in the AKP

pancreatic tumor cell lines, but their responses to TGF-β1 treatment remained intact; the loss of p16, retention of SMAD4, and upregulation of oncogenic Cyclin D1 proteins were observed; and no alteration in the KRAS/RAF/MEK signal pathway was observed in these cell lines. (C) IHC: In the AKP mice, cytoplasmic expression of p16 was initially upregulated in low-grade of IPMN lesion, but was lost in invasive carcinoma cells (arrow) while remained detectable in stromal cells (arrow head). (D) In humans, p16 loss is associated with nuclear grade of IPMN by IHC. Box-and-whisker plot shows average p16 staining intensity versus nuclear grade. The whiskers show the 5th and 95th percentiles. "+" denotes the mean. The middle bar shows the median. p=0.011. Scale bars indicate 100 μm in (C).