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► **To cite this version:**

David Vincent, Johann Gout, Nicolas Chuvin, Vanessa Arfi, Roxane M. Pommier, et al.. Tif1 γ Suppresses Murine Pancreatic Tumoral Transformation by a Smad4-Independent Pathway. American Journal of Pathology, 2012, 180 (6), pp.2214-2221. 10.1016/j.ajpath.2012.02.006 . hal-02954464

HAL Id: hal-02954464

<https://hal.science/hal-02954464>

Submitted on 1 Oct 2020

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Short Communication

Tif1 γ Suppresses Murine Pancreatic Tumoral Transformation by a Smad4-Independent Pathway

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Transcriptional intermediary factor 1 γ (TIF1 γ ; alias, TRIM33/RFG7/PTC7/ectodermin) belongs to an evolutionarily conserved family of nuclear factors that have been implicated in stem cell pluripotency, embryonic development, and tumor suppression. TIF1 γ expression is markedly down-regulated in human pancreatic tumors, and Pdx1-driven Tif1 γ inactivation cooperates with the *Kras*^{G12D} oncogene in the mouse pancreas to induce intraductal papillary mucinous neoplasms. In this study, we report that aged *Pdx1-Cre*; *LSL-Kras*^{G12D}; Tif1 γ ^{lox/lox} mice develop pancreatic ductal adenocarcinomas (PDACs), an aggressive and always fatal neoplasm, demonstrating a Tif1 γ tumor-suppressive function in the development of pancreatic carcinogenesis. Deletion of *SMAD4/DPC4* (deleted in pancreatic car-

cinoma locus 4) occurs in approximately 50% of human cases of PDAC. We, therefore, assessed the genetic relationship between Tif1 γ and Smad4 signaling in pancreatic tumors and found that *Pdx1-Cre*; *LSL-Kras*^{G12D}; *Smad4*^{lox/lox}; Tif1 γ ^{lox/lox} (alias, KSS1T) mutant mice exhibit accelerated tumor progression. Consequently, Tif1 γ tumor-suppressor effects during progression from a premalignant to a malignant state in our mouse model of pancreatic cancer are independent of Smad4. These findings establish, for the first time to our knowledge, that Tif1 γ and Smad4 both regulate an intraductal papillary mucinous neoplasm-to-PDAC sequence through distinct tumor-suppressor programs. (Am J Pathol 2012, 180:2214–2221; <http://dx.doi.org/10.1016/j.ajpath.2012.02.006>)

Transcriptional intermediary factor 1 γ (TIF1 γ ; alias, TRIM33/RFG7/PTC7/ectodermin) belongs to an evolutionarily conserved family of nuclear factors that have been implicated in stem cell pluripotency, embryonic development, and tumor suppression.¹ These proteins are characterized by the presence of a RING domain with E3 ubiquitin-ligase activity and multiple chromatin interaction domains, including B-boxes, coiled-coil domains, plant homeo domains, and bromodomains, suggesting functions in both protein degradation and modulation of

Supported by INSERM (Avenir program), the Centre National de la Recherche Scientifique (CNRS), fellowships from the Ministère de l'Enseignement Supérieur et de la Recherche of France (D.F.V., R.M.P., and D.R.) and the Association pour la Recherche sur le Cancer (D.F.V.), fellowships from the Ligue Nationale Contre le Cancer (J.G., V.A., and N.J.), and a grant from the NIH (R01 CA133557-01 to N.B.).

Accepted for publication February 7, 2012.

D.F.V. and J.G. contributed equally to this work.

The author R.L. is deceased.

This article is dedicated to the memory of our dear colleague, Régine Losson, who discovered the TIF1 family and passed away 2 years ago after a courageous fight against cancer.

Supplemental material for this article can be found at <http://ajp.amjpathol.org> or at <http://dx.doi.org/10.1016/j.ajpath.2012.02.006>.

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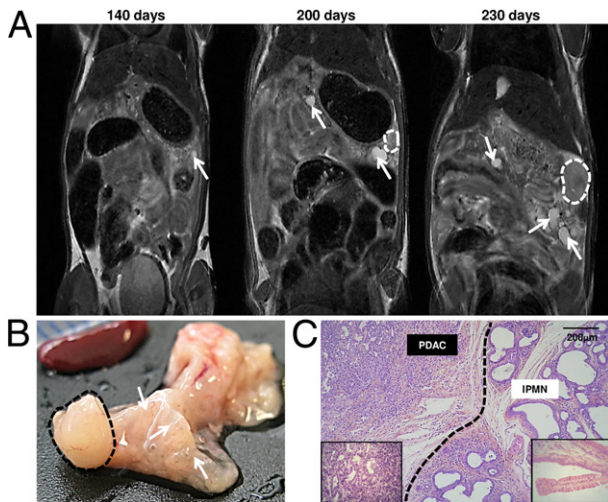


Figure 1. $Tif1\gamma$ deficiency cooperates with $Kras^{G12D}$ to drive an IPMN-PDAC sequence. **A:** Longitudinal magnetic resonance imaging study (from 140 to 230 days) of a KTT mouse. Cystic lesions (hypersignal, **arrow**) and a firm mass (isosignal, **dotted line**) are indicated. **B:** Macroscopic analysis of the KTT pancreas after dissection at 230 days. **Dotted line,** PDAC; **arrows,** IPMN lesions. **C:** Histological section of the KTT pancreas stained with hematoxylin-phloxin-saffron. The **dotted line** separates PDAC from the IPMN environment.

transcription. $TIF1\gamma$ interacts with transforming growth factor β (TGF- β) signaling SMAD transcription factors.²⁻⁵ Overall, the potential role of $TIF1\gamma$ in the TGF- β -SMAD signaling pathway is of high significance given the importance of this pathway in tumor suppression. TGF- β is a secreted polypeptide belonging to a wide family of cytokines and growth factors, including TGF- β s, bone morphogenetic proteins, and activins. TGF- β signaling involves two serine-threonine kinase receptors, type I and II TGF- β receptors. After binding to its receptors, TGF- β induces the phosphorylation of the type I TGF- β receptor, whose serine-threonine enzymatic activity is activated to allow the phosphorylation of receptor-regulated SMAD2 and SMAD3 (R-SMADs). Phosphorylated R-SMADs then interact with SMAD4. The R-SMADs-SMAD4 complex accumulates within the nucleus, binds to DNA, and activates the transcription of target genes. TGF- β also activates noncanonical pathways that are independent of SMAD proteins.

Pancreatic cancer is a particularly relevant context for exploring the interplay of $TIF1\gamma$ and TGF- β -SMAD function because this malignancy is characterized by a high rate of inactivating genetic alterations in the TGF- β -SMAD4 pathway. Pancreatic ductal adenocarcinoma (PDAC) is an aggressive neoplasm; it is always fatal and is the fifth most common cause of death by cancer in the Western world. The median age of patients diagnosed as having PDAC is 65 to 70 years. PDAC, which affects the exocrine pancreas, accounts for >80% of pancreatic cancers. PDAC arises from precursor lesions with ductal differentiated features. The precursors of the disease are divided into three groups: pancreatic intraepithelial neoplasm, intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm. Both human patients and mouse models with these types of lesions have an increased risk of developing a PDAC.

We previously developed a genetically engineered mouse model and showed that targeted pancreatic conditional inactivation of $Tif1\gamma$ cooperated with activated $Kras$ to induce IPMNs.⁶ Indeed, $Pdx1-Cre; LSL-Kras^{G12D}; Tif1\gamma^{lox/lox}$ mice (KTT) develop IPMNs with a short latency and a full penetrance ($n = 12$; age range, 19 to 189 days). However, none of these KTT mice presented obvious signs of malignant transformation reminiscent of PDAC.

In the present study, we explored the susceptibility of KTT aging mice (aged >200 days) to develop PDAC. We also investigated the impact of $Smad4$ deletion, which occurs in approximately 50% of human PDACs, in the IPMN-PDAC sequence resulting from combined $Kras$ activation and $Tif1\gamma$ deficiency in the murine pancreas.

Materials and Methods

Mice

$Tif1\gamma^{lox/lox}$,⁷ $LSL-Kras^{G12D}$,⁸ $Smad4^{lox/lox}$,⁹ and $Pdx1-Cre^{10}$ alleles, provided by other groups, were previously described. Mice were maintained in a specific pathogen-free animal facility at the AniCan Plateform, Centre Léon Bérard (Lyon, France), and handled in compliance with the institutional guidelines. All procedures were approved by an ethics committee under governmental regulatory authority (Comité d'Evaluation Commun au Centre Léon Bérard, à l'Animalerie de transit de l'ENS, au PBES et au laboratoire P4).

Histological Data

Histological experiments were performed as previously described.⁶ The primary antibodies used are: mouse monoclonal antibodies against $Tif1\gamma$ (1:500; Euromedex, Souffelweyersheim, France), Mucin-5ac (clone 45M1, 1:100; Thermo Scientific, Illkirch, France), chymotrypsin (1:1000; AbD Serotec, Colmar, France), E-cadherin (1:700; BD Biosciences, Le Pont-De-Claix, France), and proliferating cell nuclear antigen (1:100; Santa Cruz Biotechnology, Santa Cruz, CA); rat monoclonal antibodies against cytokeratin-19 (1:50, Troma-III-s; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and F4/80 (1:100; Acris Antibodies, Herford, Germany); goat polyclonal antibodies against vimentin (1:100; Santa Cruz Biotechnology); and guinea pig polyclonal antibodies against α -smooth muscle actin (α -SMA, 1:100; DakoCytomation, Trappes, France). For immunofluorescence, histological section cells were incubated with rabbit polyclonal anti-Glut2 antibody (1:100; Millipore, Molsheim, France) and, finally, with secondary-labeled antibody (goat anti-rabbit-IgG Alexa Fluor 488 nm, 1:200; Invitrogen, Cergy-Pontoise, France). Alcian blue staining was performed in acidic pH conditions.

Magnetic Resonance Imaging

The magnetic resonance imaging experiments were performed as previously described.⁶

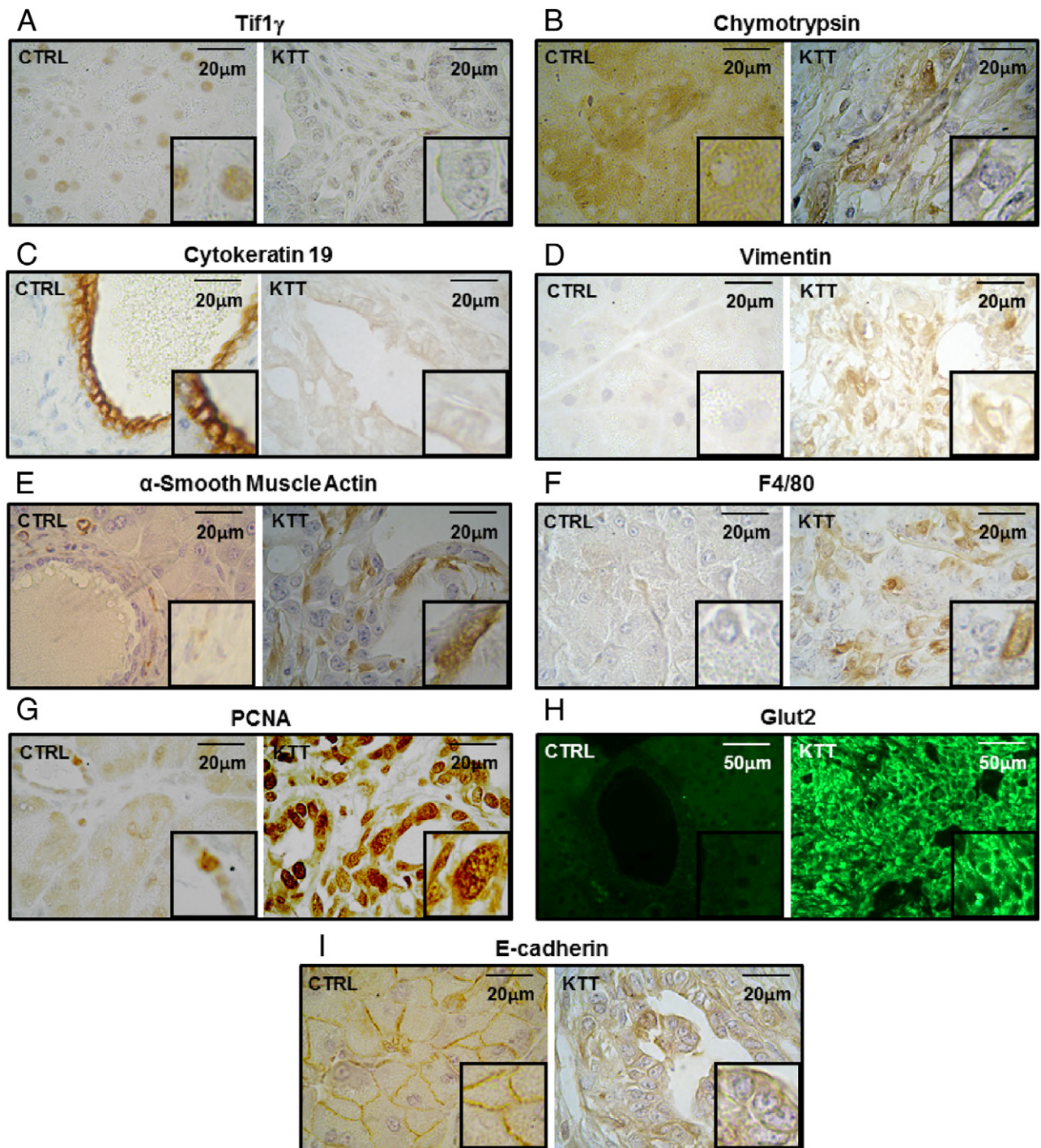


Figure 2. Histological characterization of KTT pancreatic tumors. IHC analysis reveals a complete loss of Tif1 γ expression in the epithelial compartment (A). KTT tumors exhibit an altered differentiation status both in the acinar (chymotrypsin, B) and ductal (cytokeratin-19, C) compartments. Markers of activated fibroblasts and/or myofibroblasts [vimentin (D) and α -SMA (E)] can be detected in the stroma of KTT tumors. Interestingly, some vimentin-positive, but α -SMA-negative, cells were seen lining the lumen, suggesting that some tumor cells express the vimentin marker. Tumor-associated macrophages are also seen in KTT tumors (F4/80, F). Proliferative index [proliferating cell nuclear antigen (PCNA), G] and metabolic activity (Glut2, H) markers are both highly overexpressed in KTT tumors. These PDACs present a dedifferentiated phenotype, generally associated with disorganization of E-cadherin (I) from membrane toward the inside cellular compartment. CTRL, control.

Statistical Analysis

Statistical analyses were performed using the Fisher exact test. Differences were considered significant when $P < 0.05$.

Results

We generated aging KTT mice that were euthanized between the age of 200 days and 1 year ($n = 3$). As described later, two of three of these mice presented with

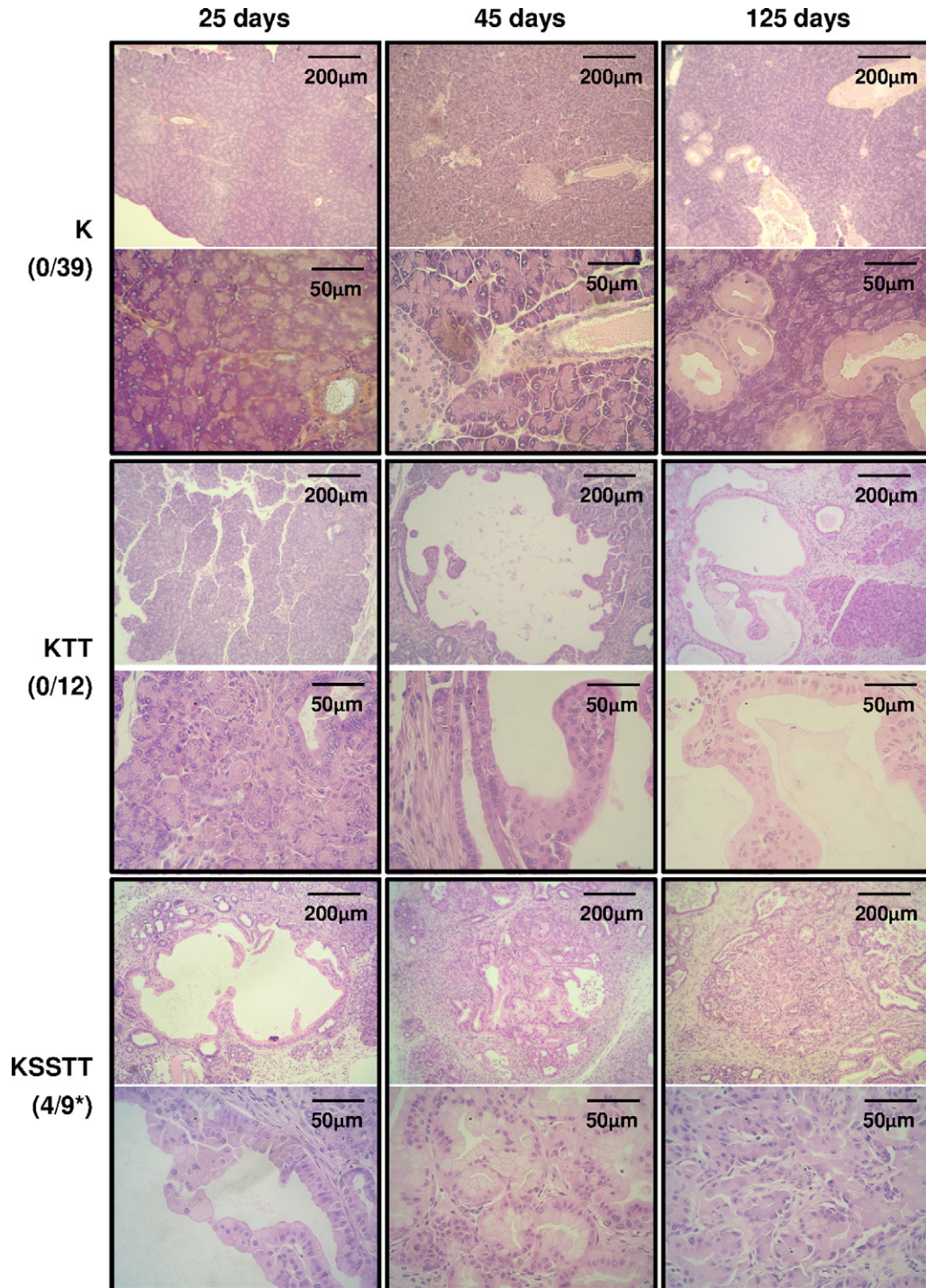


Figure 3. Deletion of *Smad4* in KTT mice accelerates PDAC development. Histological sections of K, KTT, and KSSTT mutant mouse pancreas were stained with hematoxylin-phloxinsaffron from 25 to 125 days. For each panel pair, low (**upper**) and high (**lower**) magnifications are shown. For each genotype, the number of mice developing PDACs before the age of 200 days is represented as a fraction number (in parentheses). The *P* value was determined by the exact Fisher test. **P* < 0.05.

PDAC, whereas none of the *Pdx1-Cre; LSL-Kras^{G12D}* (K; *n* = 9) or *Pdx1-Cre; Tif1 $\gamma^{lox/lox}$* (TT; *n* = 6) control mice developed PDAC during this time frame (see Supplemental Table S1 at <http://ajp.amjpathol.org>). The longitudinal analysis of one KTT mouse by magnetic resonance imaging showed gradual growth of cystic lesions (hyper-signal), with the onset at 200 days of age of a firm and solid mass (isosignal) that developed into the tail of the

pancreas (Figure 1A). Thirty days later (at the age of 230 days), the size of this solid lump had dramatically increased, prompting us to euthanize the animal. Macroscopic analysis of the pancreas from this animal confirmed the presence of multifocal cystic lesions (smooth texture), colonizing the entire organ, and the presence of a voluminous and firm tumor (tough texture), which had invaded the tail of the pancreas (Figure 1B). Microscopic

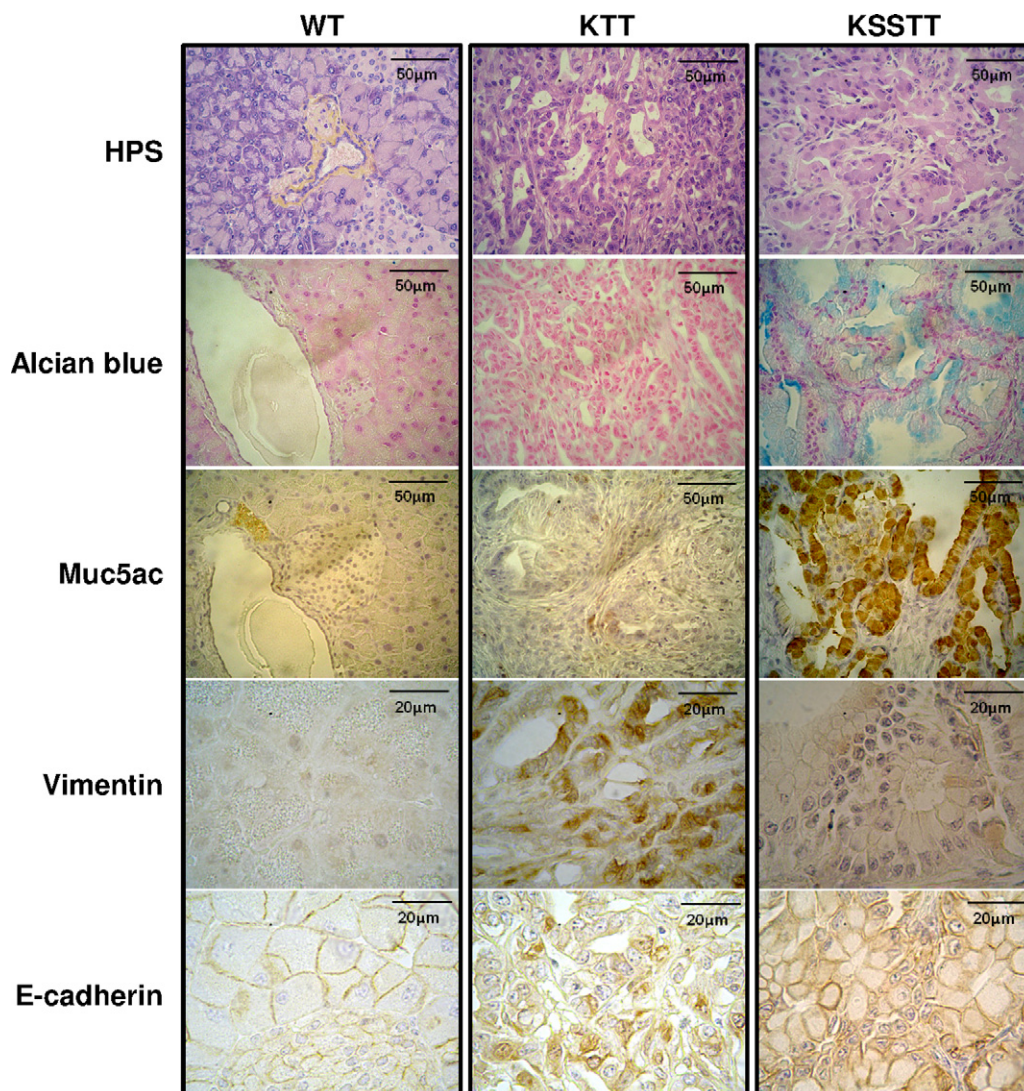


Figure 4. KTT and KSSTT pancreatic tumors display different histological features. Histological sections of 365-day-old control [wild type (WT)], 231-day-old KTT, and 126-day-old KSSTT pancreas samples stained with hematoxylin-phloxinsaffron or Alcian blue (mucin specific) or subjected to IHC for Mucin-5ac (Muc5ac), vimentin, or E-cadherin.

analysis of the pancreatic nodule that developed in this KTT mouse revealed a poorly differentiated carcinoma (Figure 1C), found adjacent to epithelial cysts reminiscent to human IPMNs. We observed focal destruction of the pancreatic parenchyma by a malignant epithelial proliferation made of irregular glands and cellular cords. Tumor cells are irregular in size and shape, with abundant basophilic cytoplasm and irregular nuclei. Mitotic features are common. The tumoral stroma presents with multiple inflammatory cells but only a low level of collagen between tumor cells. Histological analysis of the two other KTT mice sacrificed at the ages of 256 and 342 days revealed the presence of a small focus with PDAC and the presence of multiple large high-grade IPMNs with some papillary protrusions (data not shown). Altogether, these data indicate that KTT mice develop IPMNs with short latency and revealed that these tumors have high malignant potential.

To carefully characterize the pathological features of PDAC arising in KTT mice, we performed immunohistochemistry (IHC) and immunohistochemistry experiments, which allowed us to underline the hallmarks of PDAC (Figure 2). In accordance with the expression pattern of the *Pdx1-Cre* transgene (in all pancreatic epithelial lineages¹⁰), we observed a complete loss of Tif1 γ expression in the epithelial compartment of KTT tumors, whereas residual expression could be detected in the microenvironment surrounding the cancer cells (mesenchymal cells and immune infiltrates) (Figure 2A). KTT tumors have lost their acinar differentiation (chymotrypsin negative) (Figure 2B). Cytokeratin 19, a marker that is normally only expressed at the membrane of the cells lining the duct in wild-type pancreas, presents a faint, but diffuse, cytoplasmic localization in KTT tumors, attesting to the altered ductal differentiation of these tumors (Figure 2C). We next explored the tumor-associated stroma.

Indeed, it is well documented that PDAC is characterized by an abundant stroma (desmoplastic stroma), the epithelial cancer cells eventually representing <10% of the tumor. Positive staining for vimentin (Figure 2D) and α -SMA (Figure 2E) confirmed the presence of a fibroblastic/myofibroblastic stromal compartment in KTT tumors. Interestingly, some vimentin-positive cells (but α -SMA negative) were seen lining the lumen, suggesting that some tumoral cells express the vimentin marker. We also detected tumor-associated macrophages (F4/80-positive cells) in KTT tumors (Figure 2F). To assess the proliferative index and the metabolic status of KTT tumors, we examined the expression of proliferating cell nuclear antigen (Figure 2G) and glut2 (Figure 2H) markers, which are both highly expressed in KTT tumors. The poorly differentiated state is generally associated with disorganization of E-cadherin, a membrane protein ensuring the maintenance of proper cell-cell contact. IHC against E-cadherin in KTT PDACs revealed a severe disorganization of the epithelial architecture, with a clear delocalization of E-cadherin from the membrane to the intracellular compartment (Figure 2I).

SMAD4 (alias, *DPC4*, deleted in pancreatic carcinoma, locus 4) is involved in TGF- β signaling and is a major tumor suppressor deleted in approximately 50% of PDACs. TIF1 γ has been implicated as a modulator of TGF- β signaling, potentially either augmenting or compromising SMAD protein function. To examine the genetic interactions between *Tif1 γ* and *Smad4*, we generated conditional null mice for both *Smad4* and *Tif1 γ* in a *Kras*-driven mouse model of pancreatic cancer. We observed that *Pdx1-Cre; Smad4^{lox/lox}; Tif1 γ ^{lox/lox}* (SSTT; $n = 18$) double-null mice do not develop pancreatic lesions (all ages, $n = 18$; aged >125 days, $n = 8$) (see Supplemental Table S1 at <http://ajp.amjpathol.org>). We next generated *Pdx1-Cre; LSL-Kras^{G12D}; Smad4^{lox/lox}; Tif1 γ ^{lox/lox}* mice harboring pancreatic inactivation for both *Smad4* and *Tif1 γ* in a *Kras^{G12D}* background (KSSTT; $n = 9$) (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Interestingly, the two KSSTT mice that we euthanized at the age of 25 days presented high-grade IPMN lesions. Moreover, 44% (4/9) of KSSTT mice that we euthanized before the age of 200 days had developed a PDAC (Figure 3). More important, PDAC developing as soon as the age of 46 days has never been reported in K or KTT mice. Thus, double-null homozygous inactivation of *Tif1 γ* and *Smad4*, in a *Kras^{G12D}* background, induces the formation of PDACs at a higher frequency and a shorter latency than any other combined mutations. We have also generated *Pdx1-Cre; LSL-Kras^{G12D}; Smad4^{lox/+}; Tif1 γ ^{lox/lox}* mice (KSTT; $n = 7$) harboring *Tif1 γ* homozygous inactivation and a *Smad4* heterozygous inactivation. Interestingly, >50% (4/7) of the KSTT mice develop PDACs in an intermediary time frame to what is observed in KTT and KSSTT mice (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

Histological analysis of these tumors revealed that KSSTT tumors retained more differentiated features compared with KTT tumors. Indeed, KSSTT showed strong Alcian blue staining and Mucin-5ac immunoreactivity, attesting a well-differentiated mucinous phenotype. In addition, KSSTT tu-

mors retained a clear epithelial differentiation, as attested by an intact E-cadherin intercellular network and the absence of vimentin expression (Figure 4).

Discussion

By using the *Pdx1-Cre; LSL-Kras^{G12D}; Tif1 γ ^{lox/lox}* mouse model, we previously reported that the inactivation of *Tif1 γ* could cooperate with the *Kras^{G12D}* oncogene to induce IPMNs with full penetrance by the age of 189 days.⁶ Herein, we extended this study by demonstrating, in aging KTT mice (aged >200 days), that homozygous inactivation of *Tif1 γ* significantly potentiates the capacity of *Kras^{G12D}* to induce PDACs, supporting the potency of IPMNs to evolve toward aggressive lesions, as observed in humans. This observation reinforces the existence of a tumor-suppressive pathway, orchestrated by *Tif1 γ* , to prevent malignant transformation of premalignant lesions induced by activated *Kras*. However, no direct communication between the tumor itself and a pre-existing IPMN was proved by the pathological examination. Whether PDACs directly arise from existing precancerous cystic lesions remains an open question in the field.

The mechanism by which TIF1 γ exerts its tumor-suppressive effect remains elusive. TGF- β signaling is classically considered as tumor suppressive, via its anti-proliferative and pro-apoptotic functions, to prevent normal or premalignant cells to evolve toward a malignant state. This is well illustrated by the high rate of inactivating genetic alterations in the TGF- β -SMAD4 pathway¹¹ and by mouse models showing that *Smad4*-homozygous inactivation, in combination with activated *Kras*,¹²⁻¹⁴ causes the development of cystic lesions with a higher potential to progress toward PDAC, compared with mice expressing activated *Kras* alone (*Pdx1-Cre; LSL-Kras^{G12D}*).¹⁵ To address the genetic relationship between *Tif1 γ* and *Smad4* in pancreatic tumors, we generated KSSTT double-null homozygous mutant mice. We observed a pronounced genetic interaction between *Smad4* and *Tif1 γ* inactivation, demonstrating that both molecules act in separate pathways to suppress a premalignant-to-malignant *Kras*-driven pancreatic tumor. It is, however, possible that the capacity of *Tif1 γ* to constrain the premalignant-to-malignant progression depends on the modulation of TGF- β signaling arms independent of SMAD4. This could involve R-SMADs or noncanonical TGF- β pathways. Indeed, it has been proposed in other cell systems that TIF1 γ /R-SMAD complexes could positively mediate TGF- β functions,^{3,16,17} through the regulation of expression of a different set of genes by R-SMAD-TIF1 γ and R-SMAD-SMAD4 complexes.¹⁸ This hypothesis is consistent with the phenotype observed in *Pdx1-Cre; LSL-Kras^{G12D}; T β R11^{lox/lox}* mice,¹⁹ in which canonical and noncanonical TGF- β pathways are abrogated, with a phenotype resembling the one observed in KSSTT mice. Finally, TIF1 γ could prevent the premalignant-to-malignant progression through mechanisms totally unrelated to TGF- β signaling. Indeed, it may result from the modulation of gen-

eral programs affecting broader genomic functions. For instance, TIF1 γ and related family member TIF1 α were involved in chromatin remodeling²⁰ and transcription elongation,^{17,21} and TIF1 β was involved in DNA repair.²²

Once the malignant transformation has occurred by bypassing its anti-proliferative and pro-apoptotic effects, TGF- β acquires oncogenic properties and eventually facilitates tumor progression. Even if our results clearly argue for a role of Tif1 γ to constrain the transition between a premalignant and a malignant state through a mechanism independent of Smad4 activity, we cannot exclude that Smad4 function may facilitate tumor aggressiveness after the premalignant state is overrun and the premalignant state is achieved (in malignant tumors that retained a functional Smad4). In such cases, Smad4 may facilitate local invasion and metastatic dissemination by inducing epithelial-to-mesenchymal transition (EMT). This may reflect the capacity of TIF1 γ to constrain invasiveness programs, such as EMT, as recently demonstrated *in vitro* in human mammary epithelial cells.¹⁸ Consistent with this, the *Pdx1-Cre; LSL-Kras^{G12D}; Ink4a/Arf^{lox/lox}; Smad4^{lox/lox}* mice¹² develop well-differentiated PDAC, with attenuated invasive properties, compared with the poorly differentiated tumors observed in *Pdx1-Cre; LSL-Kras^{G12D}; Ink4a/Arf^{lox/lox}* mice. In our study, even if PDACs developing in KSTT mice arise with a shorter latency and a higher penetrance compared with KTT mice, they present a well-differentiated phenotype compared with the poorly differentiated phenotype of the PDACs we observed in KTT mice. This suggests a tumor-suppressive role of Tif1 γ to limit the aggressiveness of Smad4-positive cancer cells by compromising their capacity to undergo EMT. It may result in a lower capacity to generate metastases. The molecular mechanism occurring *in vivo* may rely on the capacity of TIF1 γ to mono-ubiquitinate SMAD4 and limit SMAD4 nuclear accumulation, as demonstrated in other systems.^{2,4}

In conclusion, we demonstrate, in this study, that *Tif1 γ* inactivation cooperates with activated *Kras* to potentiate the premalignant-to-malignant transition to promote the onset of poorly differentiated tumors. We also evidence that the tumor-suppressive role of Tif1 γ during the premalignant-to-malignant progression is independent of Smad4 activity. Finally, we noticed that the absence of Smad4 could redirect Tif1 γ -negative pancreatic tumors toward a well-differentiated phenotype, consistent with an effect of Tif1 γ to inhibit Smad4-dependent EMT. Then, Tif1 γ exerts tumor-suppressive functions independently of Smad4 in the premalignant-to-malignant transition but may also exert tumor-suppressive functions dependent on Smad4 later on during tumor progression.

Acknowledgments

We thank the specific pathogen-free animal facility at AniCan Platform, Centre Léon Bérard and each of its members for technical assistance and animal care and Dr. David Wotton for helpful discussion.

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