Enhancement of TGF-β Signaling Responses by the E3 Ubiquitin Ligase Arkadia Provides Tumor Suppression in Colorectal Cancer

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Abstract

TGF-β signaling provides tumor protection against colorectal cancer (CRC). Mechanisms that support its tumor-suppressive properties remain unclear. The ubiquitin ligase Arkadia/RNF111 enhances TGF-β signaling responses by targeting repressors of the pathway for degradation. The corepressors SnoN/Ski, critical substrates of Arkadia, complex with the activated TGF-β signaling effectors Smad2/3 (pSmad2/3) on the promoters of target genes and block their transcription. Arkadia degrades this complex including pSmad2/3 and unblocks the promoter. Here, we report that Arkadia is expressed highly in the mouse colonic epithelium. Heterozygous Akd¹⁻/⁻ mice are normal but express less Arkadia. This leads to reduced expression of several TGF-β target genes, suggesting that normal levels of Arkadia are required for efficient signaling responses. Critically, Akd¹⁻/⁻ mice exhibit increased susceptibility to azoxymethane/dextran sulfate carcinogen–induced CRC, as they develop four-fold more tumors than wild-type mice. Akd¹⁻/⁻ tumors also exhibit a more aggressive pathology, higher proliferation index, and reduced cytostasis. Therefore, Arkadia functions as a tumor suppressor whose peak expression is required to suppress CRC development and progression. The accumulation of nuclear SnoN and pSmad2, along with the downregulation of TGF-β target genes observed in Akd¹⁻/⁻ colon and tumors, suggest that tumor-suppressing properties of Arkadia are mediated by its ability to derepress TGF-β signaling. Consistent with this likelihood, we identified mutations in primary colorectal tumors from human patients that reduce Arkadia function and are associated with the accumulation of nuclear SnoN. Collectively, our findings reveal that Arkadia enhances TGF-β signaling responses and supports its tumor-suppressing properties in CRC. Cancer Res 71(20): 6438–49. ©2011 AACR.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide and accounts for over 600,000 deaths each year (1). Mutations that inactivate TGF-β signaling components are associated with CRC, consistent with a role of this pathway in tumor suppression (2, 3). Specifically, mutation or deletion of the receptor genes TGFβRI and TGFβRII, and the effector genes SMAD2, SMAD3, and SMAD4 of the pathway, have been reported in CRC (2, 4). Germ line mutations of SMAD4 are also associated with juvenile polyposis in the colon (5). In addition, mouse models in which these core signaling components are inactivated result in an increased susceptibility to develop adenocarcinomas under oncogenic stress (3). However, analyses of tumor samples have revealed that the TGF-β pathway also exhibits oncogenic properties in CRC, as it promotes survival, invasion, and metastasis (6). Mechanisms that impair TGF-β–mediated tumor suppression but do not abolish signaling may allow TGF-β–mediated metastasis to occur more frequently later in tumorigenesis. Regulators of such mechanisms could be used as early markers for susceptibility and prognosis and help in both the choice of drug treatment and in the development of novel anticancer agents.

The TGF-β ligands, including TGF-β1/2/3, Nodal and Activin, bind to specific cell surface receptors and activate, via phosphorylation, the Smad2/3 effectors (pSmad2/3). pSmads complex with Smad4 and translocate to the nucleus where they function as transcription factors (7, 8). Within the constantly regenerating colonic epithelium, the normal function of the TGF-β pathway is to maintain homeostasis by counteracting proliferation and promoting differentiation and apoptosis (9). TGF-β factors specify cell fate and differentiation.
in a dose-dependent fashion during development (10). However, reduction of TGF-β signaling in the adult colonic epithelium and its involvement in CRC is not clear.

TGF-β signaling is tightly regulated by both extracellular and intracellular mechanisms. Key intracellular regulators include the inhibitors Smads6/7, which mediate the degradation of receptors and interfere with the phosphorylation of effector Smads, and the nuclear corepressors SnoN and Ski, which directly interact with the effector Smad proteins and recruit a corepressor complex containing histone deacetylase and nuclear receptor corepressor to targeted gene promoters to block expression (11). Gene amplification and overexpression of SMAD7 and SNON/SKI proteins have also been linked to CRC, attributed to loss of TGF-β signaling (12, 13). We previously showed that Arkadia, a nuclear RING domain E3 ubiquitin ligase, is a positive regulator of the TGF-β/Nodal signaling branch. It mediates the ubiquitin–proteasome degradation of all the abovementioned negative regulators of the pathway and therefore constitutes a potent “derepressor” that enhances TGF-β target gene transcription (14–17). Degradation of SnoN/Ski by Arkadia depends upon their specific interaction with pSmad2/3 (15, 18, 19). As Arkadia also interacts and degrades pSmad2/3, its function results in clearing the promoters from used/blocked effectors, thereby allowing fresh effectors to bind and activate transcription (20). Consistent with this, the absence of Arkadia results in increased levels of stable SnoN/Ski and pSmad2/3, but as these are together in a complex, the promoters of target genes are occupied by stable, repressed pSmad2/3 leading to repression (20). Arkadia is broadly expressed in the mouse embryo and its absence leads to loss of a subset of Nodal signaling responses necessary for the development of anterior/head structures, which are also lost with the genetic reduction of Nodal (20–22). However, whether Arkadia enhances TGF-β signaling responses in the adult colonic epithelium and how this affects CRC development is unknown.

Using a deep sequencing screen of human Arkadia (AKD) mRNA from tumors of patients with CRC, we identified somatic mutations that reduce AKD function. We showed that reduction in Arkadia levels increased susceptibility to develop CRC in a mouse model and showed that this mechanism involves increased stability of SnoN and pSmad2 and a reduction of TGF-β–mediated target gene transcription. Collectively, our data reveal that Arkadia is required for peak efficiency of a subset of TGF-β transcriptional responses in the colonic epithelium and in colorectal tumors and thereby supports the tumor-suppressive arm of this pathway.

Materials and Methods

Deep sequencing

Total RNA was extracted from formalin-fixed, paraffin-embedded tumor and adjacent normal tissue sections as previously described (23) and reverse transcribed. Five to 6 overlapping PCR amplifications spanning the 300-bp C-terminus of AKD were conducted per patient/tissue type; each representing an “amplicon library” (primer sequences in Supplementary Section). Preparation of DNA-carrying beads was conducted as previously described (24). Beads were purified and loaded onto a 16-gasket picotiter plate for high-throughput pyrosequencing using the GS20 454-sequencer (Roche). Each amplicon library yielded an average of 4,000 sequencing reads. Data were analyzed as previously described (24). The Ensembl entry ENST00000380504 was used as the reference sequence for AKD (corresponding to protein Q6ZNA4-1).

Plasmid construction and cell culture assays

Site-directed mutagenesis to generate the different point mutations was conducted on a full-length human AKD cDNA clone (Invitrogen; ID: 30336202; see Supplementary Section for primer details). Fragments were subsequently PCR amplified and cloned in-frame into the expression plasmid pTriex2-GFP as Xhol-digested products (20). Transfections and luciferase assays were conducted in HEK293T cells (Cancer Research UK) as previously described (15). For immunoblotting, cells were harvested 24 hours posttransfection and lysates were analyzed for green fluorescent proteins (GFP). The HEK293T cell line was authenticated in July 2011 by the Health Protection Agency using short tandem repeat multiloci genotyping.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted as previously described (25) using the following rabbit polyclonal antibodies: Arkadia (1:100; LifeSpan BioSciences, Inc.); SnoN, H-317 (1:80; Santa Cruz); and pSmad2 (1:50; Millipore). The ABC–DAB detection system was used (ABC detection kit, Pierce; DAB substrate, Thermo Scientific). Blocking solution was used instead of primary antibodies for negative controls. Immunoreactivity was graded on a scale of 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong) according to intensity of staining and percentage of immunopositive cells as previously described (26). All sections were counterstained with hematoxylin.

Immunoblotting

Experiments were carried out as described previously (20), using the following rabbit polyclonal antibodies: GFP (1:1,000; Invitrogen); pSmad2 (1:500; Cell Signaling); SnoN, H-317 (1:5,000; Santa Cruz); p21, C-19 (1:200; Santa Cruz); and Histone H3 (H3), ab-1791 (1:10,000; Abcam). The following monoclonal antibodies were used: rabbit Smad2 (1:1,000, Epitomics); mouse proliferating cell nuclear antigen (PCna), PC-10 (1:3000; Santa Cruz), and mouse active β-catenin (anti-β-cat3). 8E7 (1:500, Millipore). Densitometric quantification of bands was conducted using ImageJ software.

Colorectal tumor induction protocol and histologic analysis

Twenty-week old wild-type and Akd−/− mice in a 129Svcc inbred genetic background were injected with a single intraperitoneal injection of the carcinogen azoxymethane (AOM; 7.4 mg/kg; Sigma). After one week, mice were subjected to 2% dextran sodium sulfate (DSS; molecular weight: 36,000–50,000; MP Biomedical) in their drinking water for a period of 5 days. Two more cycles of the 5-day DSS treatment were given, each separated by a 16-day period on normal drinking water. Twelve
Arkadia mutations in selected human colorectal tumors with high SNON

We recently described a cohort of primary human CRCs, of which 83 of 87 (95.4%) overexpressed SNON (26). Nuclear SNON expression was identified in 42 of 87 (48.3%) of these tumors and, importantly, this correlated with advancing tumor grade. Interestingly, we showed that SNON accumulation in these tumors was not a result of elevated SNON mRNA, suggesting increased protein stability. Because SNON is a substrate of AKD, itself a nuclear protein, it is possible that inactivating mutations in AKD may account, in part, for this stable nuclear SNON phenotype. To test this possibility, we conducted a deep sequencing screen for mutations in AKD mRNA extracted from human CRC paraffin-embedded tumors. We selected 5 CRC patients with tumors displaying the highest levels of nuclear SNON protein but a relatively low level of SNON mRNA expression (Fig. 1A; ref. 26). Normal expression levels of AKD mRNA were present in these samples (data not shown).

We hypothesized that mutations which inactivate the ubiquitin ligase activity of AKD, but do not disrupt substrate binding, would protect the substrates from ubiquitination and subsequent degradation and therefore prevent wild-type AKD function. The domains that are required for the activity of AKD are located in the highly conserved C-terminal 100 amino acids. This region comprises the NRG, which is at the end of the domain required for substrate (pSMAD2 and SNON/SKI) recognition, followed by a conserved TIER domain (of unknown function), a nuclear localization signal (NLS), and a RING domain, required for the ubiquitin ligase activity (20). Inactivation of the RING domain or deletion of the TIER domain in Arkadia converts its function to dominant-negative, as shown in luciferase reporter experiments carried out in HEK293T cells using the SMAD-dependent reporter CAGA12-Luc (Supplementary Fig. S1). Interestingly, the COSMIC database has catalogued 2 different point mutations at the C-terminal of AKD from ovarian cancer (27). This supported our hypothesis that mutations in the C-terminus are more likely to affect AKD function and led us to focus the mRNA sequencing screen on the last 300 bp of AKD.

The paraffin-embedded tumor sections that we used for our sequencing analysis comprised a heterogeneous population of cells including both tumorigenic epithelial cells and nontumorigenic stromal cells such as fibroblasts and inflammatory cells. In addition, not all cells exhibited an accumulation of nuclear SNON and therefore a potential mutation in AKD. Furthermore, as AKD mutations may arise from only 1 allele in these cells, they are likely to be represented at a low frequency in the sample. To determine the true frequency of such mutations in the mRNA (cDNA), we applied the GS20 system to achieve thousands of bidirectional sequencing reads for the C-terminal of AKD from each sample (24). Mutations were identified in 2 of the 5 patients (patients 6 and 32; Supplementary Table S1). Flowgram analysis was conducted to validate these mutations (Supplementary Fig. S2). Table 1 summarizes 4 of these mutations identified in the screen, each corresponding to a conserved amino acid residue (Fig. 1B).

We tested the functionality of each of these mutations in HEK293T cells using the CAGA12-Luc reporter assay (Fig. 1C). Overexpression of GFP-tagged AKD carrying the P908S, D937N, or T943I mutation enhanced reporter expression to a similar level as that of wild-type GFP-AKD (which is >2-fold above endogenous signaling, as assessed by overexpression of empty vector controls). In contrast, the Q999STOP mutant reduced signaling 3-fold compared with controls (Fig. 1C), suggesting that it exhibits a dominant-negative function similar to the control RING-W972R mutant AKD. Consistent with our hypothesis, this mutation introduces a STOP codon at the end of the substrate recognition domain, resulting in a truncated protein lacking the enzymatic RING domain. The P908S mutation was observed at the highest frequency (9.94%). As this is positioned within the NLS of AKD, we examined the localization of its GFP-tagged form in HEK293T transfections but found it to be nuclear like wild-type AKD (data not shown).

We have previously shown that deletion of the RING or NRG stabilizes the normally unstable wild-type Arkadia (20). By coexpressing a GFP control plasmid with each GFP-tagged AKD mutant in HEK293T cells, we observed an equal abundance of short GFP in all lysates following immunoblotting. However, only the GFP-Q899STOP AKD mutant gave a larger stable and visible band (Fig. 1D). The stabilization of Q899STOP AKD most likely contributes to its ability to strongly suppress wild-type AKD function when this mutation occurs in 1 allele. Considering that only a proportion of tumor cells displayed stabilized nuclear SNON (26), combined with the basis of screening for single-allele mutations in a heterogeneous tumor sample, the observed frequency of 2.76% for the Q899STOP mutation has a good association to be causal to the accumulation of SNON.
Expression from both alleles of Arkadia in the colonic epithelium maintains peak levels of TGF-β target gene expression

We decided to directly test the role of Arkadia in a mouse model for CRC, and for this, we first examined its expression in the colon. We used Akd^+/− gene trap mice expressing β-geo under the endogenous promoter (21) to show that Arkadia is highly expressed in the crypt compartments of the colon (Fig. 2A and B). IHC with an Arkadia antibody showed a defined nuclear distribution of Arkadia protein in epithelial cells along the colon. We used Akd^+/− gene trap mice expressing β-geo under the endogenous promoter (21) to show that Arkadia is highly expressed in the crypt compartments of the colon (Fig. 2A and B). IHC with an Arkadia antibody showed a defined nuclear distribution of Arkadia protein in epithelial cells along
the crypt (Fig. 2C). Both methods showed weaker expression in
the surface epithelium. These expression data suggest a role for
Arkadia in the colonic epithelium, where TGF-β is involved in
maintaining tissue homeostasis.

The gene trap insertion terminates transcription of the
Akd allele before the first exon (21) and quantitative PCR (qPCR)
confirmed that Akd+/−/− colons express half the level of Arkadia
compared with wild-type colons (Fig. 2D). Analysis of TGF-β
target gene expression revealed significant downregulation
of genes such as p15^ink4a, Pai-1 (Serpine1), Smad7, and
Timp1 (Pmea1) in the Akd+/−/−/− colon, whereas targets, such as SnoN
and p21^waf1, showed no difference (Fig. 2E). Immunoblotting
revealed higher levels of pSmad2 in Akd+/−/− than in wild-type
colon (Fig. 2F). We also conducted IHC for SnoN and observed
higher levels in Akd+/−/− than in wild-type colon (Fig. 2G and H).
These observations are consistent with the known molecular
function of Arkadia to degrade SnoN–Smad complexes and
enhance TGF-β target gene expression (14, 15, 20), as it does
in embryos, embryonic stem, and other cell lines (14, 20).

Interestingly, the above data suggest that even a reduction
of Arkadia function by the loss of 1 wild-type allele is
associated with the reduction of TGF-β signaling responses,
which was not known before. Multipotent stem cells reside
at the base of the crypts and give rise to proliferating
ductor epithelial cells that migrate upward before finally
acquiring a differentiated epithelial phenotype at the surface
with TGF-β signaling counteracting proliferation and
maintaining homeostasis (28). Notably, the increase of SnoN in
the Akd+/−/− colon occurs predominantly in the more differ-
entiated cells of the surface epithelium (compare Fig. 2G and
H). This suggests that the downregulation of TGF-β signaling
responses occurs within the epithelium and most likely
impairs the maintenance of balance between proliferation
and differentiation.

Akd+/−/− mice exhibit increased susceptibility to CRC

To determine whether loss of 1 allele of Arkadia increases
susceptibility to develop tumors, we induced CRC using
the AOM/DSS method (29, 30) in age-matched wild-type and
Akd+/−/− mice in 2 different genetic backgrounds (129Svcc
inbred and 129Svcc/CD1 hybrid) under a specific pathogen-
free environment (Fig. 3A). Azoxymethane is a potent carcino-
gen that reproduces the activating mutations in β-catenin
and KRAS (31, 32) and the inactivating mutations in adenom-
atosus polyposis coli (APC) that are observed in human CRC
(33, 34). Crucially, 5 of 12 (42%) wild-type mice did not develop
any tumors throughout the duration of the protocol, whereas
all Akd+/−/− mice developed tumors (Fig. 3B). Akd+/−/− mice
developed almost 4-fold the number of colorectal tumors as
wild-type mice in a 129Svcc background (number of Akd+/−/− tumors,
mean ± SEM = 7.6 ± 1.0; P = 0.0001; Fig. 3C, left). We found very similar results in the 129Svcc/CD1 background,
with a near 3-fold increase in tumor multiplicity in Akd+/−/− mice
( P = 0.0053, Fig. 3C, right). These results show that
Akd+/−/− mice are more susceptible to carcinogenesis and that
wild-type levels of Arkadia provide a protection against CRC.
This supports the hypothesis that mutations that simply
reduce AKD function in patients may contribute to the develop-
ment of CRC.

Enhanced tumor progression in Akd+/−/− mice

To characterize the tumor pathology from wild-type and
Akd+/−/− mice, all 23 tumors from wild-type mice and a total of
24 tumors from Akd+/−/− mice from a 129Svcc background were
subjected to histopathologic analysis. The lesions identified
in the colon of treated mice included tubular adenomas with low-
or high-grade dysplasia, intramusculosal carcinomas, and
invasive carcinomas (Table 2). Although the majority of tumors
from wild-type mice developed to intramusculosal carcinomas,
a significant proportion of tumors (13%) did not develop beyond
adenomas with high-grade dysplasia (Fig. 3D–F). In contrast,
all (24 of 24) Akd+/−/− tumors analyzed developed to intramu-
scosal carcinomas (Fig. 3G), with 1 Akd+/−/− mouse even har-
boring an invasive carcinoma (Fig. 3H). We also observed a
vessel invasion within a carcinoma from another Akd+/−/−
mouse (Fig. 3I).

The above analysis shows that lesions progress more rapidly
through the adenoma–carcinoma sequence in Akd+/−/− mice,
which may explain why a high proportion of wild-type mice did
not develop any gross tumors. Statistical analysis confirmed
that loss of 1 allele of Akd significantly correlated with the
progression of lesions from adenoma to intramusculosal
and invasive carcinomas ( P = 0.03; Table 2).

Tumors from Akd+/−/− mice exhibit reduced TGF-β–
mediated cytostasis and increased proliferation

We next examined the tumors at a molecular level to identify
the mechanisms involved in their enhanced progression. We
analyzed the expression of various genes by conducting
immunoblot and qPCR analysis on proteins and RNA extracted
from 6 wild-type (T^+/-T^+), 6 Akd+/−/− (T^+/-T^−) tumors
(Fig. 4A–C). Using the proliferation marker Pcna, we found that
tumors from Akd+/−/− mice (hereafter termed Akd+/−/− tumors)
$
\text{Table 1. Summary of the Q899STOP, P908S, D937N, and T943I mutations of AKD identified in the deep sequencing screen}

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Codon change</th>
<th>Amino acid change</th>
<th>Total frequency, % (reads)</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAG&gt;TAG</td>
<td>Q899STOP</td>
<td>2.76 (4,167)</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>CCA&gt;TCA</td>
<td>P908S</td>
<td>9.94 (4,167)</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>GAC&gt;AAC</td>
<td>D937N</td>
<td>4.30 (3,833)</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>ACT&gt;ATT</td>
<td>T943I</td>
<td>1.59 (3,833),1.76 (5,282)</td>
<td>32,6</td>
</tr>
</tbody>
</table>
tumors from wild-type mice (hereafter termed wild-type tumors; Fig. 4A).

As activation of Wnt/β-catenin signaling is critically involved in the proliferation of the regenerating colonic epithelium and in CRC, we examined whether differences in its activation underpinned this hyperproliferation. An antibody that specifically detects the active nuclear form of β-catenin (35) showed no difference between Akd+/−/C0 and wild-type tumors (Fig. 4A). Furthermore, the expression of the Wnt/β-catenin target oncogene c-Myc was also not significantly different between Akd+/− and wild-type tumors (Fig. 4C). These data suggest that an alternative mechanism underlies the more aggressive pathology observed in Akd+/− tumors.

As reduction in cytostasis can also lead to hyperproliferation and as this critically depends on TGF-β signaling in the colonic epithelium, we examined the levels of the p21 WAF cyclin-dependent kinase inhibitor in tumors (36). Indeed, this factor was significantly lower in all 6 Akd+/− tumors and in 2 of 6 wild-type tumors (Fig. 4D).
type tumors (Fig. 4B). However, we did not observe a corresponding decrease in mRNA expression of p21WAF in Akd\(^{+/−}\) tumors (Fig. 4C), suggesting a possible reduction in p21WAF protein stability in these tumors. In contrast, transcription of the cytostatic target p15\(\text{INK4b}\) was significantly reduced in Akd\(^{+/−}\) tumors (Fig. 4C). This reduction in p15\(\text{INK4b}\) expression preexists in the colon of untreated Akd\(^{+/−}\) mice (Fig. 2E). Together, the data suggest that reduced cytostasis is responsible for the hyperproliferation and enhanced progression of Akd\(^{+/−}\) tumors.

Furthermore, examination of the levels of Arkadia’s substrate SnoN showed that it is elevated in tumors and adjacent normal colon of Akd\(^{+/−}\) mice compared to wild-type mice (Fig. 4A and B). SnoN mRNA expression, however, was not significantly different between Akd\(^{+/−}\) and wild-type tumors (Fig. 4C), suggesting an increased stability of the SnoN protein in Akd\(^{+/−}\) tumors. Consistent with the molecular function of Arkadia, pSmad2, which complexes with SnoN and is also degraded by Arkadia, was also increased in Akd\(^{+/−}\) tumors (Fig. 4A and B). As the expression of the TGF-\(\beta\) target gene PAI-1, like p15\(\text{INK4b}\), was also reduced in Akd\(^{+/−}\) tumors (Fig. 4C), collectively, the above data strongly suggest that repression of the pathway is increased because of the reduction or loss of Arkadia from cells of Akd\(^{+/−}\) tumors.

Enhanced tumor progression in Akd\(^{+/−}\) mice is associated with reduction and not loss of Arkadia function

To address the distribution and level of TGF-\(\beta\) pathway repression in tumors, we conducted IHC to visualize SnoN and pSmad2 proteins in 6 Akd\(^{+/−}\) tumors and 4 wild-type tumors. We found that pSmad2 staining was stronger in Akd\(^{+/−}\) tumors.
and no difference in the cellular localization of pSmad2 in wild-type tumor expresses, which may vary between tumors. There was levels also depend on the amount of active ligand that each signaling in these cells (Fig. 5B and D).

significantly (score 1 in 3 of 4 tumors). This difference was statistically whereas expression in wild-type tumors was frequently weak (score 2 in 2 of 6 tumors) or strong (score 3 in 4 of 6 tumors), nuclear in Akd was cytoplasmic in wild-type tumors (3 of 4 tumors) and largely

levels in Akd were found to be nuclear in the cells that constitute the lining of the tubular structures of the adenocarcinoma from Akd mice, suggesting increased repression of TGF-β signaling in these cells (Fig. 5E and D). Both SnoN and pSmad2 were found to be nuclear in the cells that constitute the lining of the tubular structures of the adenocarcinoma from Akd mice, suggesting increased repression of TGF-β signaling in these cells (Fig. 5E and D).

To examine whether complete loss of Arkadia expression is associated with the enhanced progression of Akd tumors, we conducted qPCR on wild-type and Akd tumors and found that Akd tumors consistently express Akd but at only half the level of wild-type tumors (Fig. 4C). To determine whether this reduction is an average expression of all tumor cells rather than complete loss in a subset of cells, we conducted IHC for Arkadia in tumors (this antibody is raised against the C-terminus of AKD and is expected to recognize functional Arkadia proteins). We observed the presence of nuclear Arkadia protein in almost every cell lining the tubular structures of tumors from both genotypes (Fig. 5E and F), even in the most aggressive Akd tumors (Fig. 5F shows Arkadia staining on the same invasive adenocarcinoma shown in Fig. 3H). Therefore, even in the most advanced tumors, Arkadia is not lost and this therefore cannot account for the enhanced growth of Akd tumors. Collectively, the above data suggest that reduction of Arkadia rather than loss is responsible for the enhanced tumor progression.

Our findings also mirror reports that Smad4 and Tgfb1 haploinsufficiency promotes CRC development in a genetic background carrying Apc mutations (37–40), which alone causes adenomas (37, 41). Together, these studies emphasize that gene dosage of important components of the TGF-β pathway acts as key determinants in CRC susceptibility and supports the hypothesis that the tumor-suppressing properties of this pathway depend on its peak levels. Furthermore, TGF-β signaling responses and cytostasis are reduced in Akd mice, suggesting that Arkadia's tumor-suppressing properties in both the normal colonic epithelium and colorectal tumors are mediated by the enhancement of this pathway.

**Table 2.** Histopathology of wild-type and Akd colorectal tumors

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Tumors</th>
<th>Tumors analyzed</th>
<th>Adenomas</th>
<th>Intramucosal carcinomas</th>
<th>Invasive carcinomas</th>
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<td>5</td>
<td>—</td>
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<tr>
<td>Total</td>
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<td>23</td>
<td>3 (13%)</td>
<td>20 (87%)</td>
<td>—</td>
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<tr>
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<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Akd_7</td>
<td>10</td>
<td>5</td>
<td>—</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>24</td>
<td>—</td>
<td>23 (96%)</td>
<td>1 (4%)</td>
</tr>
</tbody>
</table>

NOTE: Tumors from all wild-type (n = 12) and all Akd (n = 7) mice (129Sv background) were subjected to histopathologic analysis. Colonies were examined along the entire proximal-distal axis. Abbreviations: n/a, not applicable; VI, vessel invasion (found in 1 tumor).
promote metastasis and progression. What regulates these different downstream TGF-β signaling effects remains largely unknown. In this study, we provide evidence that the E3 ubiquitin ligase Arkadia, whose major function is to enhance TGF-β signaling by degrading negative regulators of the pathway, exhibits tumor-suppressing properties in CRC (Fig. 3 and Table 2). We found that 2 wild-type alleles of Arkadia are required to maintain sufficiently low levels of these negative regulators (and therefore a high activity of the TGF-β pathway) and lower the susceptibility for CRC development and progression under oncogenic stress in mice (Figs. 4 and 5). We did not find evidence of complete loss of Arkadia in invasive tumors from Akd<sup>−/−</sup> mice, suggesting that the tumor-suppressing properties of TGF-β signaling depend on enhanced downstream responses. Furthermore, we screened for mutations that lower AKD function in patients with CRC exhibiting stabilization of SNON (26), a key repressor and substrate for AKD ubiquitination and degradation. We found several point mutations in the C-terminal functional domains of AKD that may account for the nuclear accumulation of SNON (Fig. 1 and Table 1). As this is associated with a more advanced tumor grade, AKD emerges as a likely tumor suppressor in CRC.

In the regenerating colonic epithelium, homeostasis is thought to be maintained by the Wnt/β-catenin pathway that promotes proliferation of epithelial cells (and therefore exhibits oncogenic potential) and the TGF-β pathway, which promotes differentiation, cytostasis, and apoptosis (tumor-suppressive properties; ref. 9). Mutations in Wnt/β-catenin signaling, such as the constitutive activation of β-catenin or the inactivation of APC that regulates β-catenin activity, lead to hyperproliferation and adenoma formation. However, for progression to CRC, it is clear that there is a requirement for cooperation with other pathways (3, 28, 42). Mutations that inactivate TGF-β signaling have been shown to contribute to CRC progression (37–39, 41). However, whereas activation of Wnt/β-catenin can be generated in a single hit (i.e., 1 allele of either APC or β-catenin), inactivation of a component of the TGF-β pathway usually has to occur in both alleles in the same cell. This is a very rare event and most likely occurs late after the accumulation of mutations that increase genomic instability. Gene amplification of negative regulators of the TGF-β pathway, such as SMAD6/7 and SNON/SKI, has been found in CRC (12, 13). However, amplification rarely occurs early enough...
to correspond to a tumor-promoting event, nor have single mutations leading to the overexpression of these factors been described.

In contrast, as Arkadia mediates the degradation of all the above negative regulators, and not only that of SNON, the simple reduction of its function by point mutations may lead to repression of TGF-β signaling responses and constitute an early tumor-promoting event. This is supported in this study by the identification of several missense mutations in AKD from CRC patients with stabilized nuclear SNON. One mutation, in particular Q899STOP, introduces a stop codon at the C-terminus that eliminates the final 100 amino acids harboring the ubiquitin ligase activity, but preserves the substrate recognition domains (Fig. 1B). This truncated AKD is more stable (Fig. 1D), binds to the substrates, and protects them from ubiquitination by wild-type AKD (Fig. 1C). In fact, a mutation that introduces a stop codon anywhere in the C-terminus between residues 889 and 978 (essential for enzymatic activity; Fig. 1B) would be expected to disrupt AKD function and exhibit a dominant-negative effect. Other missense mutations within this highly conserved region may also inactivate the enzymatic activity of Arkadia and result in a dominant-negative effect. The COSMIC database of somatic mutations in cancer reports 2 missense mutations in AKD (R904S and K915I), both in heterozygosity from primary ovarian tumors (27). The R904S mutation is located in the center of the TIER domain (Fig. 1B). Deletion of the TIER domain results in a dominant-negative form of Arkadia (Supplementary Fig. S1), suggesting that R904S AKD may also act in this fashion. Therefore, each AKD allele exhibits a large region sensitive to single-hit mutations, which could act as an early event in the progression of adenomas to CRC and perhaps in other tumors that depend on TGF-β tumor suppression.

In our carcinogenesis model, both wild-type and Akd+/− tumors displayed a similar level of nuclear β-catenin. However, only Akd+/− tumors showed nuclear accumulation of SnoN and pSmad2 (Fig. 5), suggesting that repression of TGF-β signaling by SnoN, and most likely Ski (not examined in this study), is associated with susceptibility to CRC development and increased progression. In human CRCs, we also reported a correlation between nuclear SNON/SKI accumulation and nuclear β-catenin (26). This suggests that tumors with Wnt/β-catenin activation, combined with mutations that increase SNON, lead to a more aggressive CRC pathology. Therefore, as mutations inactivating just 1 allele of AKD can lead to stable nuclear SNON, combination with nuclear β-catenin may provide an early prognostic marker.

![Figure 5. IHC for SnoN, pSmad2, and Arkadia in wild-type (wt) and Akd−/− tumors. Representative sections of adenocarcinomas stained with anti-pSmad2: (A) wild-type and (B) Akd−/−; anti-SnoN: (C) wild-type and (D) Akd−/−; and anti-Arkadia: (E) wild-type and (F) Akd−/−. All images are shown at 40× magnification; scale bars, 25 μm.](image-url)
In this study, we have shown a strong association between susceptibility to CRC and repression of TGF-β signaling responses caused by loss of 1 allele of Arkadia in mice and a dominant-negative functioning mutant of AKD from a human CRC patient. Interestingly, in Akd+/− colons and tumors, expression of the TGF-β target genes p21WAF and SnoN are not obviously affected, whereas p15INK4b, Pat-1, Tmepai, and Smad7 are reduced (Figs. 2E and 4C). Similarly, in Akd-null embryos and embryonic stem cells, not all TGF-β/Nodal target genes show equally reduced expression levels (20). Most likely SnoN/Ski do not repress all target gene promoters, suggesting that their targets are involved in the tumor-suppressing function of TGF-β signaling. As mutations in AKD do not completely inactivate all TGF-β signaling responses, this will increase the risk of TGF-β-mediated metastasis later, which most likely involves a different subset of target genes. Collectively, our data suggest that somatic mutations in a single AKD allele that reduce its function could act as an early tumor-promoting event in human CRC development and progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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