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Nuclear Localization of Dpc4 (Madh4, Smad4) in Colorectal Carcinomas and Relation to Mismatch Repair/Transforming Growth Factor-β Receptor Defects

Elizabeth Montgomery,* Michael Goggins,‡ Shibin Zhou,† Pedram Argani,* Robb E. Wilentz,* Manju Kaushal,* Susan Booker,† Katharine Romans,* Parul Bhargava,* Ralph H. Hruban,* and Scott E. Kern†

From the Departments of Pathology, Oncology,† and Gastroenterology,‡ The Johns Hopkins Medical Institutions, Baltimore, Maryland*

The tumor-suppressor protein Dpc4 (Smad4, Madh4) regulates gene expression. On binding of an extracellular ligand of the extensive transforming growth factor (TGF) superfamily to its cognate receptor complex, latent cytoplasmic Dpc4 is activated and translocated into the nucleus to function as part of various DNA-binding transcriptional activator complexes. The most relevant ligand/receptor pair to control the tumor suppressive function of Dpc4 remains uncertain, but is usually assumed to be TGF-β and its heteromeric receptor. We exploited a fortuitous experiment of nature to directly test this hypothesis: the TGF-β type II receptor gene is inactivated by mutation in nearly all colorectal carcinomas having microsatellite instability, as seen in hereditary nonpolyposis colorectal cancer (HNPCC) and in sporadic medullary colorectal cancers. Using a specific and sensitive immunohistochemical label for Dpc4, we examined nuclear localization of Dpc4 in 13 HNPCC, six medullary, and 41 sporadic nonmedullary colorectal carcinomas. In agreement with published rates, two (5%) of 41 sporadic tumors showed complete loss of Dpc4 protein, indicative of genetic inactivation. All 13 HNPCC and six medullary tumors had intact cytoplasmic and nuclear Dpc4 localization. The *TGFBR2* gene was sequenced in three of the cancers from patients with HNPCC, and all of these harbored inactivating mutations. The specificity of the immunohistochemical assay was demonstrated in xenograft tumors of syngeneic cell lines that differed in *DPC4* genetic status because of an engineered gene knockout. Thus, nuclear localization of Dpc4 can be maintained in cells with inactivated TGF-β type II receptors, suggesting the persistence of tumor-suppressive action of an upstream signaling input, most likely a ligand/receptor complex distinct from TGF-β. Identification of the relevant input would be expected to have implications for the understanding of tumorigenesis and the design of rational biological therapy. *(Am J Pathol 2001, 158:537–542)*

Dpc4 is a phosphoprotein with sequence-specific DNA binding abilities and it is able to form complexes with a considerable variety of additional proteins. Somatic inactivation of this tumor-suppressor protein is common in pancreatic ductal adenocarcinomas and, albeit at lower frequencies, in multiple other tumor systems including colorectal cancer.[1,2] Germline *DPC4* gene mutations cause juvenile polyposis.[3]

The *TGFβ* genes form part of a large superfamily of extracellular ligands, and the corresponding receptors of each type of ligand also constitute distinct gene families. Signal transduction is initiated when a ligand binds and stabilizes the formation of the corresponding heterodimeric receptor pair. Transforming growth factor-β (TGF-β) has long been studied for its tumor-suppressive properties in a variety of cancer types and a lack of TGF-β-responsiveness has been demonstrated in many tumor lines. The importance of this signaling pathway in neoplasia is most strongly supported by the finding of biallelic inactivating mutations in the *TGFBR2* gene in nearly all colorectal carcinomas having microsatellite instability.[4–6]

A number of *Smad* genes mediate functions of the TGF-β superfamily (including those of bone morphogenic protein, activin, and TGF-β).[7–9] For example, Smad2 mediates TGF-β signals and *Smad2* mutations have been identified in occasional colorectal cancers. Likewise, *DPC4* is a common mediator of multiple TGF-β superfamily pathways. As depicted in Figure 1, ligand binding to a heterodimeric receptor activates a serine-threonine ki-

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Address reprint requests to Scott E. Kern, M.D., The Johns Hopkins University, 451 Cancer Research Building, Baltimore, MD 21231. E-mail: sk@jhmi.edu.

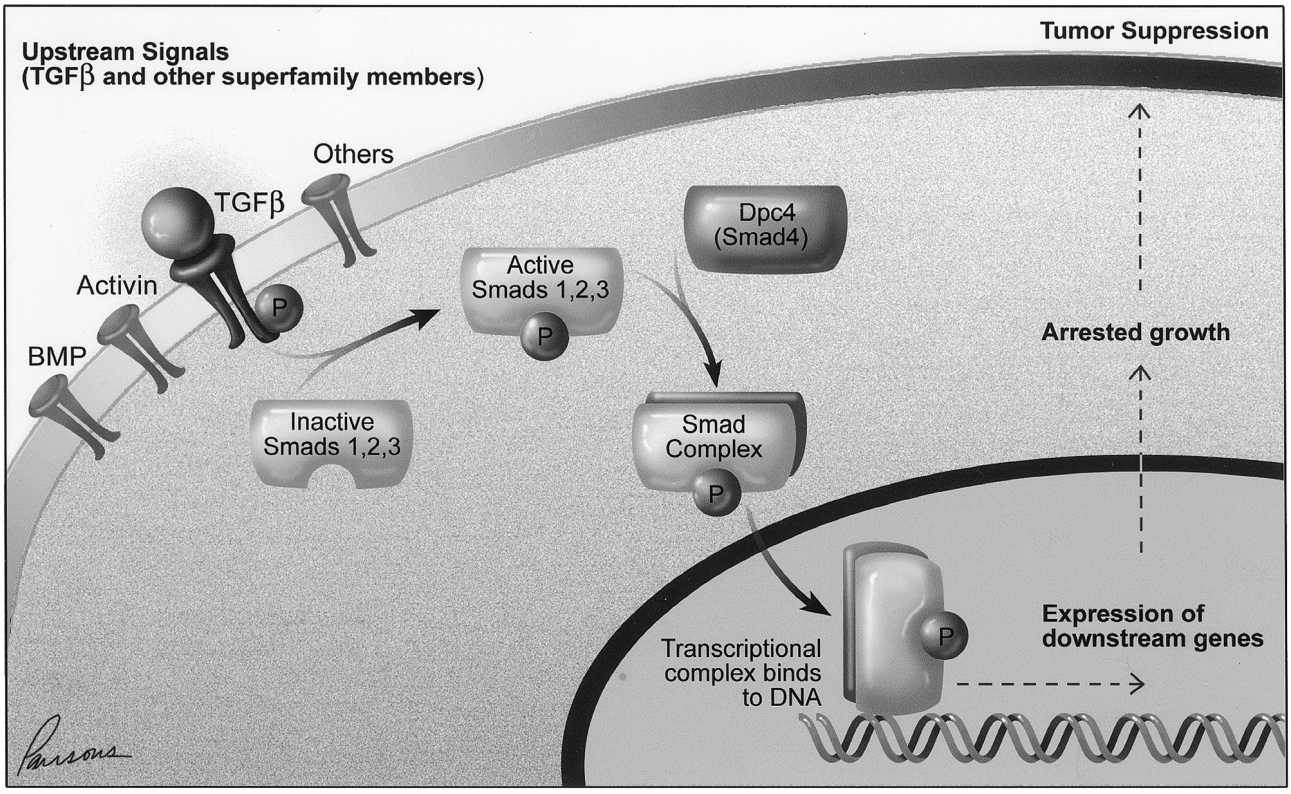


Figure 1. Diagram showing interrelationships between TGF-β superfamily members. Although TGF-β itself is known to lead to activation of Dpc4 in some systems, the data presented here support the presence of other input as well. The figure is schematic; the receptor is heterodimeric and requires additional proteins for efficient signaling, and the Smad complex also comprises multiple proteins to accomplish specific functions.

nase functional group. Certain Smad proteins interact with the receptors transiently, become phosphorylated, and then form a complex with Dpc4. These activated complexes are translocated into the nucleus where their binding to DNA stimulates the transcription of nearby genes.[10] There is now a considerable body of literature concerning experimental systems that confirms these biochemical relationships.[9] It was therefore attractive to assume that Dpc4 functioned as a tumor suppressor (ie, it was selectively inactivated in tumors) primarily because it mediated the TGF-β signals.

The identification of a sensitive and specific means of Dpc4 immunolocalization provided a new method to investigate these relationships directly in human tumors. A monoclonal antibody was recently validated as providing an *in situ* assay that closely mirrors the genetic status of the *DPC4* gene.[11] Tissues with wild-type *DPC4* status harbor nuclear and cytoplasmic labeling of Dpc4 consistent with our understanding of Dpc4 function. We used this immunohistochemical system to study Dpc4 localization as an assay for its functional inactivation in colorectal cancers, particularly in those expected to exhibit genetic inactivation of TGF-β signaling.

Materials and Methods

Xenografted Syngeneic Tumors

To generate xenograft tumors, actively growing cell lines were harvested and resuspended in serum-free McCoy's 5A medium (Life Technologies, Inc., Grand Island, NY) at 5×10^7 cells per ml. The athymic nude mice (strain nu/nu; Harlan, Indianapolis, IN) were then injected subcutaneously with 0.1 ml HCT116 cells on one flank and with cells from cell line 5-60 or cells from cell line 5-18 on the other. HCT116 cells are known to have a truncating mutation of *TGFBR2* but are $DPC4^{+/+}$.[4] In contrast, the cell line 5-60 was prepared from HCT116 cells that have been genetically modified so they not only have a truncating mutation of *TGFBR2* but are also $DPC4^{+/-}$. The cell line 5-18 was prepared from HCT116 cells and they were genetically modified so they lack *TGFBR2* and are $DPC4^{-/-}$. The preparation of these cell lines has been previously described.[9] Fourteen days after injection, the animals were sacrificed. The tumors were removed and immediately preserved in 10% buffered formalin. Immunohistochemistry using the anti-Dpc4 antibody was then performed as below on each of the harvested tumors.

Specimen Selection

Slides and blocks of colorectal cancers resected from 13 patients with hereditary nonpolyposis colorectal cancer (HNPCC)[12] and from six patients with a medullary tumor phenotype (an appearance that has been associated with mismatch repair defects) were retrieved from the surgical pathology files of The Johns Hopkins Hospital. The 13 patients with HNPCC were previously shown to have DNA mismatch repair defects.[12] Medullary tumor phenotype was defined using criteria of Jass and col-

leagues[13] and Kim and colleagues.[14] These tumors appeared poorly differentiated with solid sheets of tumor cells, prominent intratumoral lymphocytic infiltration, and an intense peritumoral lymphoid response. Also retrieved and sectioned were materials from 41 sporadic colorectal carcinomas and their associated adenomas ($n = 14$) and lymph node metastases ($n = 9$).

Immunohistochemistry

Unstained 5-μm sections were cut from the paraffin blocks and deparaffinized using standard methods. Slides were treated with sodium citrate buffer (HIER buffer; Ventana-Bio Tek Solutions, Tucson, AZ) and steamed at 80°C. After cooling for 5 minutes, slides were labeled with monoclonal antibody to Dpc4 (clone B8; Santa Cruz Biotechnology, Santa Cruz, CA) using the Bio Tek-Mate 1000 automated stainer (Ventana-Bio Tek Solutions). Each slide was labeled with a 1:100 dilution of the antibody. The anti-Dpc4 antibody was detected using a biotinylated secondary antibody and 3,3′-diaminobenzidine as the chromagen with hematoxylin counterstaining.

Slides were reviewed by three of the authors (EM, REW, RHH) and recorded as positive or negative for both nuclear and cytoplasmic labeling as has been described.[11] For a slide to be interpreted as negative, no expression could be visible. Focal labeling was interpreted as positive. Normal colonic epithelium served as a positive control, and the primary antibody was omitted in negative controls. Pancreatic carcinomas with known Dpc4 genetic status were also included as positive and negative controls.[11]

Epstein-Barr Virus (EBV) Testing

In situ hybridization was performed to detect EBV using a fluorescein-conjugated oligonucleotide probe to the EBV early RNA transcripts (Novocastra, Newcastle, UK).[15] After overnight incubation, probe binding was detected using an *in situ* hybridization detection kit (Novocastra) that uses an alkaline-phosphatase-conjugated rabbit F(ab′) anti-fluorescein isothiocyanate fragment followed by 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate chromogen. Positive control tissues yielded blue/black nuclear staining.

Assessment of TGF-β Receptor Mutations

Sufficient archival material was available from three of the HNPCC colorectal cancers with nuclear Dpc4 expression for DNA sequencing. DNA was isolated from formalin-fixed paraffin-embedded tissues as previously described.[16] Evidence for mutations of the polyA tract of the *TGFBR2* gene was determined by polymerase chain reaction amplification and end-labeling of the 5′ primer. The radiolabeled 73-bp polymerase chain reaction products were resolved on a 6% polyacrylamide gel. Constitutional DNA from the duodenum of two patients with pancreatic cancer served as negative controls and two pancreatic cancers with known polyA tract mutations[17] served as positive controls.

Results

Xenografted Specimens Prepared from Cell Lines with Known Genotype

The tumors prepared from HCT116 cells (which are known to have a truncating mutation of *TGFBR2* but are *DPC4*$^{+/+}$) had both nuclear and cytoplasmic labeling for Dpc4 (Figure 2A). Those prepared from cell line 5-60 cells (lacking intact *TGFBR2* but *DPC4*$^{+/-}$) also had both nuclear and cytoplasmic labeling with the Dpc4 antibody (Figure 2B). Xenografted tumors prepared from cell line 5-18 cells (lacking intact *TGFBR2, DPC4*$^{-/-}$) showed neither cytoplasmic nor nuclear labeling with the Dpc4 antibody (Figure 2C). This result confirmed unambiguously that the nuclear labeling was because of Dpc4 protein rather than cross-reacting antigen.

HNPCC Colorectal Carcinomas and Carcinomas with Medullary Phenotype

All 13 (100%) carcinomas obtained from patients with HNPCC (Figure 3A) and all six carcinomas having medullary morphology showed both nuclear and cytoplasmic labeling for Dpc4. To assure that the six carcinomas with medullary morphology were not Epstein-Barr virus driven (a finding sometimes associated with medullary phenotype), we also performed *in situ* hybridization for Epstein-Barr virus, which was negative in all cases.

Sporadic Carcinomas

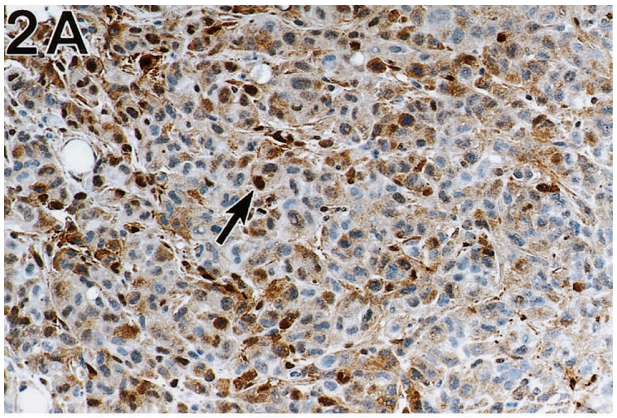
Of the 41 sporadic cancers, 39 labeled with the Dpc4 antibody, and two (two of 41; 5%) completely lacked expression of the gene product by immunohistochemistry (Figure 3B). Internal controls (normal colon epithelium and stroma) labeled in all cases. Fourteen associated sporadic adenomas all expressed the gene product. Two lymph node metastases, associated with the carcinomas that did not express Dpc4, were also negative for gene product, whereas the lymph node metastases from Dpc4-positive carcinomas (Figure 3C) were also positive.

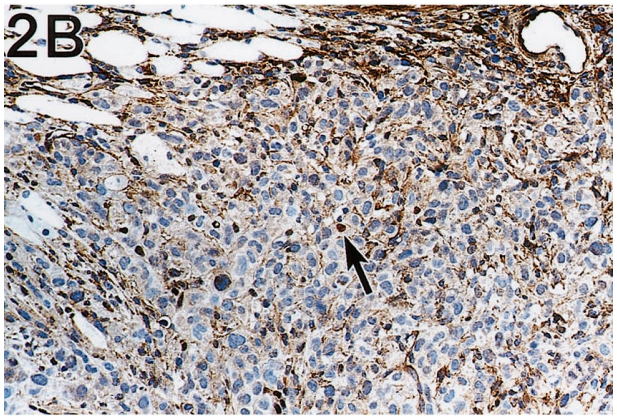
TGFBR2 Gene Sequencing

Sufficient material was available from three of the 13 carcinomas from patients with HNPCC for *TGFBR2* gene sequencing. All three cases tested had bi-allelic mutations of the polyA tract of *TGFBR2*. In all three cases, immunolabeling revealed intact nuclear localization of Dpc4 (Figure 3A).

Discussion

The findings in this study challenge the current concept that Dpc4 functions primarily to transduce TGF-β signals





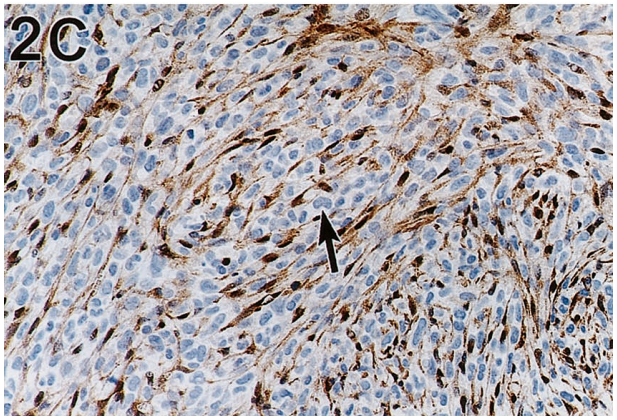
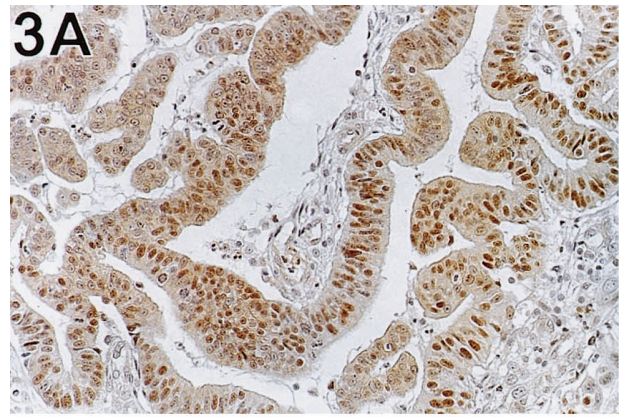
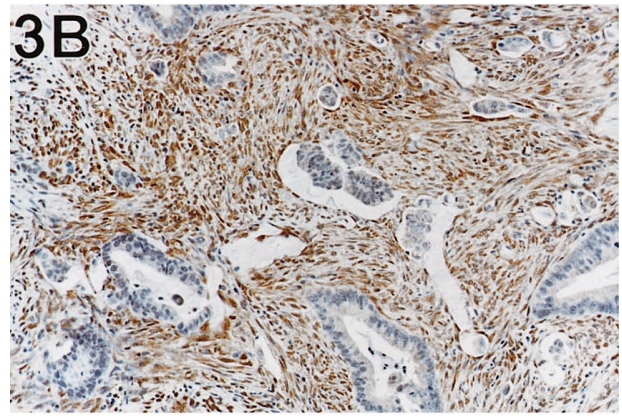


Figure 2. A: The tumors prepared from HCT116 cells (which are known to have a truncating mutation of *TGFBR2* but are *DPC4*$^{+/+}$) have both nuclear (**arrow**) and cytoplasmic labeling for Dpc4. Immunohistochemistry using anti-Dpc4 antibody, counterstained with hematoxylin. **B:** A xenograft prepared from cell line 5-60 cells (lacking intact *TGFBR2* but *DPC4*$^{+/-}$) has both nuclear (**arrow**) and cytoplasmic labeling with the Dpc4 antibody. Immunohistochemistry using anti-Dpc4 antibody, counterstained with hematoxylin. **C:** Xenografted tumor prepared from cell line 5-18 cells (lacking intact *TGFBR2* and *DPC4*$^{-/-}$) showed neither cytoplasmic nor nuclear labeling with the anti-Dpc4 antibody. The labeled cells in this photograph are ingrown stromal cells from the host animal; the large tumor cells (**arrow**) are unlabeled. Immunohistochemistry using anti-Dpc4 antibody, counterstained with hematoxylin.





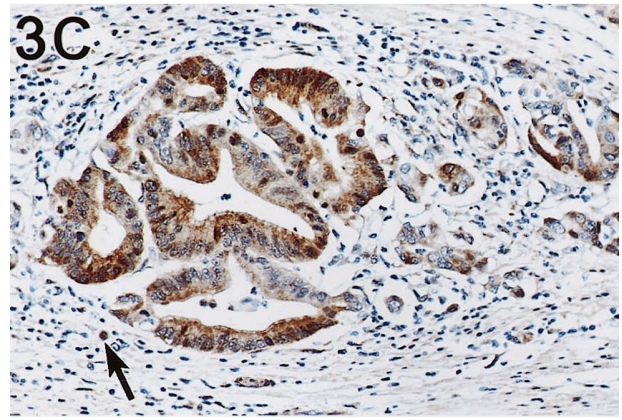


Figure 3. A: Colon carcinoma from a patient with HNPCC. This patient was shown to have a *TGFBR2* mutation. Tumor cells contain both nuclear and cytoplasmic Dpc4. Immunohistochemistry using anti-Dpc4 antibody, counterstained with hematoxylin. **B:** A sporadic colorectal carcinoma completely lacking expression of Dpc4. The stromal and inflammatory cells within the desmoplastic stroma serve as internal controls that express Dpc4. Immunohistochemistry using anti-Dpc4 antibody, counterstained with hematoxylin. **C:** Sporadic colorectal adenocarcinoma with strong expression of Dpc4 in tumor cell nuclei and cytoplasm. The lymphoid cells (**arrow**) in the lamina propria also label for Dpc4.

and highlight the need to re-explore the rationale behind accepted views on the relationship between Dpc4 and the TGF-β superfamily. Developmental systems studied in *Drosophila* and *Xenopus* have supported the concept that Dpc4 is a required mediator of TGF-β superfamily signals. In human tumors, breast carcinoma cell line 468 and colorectal cancer cell line SW480 cells have a defect in Dpc4 expression that results in their TGF-β unresponsiveness,[18–20] and somatic knockout of the *DPC4* gene in a colorectal cancer cell line resulted in TGF-β unresponsiveness.[9]

It is also known that Dpc4 can be influenced by other upstream signaling inputs. For example, developmental systems have principally concerned BMP-related li-

gands, not TGF-β itself. Similarly, the somatic knockout model of the *DPC4* gene was not only TGF-β-unresponsive but also activin-unresponsive.[9]

Indeed, there is considerable emerging evidence that raises doubts regarding, or at least complicates, the TGF-β-Dpc4 relationship. For example, *DPC4*-null pancreatic cancer and colorectal cancer cell lines do not, as a rule, lose TGF-β responsiveness, irrespective of whether one looks at transcriptional responses or growth suppression.[20,21] The *ras* genes, the MAP kinase pathway, and the MKK4-mediated stress-activated protein kinase pathway are implicated in these responses and would not involve the Dpc4 protein.[20–22] *DPC4*-null transgenic mouse cells have classes of TGF-β responses that remain intact.[23] Both pancreatic and colorectal tumors show instances in which there is co-existence of genetic inactivation of both a TGF-β receptor gene and the *DPC4* gene.[24] This would not be expected if inactivation of one were sufficient to obviate the function of the other. These data together suggest considerable branching of the pathways uniting TGF-β receptors and Dpc4 (Figure 1). Furthermore, preliminary immunohistochemical studies of pancreatic cancer also demonstrate nuclear localization of Dpc4 in the rare tumors having genetic inactivation of the TGF-β receptors (RE Wilentz, unpublished data).

The immunohistochemical labeling results in the current study raise the question of whether TGF-β responsiveness is necessary for Dpc4 function. We found intact nuclear localization of Dpc4 in human carcinomas with inactivating mutations in a TGF-β receptor gene and confirmed these observations using xenografted tumors from appropriate human cell lines. Although the mere presence of Dpc4 in the nucleus does not necessarily imply the existence of conditions sufficient for its tumor-suppressive function, there is evidence from studies of engineered cell lines that the direct manipulation of Dpc4 to relocalize to the nucleus, in the absence of exogenous administration of ligand, is sufficient to cause apoptosis and cell cycle inhibition.[25]

The accumulated data therefore suggest that TGF-β signaling is not always required for the major tumor-suppressive functions of the *DPC4* gene. It would now be important to determine whether there is a dominant signaling input to Dpc4 and what this input might be. On such an understanding would rest the better comprehension of some of the key driving forces in tumorigenesis. This, in turn, would lead to more precise identification of the molecular targets on which we might base hopes for the development of rational therapy for human cancer. Undoubtedly, additional direct observational studies of human tumors will be critical to the evaluation of competing hypotheses.

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