# The Previously Undescribed ZKSCAN3 (ZNF306) Is a Novel "Driver" of Colorectal Cancer Progression

Lin Yang,[1] Stanley R. Hamilton,[2] Anil Sood,[4] Toshio Kuwai,[1] Lee Ellis,[3] Angela Sanguino,[1] Gabriel Lopez-Berestein,[4] and Douglas D. Boyd[1]

Departments of [1]Cancer Biology, [2]Pathology, [3]Surgical Oncology, and [4]Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

## Abstract

**A relatively new view of colorectal cancer is that its development/progression reflects the contribution of a large set of altered gene products in varying combinations, each providing a "fitness advantage." In searching for novel contributing gene products using Unigene cluster data mining, we found overrepresentation of expressed sequence tags corresponding to a previously uncharacterized gene (*ZKSCAN3*) in colorectal tumors. *ZKSCAN3* was pursued for several reasons: (*a*) its sequence similarity with *bowl* required for *Drosophila* hindgut development; (*b*) it lies in a chromosomal region (6p22.1) amplified in colorectal cancer; and (*c*) its coding sequence predicts tandem $C_2H_2$ zinc finger domains present in a class of proteins gaining attention for their role in oncogenesis/tumor progression. Reverse transcription-PCR confirmed overexpression in colorectal tumor tissue compared with adjacent nonmalignant mucosa due in part to gene amplification determined by Southern blotting. Further, immunohistochemistry with an antibody generated to the predicted protein sequence revealed higher ZKSCAN3 expression in invasive compared with noninvasive tumors. Intriguingly, the ZKSCAN3 protein was also expressed in tumors wild-type for genes (*APC, p53, K-Ras*) commonly targeted in colorectal cancer. ZKSCAN3 knockdown in two independent colon cancer cell lines impaired anchorage-independent growth and orthotopic tumor growth, whereas overexpression in a third cell line had the opposite effect and increased 5-fluorouracil resistance. Liposomal delivery of a ZKSCAN3-targeting small interfering RNA reduced tumorigenicity of orthotopic colon cancer. Thus, the hitherto uncharacterized ZKSCAN3 adds to an expanding set of encoded products contributing to the progression of colorectal cancer.** [Cancer Res 2008;68(11):4321–30]

## Introduction

Sporadic colorectal cancer, afflicting 145,000 persons per year in the United States (1), largely reflects aberrantly activated pathways leading to unrestrained growth. In the Wnt pathway, APC truncations stabilize β-catenin, which translocates to the nucleus, and with T-cell factor-4 (TCF-4) and lymphoid enhancer factor-1 (LEF1) DNA-binding proteins (2) *trans*-activate target genes (3) causal for growth. Mutation-activated *K-Ras* (4) also promotes tumor growth via the mitogen-activated protein kinase (MAPK) pathway. Conversely, mutation of the type II transforming growth factor-β receptor gene (*TGF-β RII*), yielding a truncated protein, disables signaling through the *MADH*-encoded Smad transcription factors (5), thus failing to restrain growth. Paradoxically, this pathway also contributes to tumor progression (6). In the p53 pathway, mutation inactivation of the tumor suppressor renders damaged cells unable to arrest for DNA repair and these cells are not eliminated by apoptosis (7), leading to accumulated DNA damage and mutation of key genes crucial to tumor development/progression.

Notwithstanding these landmark observations, whereas the aforementioned encoded products are clearly implicated in colorectal pathogenesis, emerging studies suggest that the heterogeneity of this disease probably also involves the contribution of multiple other gene products (8, 9) acting in various combinations to promote cancer development and progression. For example, an analysis of 340 serine/threonine kinases revealed that 40% of colorectal tumors had alterations of genes in the MAPK kinase/c-jun amino-terminal kinase kinase 1 and phosphatidylinositol 3-kinase (PI3K) pathways (10). The functional consequence of at least one of these mutations in PI3KCA (encoding the catalytic subunit of PI3K) was a reduced dependence on growth factors, attenuated apoptosis, and augmented tumor invasion (11). Additionally, two independent genome-wide scans identified a novel, low-penetrance colorectal cancer susceptibility locus at chromosome 8q24 containing an uncharacterized gene (9, 12). Indeed, on analyzing the coding regions of some 18,191 genes, Wood and coworkers (8) concluded that the genomic landscape of colorectal cancer is composed of a few commonly targeted gene "mountains" with a much larger number of gene "hills" altered at low frequency, emphasizing the heterogeneity and complexity of this disease. These findings, together with the observations that mice mutated (13–15) for some of the genes (*p53, K-Ras, TGF-β RII*) commonly targeted in this disease rarely develop colorectal cancer and that Li-Fraumeni patients defective for p53 fail to show a higher colorectal cancer incidence (16), have led investigators to propose that multiple gene products, acting in various combinations, contribute to colorectal cancer development/progression. In a search for other "drivers" of colorectal cancer progression, we have identified ZKSCAN3, related to *brother of odd with entrails limited* (*bowl*) required for *Drosophila* hindgut development (17) as a novel gene product promoting the progression of this malignancy.

## Materials and Methods

**Unigene cluster analysis.** ZKSCAN3 expressed sequence tag (EST) overrepresentation, as determined by Unigene Cluster analysis,[5] is as

**Requests for reprints:** Douglas Boyd, Department of Cancer Biology, Box 173, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-563-4918; Fax: 713-563-5489; E-mail: dboyd@mdanderson.org.



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[5] http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene

follows. The approximate frequency of a Unigene cluster occurrence within each tissue is calculated[6] as follows: $A = B / C$, where $A$ is the frequency of the Unigene cluster expression within the tissue, $B$ is the number of Unigene clustered clones within the tissue, and $C$ is the total number of unique clones from the same tissue in the Unigene database. The frequencies within each tissue are summed ($D = A_1 + A_2 + A_3 + \ldots$) to find the total expression within Unigene, where $D$ is the summation of Unigene cluster expression frequencies by tissue and $A_1$, $A_2$, $A_3$, etc., is the frequency of Unigene cluster expression in tissue 1, 2, 3, etc., respectively. Normalized Unigene cluster expression within a tissue is derived by dividing the frequency of the Unigene cluster occurrence in a tissue by the summation of Unigene cluster expression frequencies by tissue (i.e., $A/D$).

**Reverse transcription-PCR analysis of resected colorectal cancers.** Total RNA was prepared from colorectal tissue (50 mg) in TRIzol (Invitrogen) according to the manufacturer's protocol. RNA (20 μg) was treated (37°C, 25 min) with 2 units/μL TURBO DNA-free DNase enzyme (Ambion), and after DNase inactivation, cDNA was synthesized with avian myeloblastosis virus reverse transcriptase. PCR was done using primers for ZKSCAN3 [100 ng each RT-5: 5′-GGCCCTGACCCTCACCCC-3′; RT-3: 5′-CAGATGTGCCGCCTCCCTCC-3′, reverse transcription-PCR (RT-PCR)], β-actin (10 ng), 1 unit Taq polymerase, and 30 amplification cycles. ZKSCAN3 primers were located in exons 6 and 7, respectively, with a PCR product of 294 bp.

**Generation of an anti-ZKSCAN3 antibody.** Hydrophobicity of the ZKSCAN3 (ZNF306) protein sequence was analyzed by a Kyte-Doolittle Hydropathy Plot. A Blast search indicated that the EGSRERFRGFRYPE was unique and this keyhole limpet hemocyanin–conjugated peptide (200 μg) was injected six times into New Zealand White rabbits. Antiserum was affinity purified using the peptide coupled to CNBr-activated Sepharose 4B and bound antibody was eluted with 0.2 mol/L glycine (pH 1.85).

**Immunohistochemistry.** After dewaxing and antigen retrieval, endogenous peroxidase was inactivated with $H_2O_2$ and slides were blocked with 5% normal horse serum/1% normal goat serum. Sections were incubated with the indicated antibodies: 1 μg/mL affinity-purified anti-ZKSCAN3 (ZNF306), an anti–β-catenin antibody (1:500; Cell Signaling Technology), and a horseradish peroxidase–conjugated secondary antibody. Immunoreactivity was detected with the 3,3′-diaminobenzidine chromogen (Research Genetics). For negative controls, the anti-ZKSCAN3 antibody was substituted with an equivalent amount of preimmune IgG. For nuclear counterstaining, we used Hoechst 33258. For histomorphometry, the percentage of ZKSCAN3-positive nuclei in four fields per patient from four independent patients was determined and differences were tested for statistical significance using the Mann-Whitney $U$ test.

***In vitro* short hairpin RNA knockdown of ZKSCAN3.** The ZKSCAN3 (ZNF306) mRNA-targeting sequence (TATCGTGCCACCTGAGAGA) cloned into the pSUPERIOR.retro.puro vector (OligoEngine VEC-IND-0010) was transfected into AmphoPack 293 cells and the medium containing the retroviral-ZKSCAN3-Short hairpin RNA (shRNA) particles was used for transductions. Puromycin-selected transduced clones (>3) showing reduced ZKSCAN3 mRNA levels were pooled.

**Soft agar assay.** Equal volumes of 1.2% melted agar (DNA grade) and 2× McCoy's medium were mixed (40°C) and dispensed into a six-well plate at 1.5 mL/well to form the bottom layer (0.6% agar). A 0.6% agar solution maintained at 40°C was mixed with an equal volume of prewarmed 2× McCoy's culture medium supplemented with the indicated cell number and 1.5 mL were dispensed into the same six-well plate to form the top layer (0.3% agar). The cultures were incubated at 37°C in a humidified incubator for 14 d.

**Orthotopic tumor model.** Cells (>95% viability; $10^6$ in 50 μL) were injected into the cecal wall of male athymic nude mice as described previously (18). All experiments were approved by the Institutional Animal Care and Use Committee.

**Liposomal encapsulation of ZKSCAN3 (ZNF306)–targeting small interfering RNA.** Small interfering RNA (siRNA) sequences were 5′-AATATCGTGCCACCTGAGAGA-3′ and 5′-AATTCTCCGAACG-TGTCACGT-3′ as ZKSCAN3-targeting and control siRNA, respectively. siRNAs were dissolved in buffer [100 mmol/L potassium acetate, 30 mmol/L HEPES KOH, 2 mmol/L magnesium acetate (pH 7.4)] to a final concentration of 1 μg/μL, heated to 90°C for 60 s, and incubated at 37°C for 60 min. Liposome preparation for *in vivo* delivery was as previously described (19). Briefly, siRNA and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Avanti) at a ratio of 1:10 (w/w) and Tween 20 were mixed in the presence of excess *t*-butanol (Sigma); vortexed; frozen in an acetone, dry ice bath; and finally lyophilized. Lyophilized liposomes were stored −20°C until needed. Before administration, they were hydrated with normal (0.9%) saline.

**Metastases model.** Viable tumor cells ($10^6$ in 0.1 mL HBSS) were injected into the exteriorized spleen. After 6 wk, the mice were killed and the presence of liver disease was determined.

**Southern blotting.** Southern blotting was done as described by us previously (20). Purified genomic DNA was *Bgl*II/*Eco*RV–digested and fragments were resolved in an agarose gel. After the transfer, the membrane was probed with radioactive cDNAs specific for *ZKSCAN3* (ZNF306) or *BAGE3* (residing at 21p11.2, a region unaltered in colon cancer); the latter was used for normalization.

## Results

**ZKSCAN3 overexpression in colorectal cancer.** In querying the Unigene Cluster database (Build 198, released 2007-01-13),[5] ESTs corresponding to a novel transcript (ZKSCAN3) were overrepresented in this malignancy. ZKSCAN3 was pursued as a potential driver of colorectal cancer progression for several reasons: (*a*) the gene lies in a chromosomal region (6p22.1) amplified in some colorectal cancers (refs. 21, 22); (*b*) a BLAST search revealed partial identity ($E = 7 \times 10^{-33}$) with the *bowl* gene required for *Drosophila* hindgut (precursor of the large intestine) elongation (17); and (*c*) the coding sequence predicts tandem $C_2H_2$ zinc finger domains present in a class of proteins gaining attention for their role in oncogenesis/tumor progression (23–25).

mRNA semiquantitation in randomly selected, sporadic colorectal cancers revealed elevated transcript levels in tumors (*) of six of nine patients compared with matched nonmalignant controls (Fig. 1*A*). To rule out tissue cellularity differences as the reason for the differential in mRNA amount, we performed immunohistochemistry using an antibody generated (and validated) to a peptide (EGSRERFRGFRYPE) unique to the predicted ZKSCAN3 protein (see Supplementary Data S1). We then performed immunohistochemistry on stage IV and II colorectal tissue microarrays (stage IV and II sections shown in Fig. 1*B* and Supplementary Data S2, respectively). Nonmalignant adjacent tissue showed diminished ZKSCAN3 immunoreactivity. Of the 11 stage IV patients, all showed ZKSCAN3-positive tumor cell nuclei. Interestingly, tumor cell nuclei (*arrows*) of invasive cancers showed pronounced ZKSCAN3 immunoreactivity (Fig. 1*B*, patients 11, 12, 13, and 15) compared with matched, noninvasive (intramucosal) tumors [histomorphometric analysis, 78 ± 17% and 14 ± 11% (average ± SD) for the two groups, respectively ($P < 0.0001$)]. Further, stage IV tumor cells showed a greater percentage (52 ± 18 and 9 ± 10, respectively; $P < 0.0001$) of ZKSCAN3-positive nuclei compared with the ZKSCAN3-positive tumors for the stage II group (Supplementary Data S2) for which 8 of 11 showed ZKSCAN3-positive tumor cell nuclei. Thus, the ZKSCAN3 expression is elevated in colorectal cancer and correlates with invasiveness.

[6] http://smd.stanford.edu/help/SOURCE/normalization.html

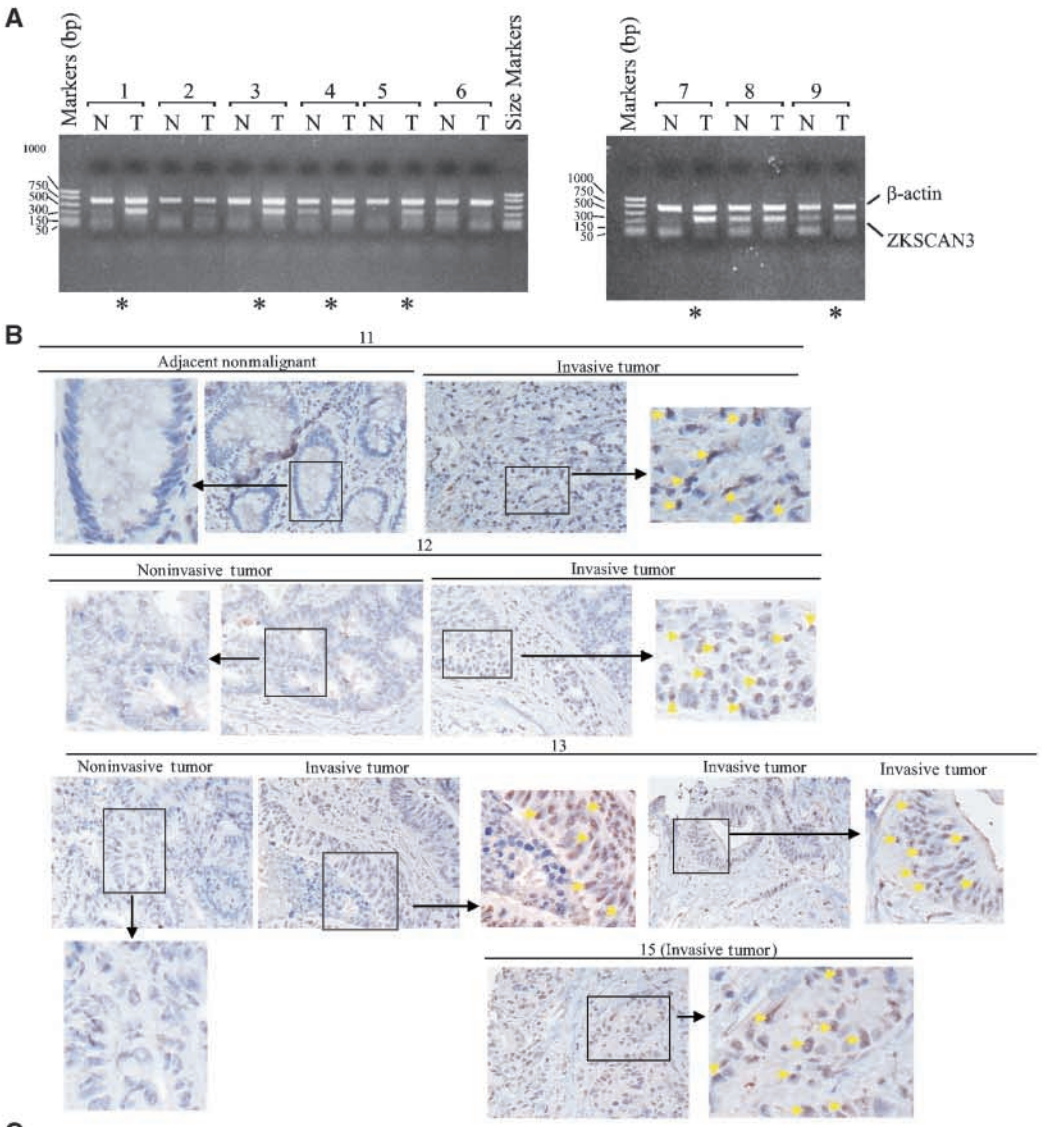


**Figure 1.** ZKSCAN3 overexpression in resected colorectal cancers. *A,* total RNA (20 μg) from colorectal tissue was analyzed by multiplex RT-PCR with β-actin and ZKSCAN3 primers spanning exons 5 and 6. *T,* tumor; *N,* nonmalignant adjacent mucosa. *B* and *C,* tissue sections were incubated with affinity-purified anti-ZKSCAN3 antibody or an anti–β-catenin antibody. After washing, sections were incubated with HRP-conjugated goat anti-rabbit antibody and immunoreactivity was visualized with DAB.

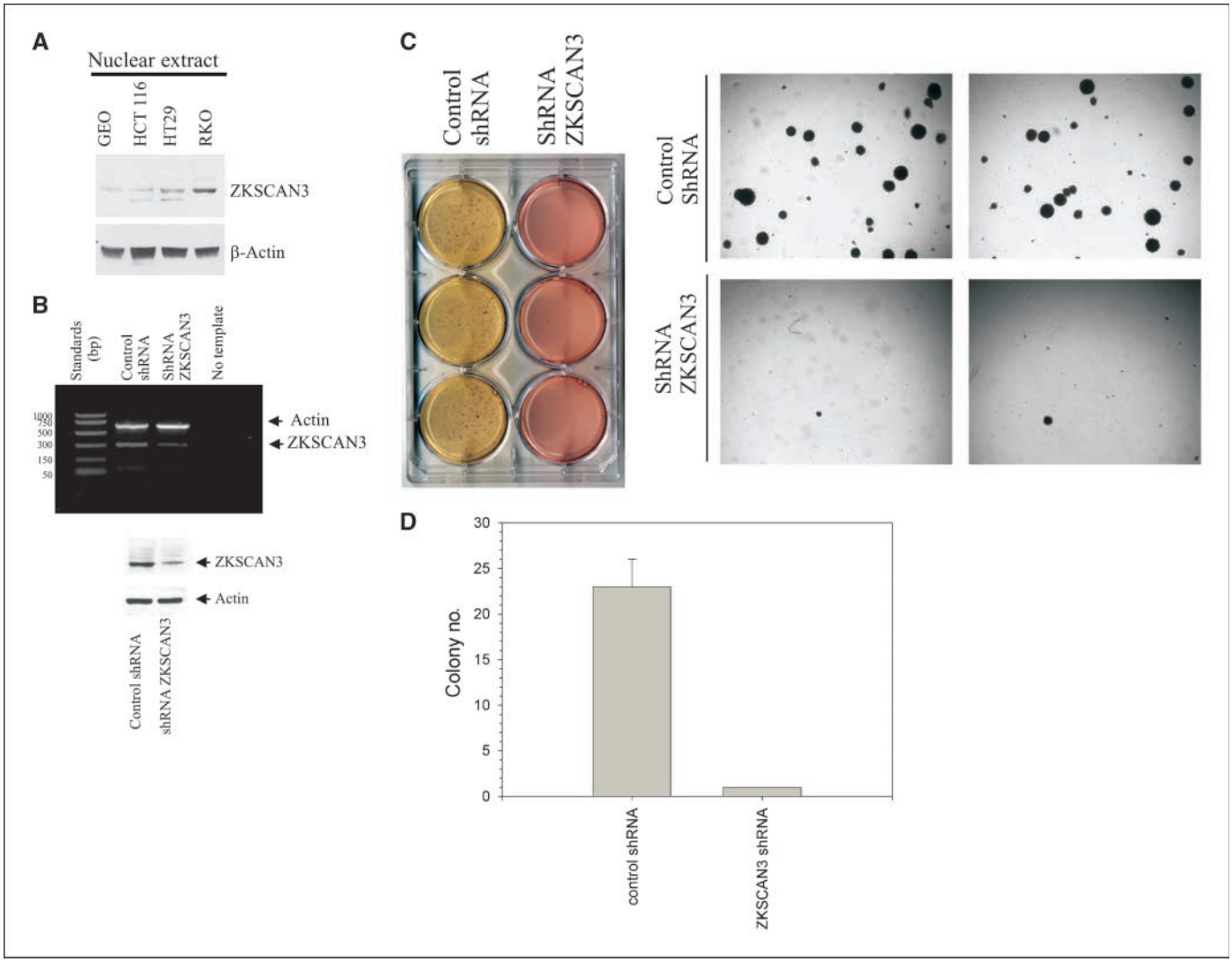


**Figure 2.** ZKSCAN3 knockdown in RKO colon cancer cells reduces anchorage-independent growth. *A,* Western blotting of nuclear extract using the affinity-purified anti-ZKSCAN3 antibody. *B* to *D,* RKO cells retrovirally transduced with either a shRNA targeting ZKSCAN3 or a control shRNA were puromycin selected, and pooled clones analyzed for ZKSCAN3 mRNA levels by multiplex RT-PCR and Western blotting (*B*) or 5,000 cells were seeded in soft agar (*C* and *D*) and colonies were counted 14 d later. *Columns,* average values of three experiments; *bars,* SD (*D*).

Interestingly, ZKSCAN3 was also expressed (Fig. 1*C*) in colorectal tumors genotyped[7] as concurrently wild-type for *APC, K-Ras*, and *p53* with four of five patients showing nuclear ZKSCAN3 (Fig. 1*C*, zoomed, *arrows*) concurrent with nonnuclear β-catenin (confirming a silent Wnt pathway).

The increased ZKSCAN3 overexpression evident in the randomly selected (nongenotyped) colorectal tumors was partly due to gene amplification as evident in Southern blotting (Supplementary Data S3). A band (Supplementary Data S3A) of the predicted size (1.27 kb) was evident and primary tumors from patients 23, 24, and 26 showed 1 to 2 additional gene copies. DNA from liver metastases also showed amplification with 4 additional copies for patient 31 and 3 extra copies for patients 32 and 34. The *BAGE3* gene (at 21p11.2 unaltered in colon cancer) was used for normalization. Thus, gene amplification results in increased ZKSCAN3 protein in some patients, consistent with a high-resolution comparative genomic hybridization (CGH) study showing gain of the chromosomal region (6p22.1) harboring the *ZKSCAN3* gene in a subset of colorectal tumors.[8]

**ZKSCAN3 knockdown or overexpression modulates colorectal cancer progression.** To determine the causal role of ZKSCAN3 in tumor progression, we then determined the effect of silencing expression of this gene on tumorigenicity using RKO colon cancer cells showing the highest endogenous ZKSCAN3 expression (Fig. 2*A*). RKO cells are wild-type for *p53, APC, K-Ras, β-catenin*, and *MADH4* (26, 27). RT-PCR and Western blotting indicated ~70% knockdown of endogenous ZKSCAN3 (Fig. 2*B*)

[7] Y. Suehiro, et al. Epigenetic-genetic interactions in colorectal carcinoma: association of G-to-A transition mutations in the APC gene with concurrent promoter hypermethylation of APC and the MGMT or hMLH1 gene. Clin Cancer Res. In press 2008.

[8] Personal communication with Dr. Manuela Gariboldi, Tumor Institute, Milan, Italy.

in RKO cells transduced with a retrovirus encoding a ZKSCAN3-targeting shRNA. Strikingly, ZKSCAN3 repression markedly reduced anchorage-independent growth (Fig. 2*C* and *D*). Note the yellow color of the pH indicator suggesting robust growth (anaerobic conditions) with control shRNA-expressing cells in contrast to the orange color (aerobic conditions) with the ZKSCAN3-knocked down cultures (Fig. 2*C*). Similar results were achieved with an independent pool of ZKSCAN3-targeting siRNAs (Supplementary Data S4). Reduced colony number was not due to slower monolayer proliferation (data not shown). Corroborating these data, nude mice injected orthotopically with RKO cells transduced with the control shRNA showed large tumors (circumscribed area), whereas cells knocked down for ZKSCAN3 formed smaller tumors (Fig. 3*A* and *B*). RT-PCR confirmed ZKSCAN3 mRNA knockdown in pooled tumor tissue from the latter group (Fig. 3*C*). We then determined if colon cancer cells bearing some of the genetic mutations common in this malignancy were also sensitive to ZKSCAN3 suppression. Indeed, HT29 mutated for *p53, APC*, and *MADH4* also showed reduced *in vitro* tumorigenicity in response to ZKSCAN3 knockdown (Supplementary Data S5).

We then determined if, conversely, forced ZKSCAN3 overexpression stimulates tumor progression. HCT 116 colon cancer cells (wild-type for *p53, APC* but mutated for *K-Ras* and

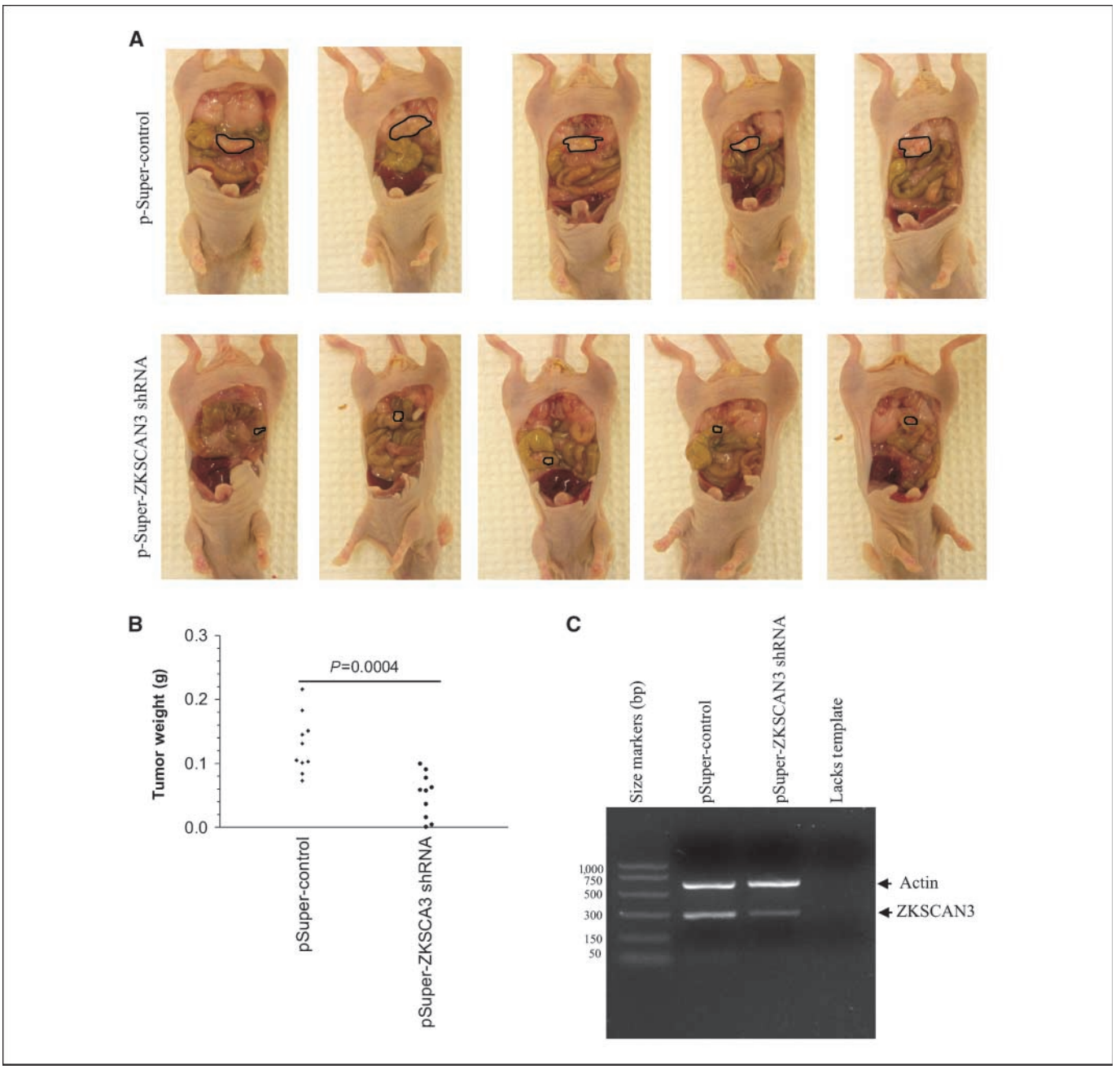


**Figure 3.** ZKSCAN3 knockdown in RKO colon cancer cells reduces tumorigenicity. RKO cells transduced and selected as in Fig. 2 were injected intracecally; tumors (circumscribed area in *A*) were harvested 4 wk later and either weighed (*B*) or portions of each were pooled and analyzed for ZKSCAN3 mRNA by RT-PCR (*C*).

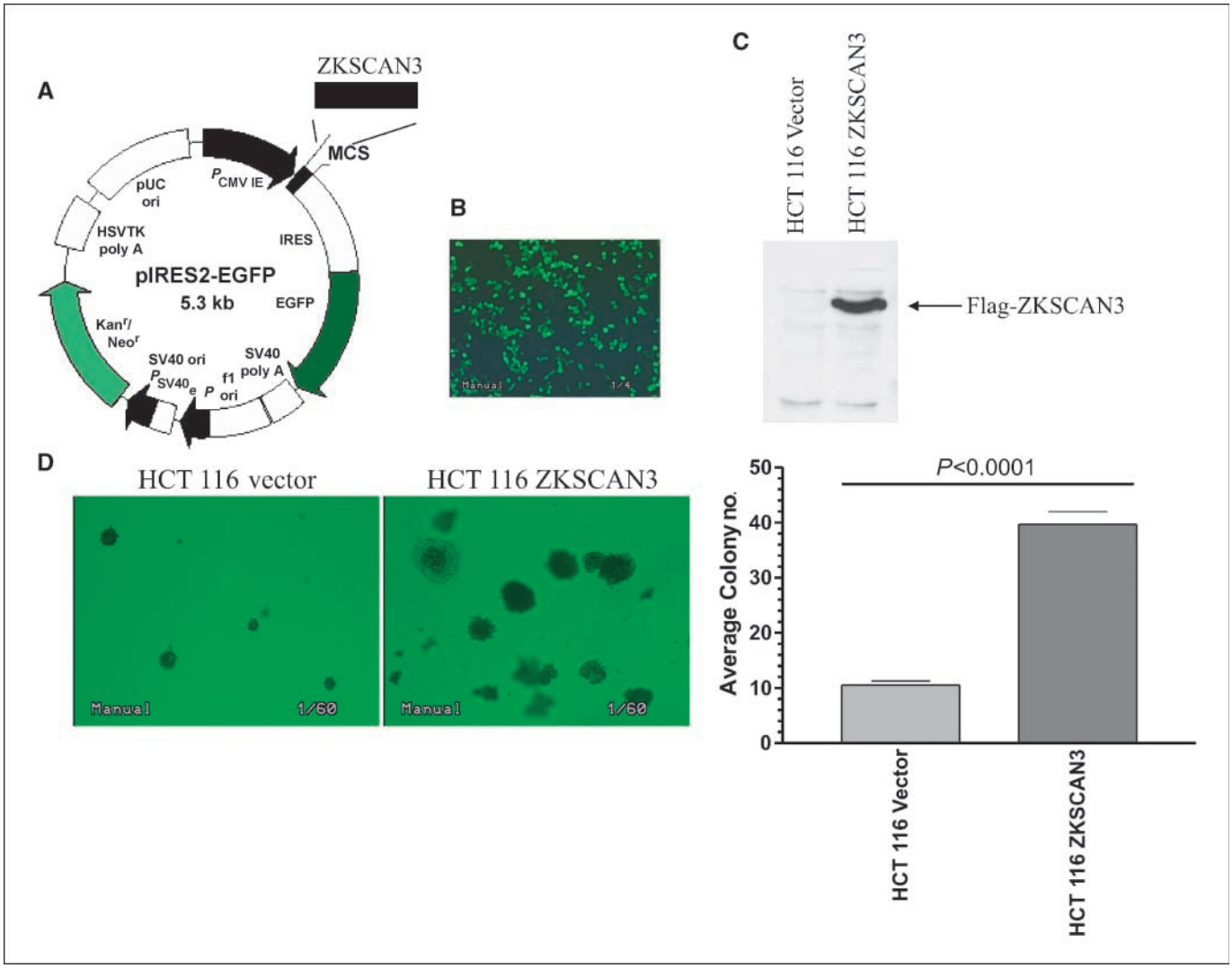


**Figure 4.** ZKSCAN3 overexpression stimulates anchorage-independent growth. $NH_2$-terminus flag-tagged ZKSCAN3 subcloned into the pIRES2-EGFP bicistronic vector (*A*) was transfected into HCT 116 cells and a G418-resistant, GFP-positive pool (*B*) analyzed for ZKSCAN3 expression (*C*) using an anti-Flag antibody. *D,* colony formation from cells (80,000) grown in agar for 14 d. *Columns,* average colony number (six fields); *bars,* SD.

*β-catenin*)[9] expressing modest ZKSCAN3 levels (Fig. 2*A*) were transfected with a Flag-tagged ZKSCAN3 subcloned into a bicistronic enhanced green fluorescent protein (EGFP)–encoding vector (Fig. 4*A*). Western blotting of G418-resistant, GFP-positive cells (Fig. 4*B*) confirmed Flag-tagged ZKSCAN3 expression (Fig. 4*C*). Anchorage-independent growth of this pooled population showed a 3- to 4-fold augmentation (Fig. 4*D*) in colony number ($P < 0.0001$). In orthotopic experiments, 5 of 5 mice inoculated with the ZKSCAN3-expressing HCT 116 pool were positive for histologically confirmed tumors compared with only 3 of 10 mice receiving the parental/vector controls (Fig. 5*A*) and tumor size was dramatically increased with the former group. RT-PCR confirmed ZKSCAN3 transcript overexpression in pooled tumor tissue from the ZKSCAN3 cDNA transfectants (Fig. 5*B*). ZKSCAN3 also modulated the later steps in tumor progression with liver tumor foci evident in more (9 of 10 versus 1 of 20) animals receiving the ZKSCAN3 cDNA-expressing cells intrasplenically compared with the parental/empty vector–bearing HCT 116 cells (Fig. 5*C*). Expression of the zinc finger protein also increased the number of liver tumor foci (Fig. 5*C*). Further, diminished sensitivity to 5-fluorouracil (5-FU), the frontline drug used in this cancer, was apparent in ZKSCAN3 cDNA-expressing HCT 116 cells (Fig. 5*D*) with ~50% of the ZKSCAN3-expressing cells surviving treatment (compared with <20% for vector controls). Together, these data suggest that ZKSCAN3 expression contributes to both early and late steps of colorectal cancer progression.

**Liposomal siRNA delivery reduces *in vivo* tumorigenicity.** Because ZKSCAN3 is expressed in colorectal tumors unaltered for *APC, K-Ras*, and *p53* (Fig. 1*C*), we determined if liposomal-delivered ZKSCAN3-targeting siRNA diminishes tumorigenesis of cells (RKO) wild-type for these genes. Mice treated with liposomal-encapsulated ZKSCAN3 siRNA were smaller (circumscribed region in Fig. 6*A, left*), demonstrating a 50% reduction ($P < 0.002$) in tumor weight compared with tumors receiving the nonsilencing siRNA (Fig. 6*A, right*). Diminished endogenous ZKSCAN3 protein was

[9] http://www.sanger.ac.uk/genetics/CGP/CellLines/

evident in tumors from animals receiving the ZKSCAN3-targeting siRNA (Fig. 6*B*), consistent with effective delivery of the siRNA (Fig. 6*C, arrows*) as determined with Alexa-tagged siRNA. This preclinical success raises the possibility that targeting this transcript in patients with ZKSCAN3-overexpressing tumors could be therapeutically beneficial.

## Discussion

Although the role of a relatively small set of genes (*p53, K-Ras, APC, β−catenin*, and *TGF-β receptor*) in colorectal cancer development and progression is well established, emerging studies (8–10, 12, 24, 25, 28, 29) have suggested that other gene products also contribute to the pathogenesis and progression of this disease by providing a "fitness advantage." Thus, a current viewpoint is that colorectal cancer progression is the consequence of various combinations of a large number of gene products, each providing some advantage with respect to tumor growth/survival. We report herein the previously undescribed ZKSCAN3, related to *bowl*, a zinc finger protein required for *Drosophila* hindgut development, as a new player in colorectal cancer, contributing to the progression of this malignancy. Interestingly, our findings that ZKSCAN3 is also overexpressed in a subset of colon cancers genotyped as wild-type for genes (*APC, K-Ras, p53*) commonly targeted in this malignancy and that interfering with its expression in colon cancer cells (RKO) wild-type for these genes suggest that ZKSCAN3 expression may also contribute to tumor progression for a subset of colorectal malignancies unaltered for these genes.

A protein homology search indicated several related proteins with sequence similarity varying between 16% and 84% (DNASTAR Lasergene MegaAlign v7). The closest relative is ZKSCAN4 with 84% similarity; however, a query of the scientific literature indicated a paucity of information as to its function and role, if any, in cancer development or progression. Nevertheless, ZKSCAN3 bears tandem repeated zinc fingers, and recent studies reporting a high mutation rate in this class of proteins in breast cancer (25) have stimulated interest in the role of this diverse set of proteins in malignancy. Kruppel-like factor 4 (KLF4) bearing 46.7% similarity

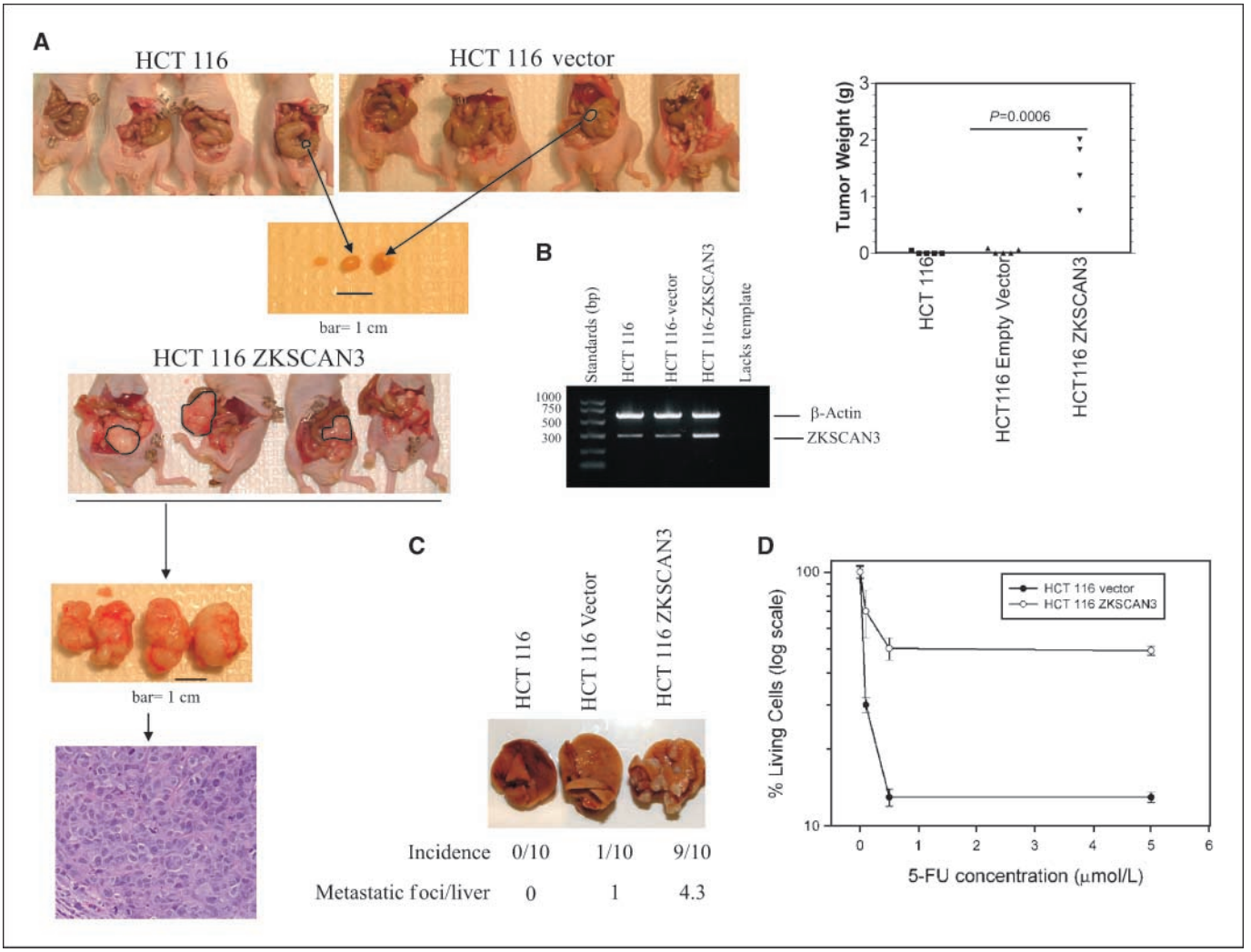


**Figure 5.** ZKSCAN3 overexpression stimulates *in vivo* tumorigenicity and tumor progression. *A,* cells ($10^6$) were injected intracecally. After 7 wk, mice were sacrificed, tumors (circumscribed area) were weighed, and differences were tested for statistical significance (unpaired *t* test). H&E-stained sections were examined histologically. *B,* ZKSCAN3 mRNA semiquantitation by RT-PCR using pooled tumors. *C,* the indicated tumor cells ($10^6$) were injected into the spleen and 6 wk later, mice were sacrificed and metastatic foci were enumerated. *D,* cells were treated with 5-FU for 6 d and trypan blue–negative cells were counted. *Points,* average values of six determinations; *bars,* SD.

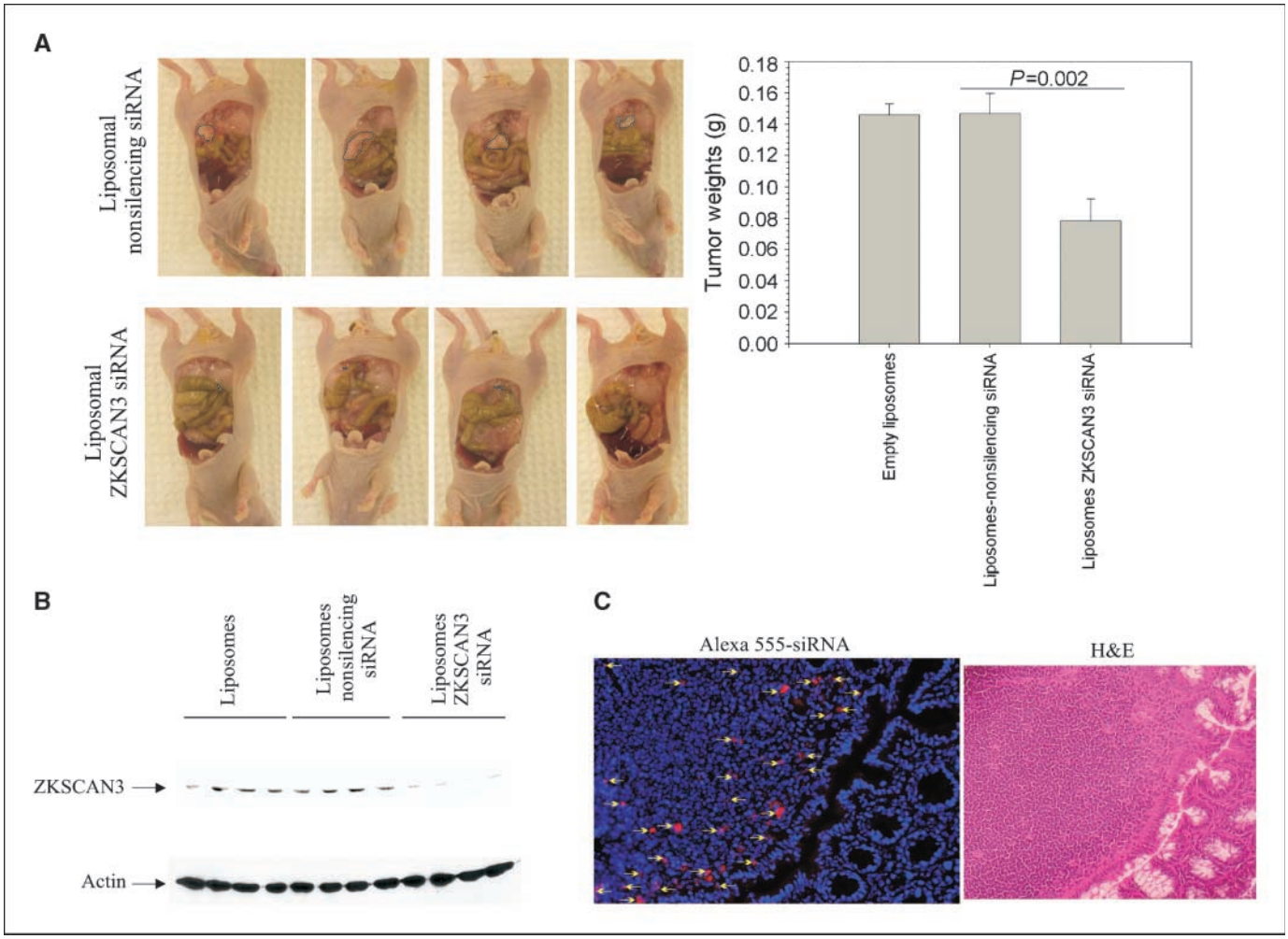


**Figure 6.** ZKSCAN3 knockdown by liposomal-delivered siRNA reduces tumorigenicity. RKO cells ($10^6$) were injected per mouse (10 per group) and after 1 wk, mice were treated via the tail vein with liposome-siRNA (150 μg/kg) 2× per week for 4 wk and sacrificed. Tumors (*A*, circumscribed area) were weighed and a portion was subjected to Western blotting (*B*). *C,* RKO cells ($10^6$) were injected; 5 wk later, Alexa 555–tagged siRNA ZKSCAN3 (150 μg/kg) was injected once via the tail vein. Mice were sacrificed 24 h later, tumors were snap frozen, and sections were either viewed for fluorescence or H&E stained.

with ZKSCAN3 promotes malignant progression in breast cancer (30), although, paradoxically, it reduces tumorigenicity of colon cancer (31). Overexpression of the zinc finger protein ZNF148, sharing 26% similarity with ZKSCAN3 in Min mice, attenuated intestinal tumor development (23); these results resemble XAF1, another tandem zinc finger repeat protein sharing 17% similarity with ZKSCAN3, which suppresses tumor growth and enhances the apoptotic response to a variety of agents (24). In contrast, and like ZKSCAN3, some zinc finger–bearing proteins augment tumorigenicity. A case in point is *TIPUH1*, sharing 41% similarity with ZKSCAN3 and encoding a 500-amino-acid protein containing 12 zinc fingers and a KRAB domain. This gene is up-regulated in hepatocellular carcinoma and induces *in vitro* tumorigenicity as evident by increased anchorage-independent growth of non-transformed cells (32).

Our data suggest that ZKSCAN3 overexpression is due, at least in part, to gene amplification, which is perhaps not surprising considering that the gene resides at chromosome 6p22.1, a region that, by previous independent CGH analyses, shows gain and amplification in some colorectal cancers (21, 22). The finding of gene amplification is reminiscent of the candidate oncogene, *ZNF217*, another zinc finger–bearing protein, the corresponding gene located in a highly amplified region on chromosome 20q1.2 (33). However, for ZKSCAN3, based on other reports, it is likely that the gain in this arm of chromosome 6p is a late event. Thus, a CGH study showed 85% Dukes D tumors showing gain in chromosome 6p in contrast to <20% tumors staged at Dukes C (22). This notion is given further credence by a meta-analysis of 895 primary and metastatic colon cancers, suggesting again that chromosome 6p gain is a late genetic event (34). Indeed, these prior reports would suggest that other mechanisms also contribute to ZKSCAN3 overexpression in the colorectal tumors. Thus, we entertain the notion that ZKSCAN3 expression is also probably regulated by posttranscriptional/posttranslational mechanisms (35, 36) and these could well contribute to its overexpression especially in the primary colorectal tumors. Certainly, altered levels of other genes, including *VEGF* (37), the matrix metalloproteinase *MMP-9* (38), and the urokinase receptor (39) achieved via posttranslational modulation has been well documented by others. Another possibility is that ZKSCAN3 levels are regulated epigenetically. However, we failed to observe any obvious CpG islands in 2 kb of upstream sequence arguing against the scenario that loss of DNA

methylation provides a means of up-regulating ZKSCAN3 expression in colorectal cancer.

As to the mechanism of action, we queried whether ZKSCAN3 intersected with the Wnt, p53, or TGF-β pathways implicated in colon cancer development/progression. However, at least in transient assays (Supplementary Data S6) using reporters for these three pathways, we found no evidence that ZKSCAN3 targets the Wnt or TGF-β modules although we observed a modest attenuation (~30%) of p53 signaling in response to ZKSCAN3 overexpression. Notwithstanding these findings, based on precedents with other zinc finger proteins and its nuclear localization evident in immunohistochemistry, ZKSCAN3 may be regulatory for gene expression. Indeed, Zfp-38, encoded by *ZKSCAN21*, and bearing 43% identity with ZKSCAN3, is a strong transcriptional activator (40). Further, ZNF 383 and ZNF 436 (51% and 43% similarity index compared with ZKSCAN3, respectively), repress transcriptional activity from synthetic promoters composed of activator protein-1 and the serum response elements (40, 41), although such a mechanism would seem counterintuitive for ZKSCAN3, which promotes tumor progression. However, other possibilities must be considered. Tandem zinc fingers also function in protein-protein interactions. The more distant relative, LIM domain only 4 protein, which induces mammary cell invasion, bears a LIM domain composed of tandem zinc fingers, the latter allowing this protein to act as an adaptor for multiprotein complex assembly (42). Protein-protein interactions also may yield protein sequestration as with XAF1, which renders the proapoptotic XIAP inaccessible (24).

Does ZKSCAN3 contribute to the progression of other cancers? Interestingly, gain in the chromosomal region (6p22.1) harboring ZKSCAN3 is pronounced in at least three nongastrointestinal tumor types. High-resolution CGH showed a gain of 6p22.1 in 5 of 19 metastatic prostate tumors,[10] in agreement with our unpublished immunohistochemistry data showing ZKSCAN3 expression in advanced prostate tumors. Similarly, high-resolution CGH showed gain of the ZKSCAN3 genomic region in ~25% and 50% of retinoblastomas and bladder cancers, respectively (43). Additionally, in unbiased expression profiling studies,[11] ZKSCAN3 expression is also increased in seminoma and various leukemias. Thus, it is worth speculating that ZKSCAN3 may also have a role in the genesis and/or progression of other malignancies.

In conclusion, we have identified the previously undescribed ZKSCAN3 as a novel driver of colorectal tumor progression and we propose ZKSCAN3 as a new addition to a growing set of gene products that, in varying combinations, stimulate colorectal tumor progression. Further, ZKSCAN3 adds to a short list of proteins in the $C_2H_2$ class of zinc finger proteins that function in tumor development and/or progression. Moreover, considering its overexpression in colorectal cancers wild-type for *APC, p53*, and *K-Ras*, a fitness advantage provided by ZKSCAN3 may be especially important for the progression of this subset of tumors.

[10] Personal communication with Dr. Pamela Paris, University of California San Francisco Comprehensive Cancer Center.

[11] Oncomine: http://www.oncomine.org/.

## Disclosure of Potential Conflicts of Interest

S.R. Hamilton: Other research support, Genentech; consultant, Novartis. G. Lopez-Berestein: Ownership interest, Bio-Path Holdings. L.M. Ellis: Commercial research grants, ImClone Systems, Sanofi-Aventis, and Amgen; speakers bureau/honoraria, Genentech. The other authors disclosed no potential conflicts of interest.

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