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Unbiased Screening for Transcriptional Targets of ZKSCAN3 Identifies Integrin β4 and Vascular Endothelial Growth Factor as Downstream Targets*[S]

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We previously described the novel zinc finger protein ZKSCAN3 as a new "driver" of colon cancer progression. To investigate the underlying mechanism and because the predicted structural features (tandem zinc fingers) are often present in transcription factors, we hypothesized that ZKSCAN3 regulates the expression of a gene(s) favoring tumor progression. We employed unbiased screening to identify a DNA binding motif and candidate downstream genes. Cyclic amplification and selection of targets using a random oligonucleotide library and ZKSCAN3 protein identified KRDGGG as the DNA recognition motif. In expression profiling, 204 genes were induced 2–29-fold, and 76 genes reduced 2–5-fold by ZKSCAN3. To enrich for direct targets, we eliminated genes under-represented (<3) for the ZKSCAN3 binding motif (identified by CAST-ing) in 2 kilobases of regulatory sequence. Up-regulated putative downstream targets included genes contributing to growth (c-Met-related tyrosine kinase (MST1R), MEK2; the guanine nucleotide exchanger RasGRP2, insulin-like growth factor-2, integrin β4), cell migration (MST1R), angiogenesis (vascular endothelial growth factor), and proteolysis (MMP26; cathepsin D; PRSS3 (protease serine 3)). We pursued integrin β4 (induced up to 6-fold) as a candidate target because it promotes breast cancer tumorigenicity and stimulates phosphatidyl 3-kinase implicated in colorectal cancer progression. ZKSCAN3 overexpression/silencing modulated integrin β4 expression, confirming the array analysis. Moreover, ZKSCAN3 bound to the integrin β4 promoter *in vitro* and *in vivo*, and the integrin β4-derived ZKSCAN3 motif fused upstream of a *tk*-Luc reporter conferred ZKSCAN3 sensitivity. Integrin β4 knockdown by short hairpin RNA countered ZKSCAN3-augmented anchorage-independent colony formation. We also demonstrate vascular endothelial growth factor as a direct ZKSCAN3 target. Thus, ZKSCAN3 regulates the expression of several genes favoring tumor progression including integrin β4.

Sporadic colorectal cancer largely reflects aberrantly activated pathways leading to unrestrained growth. In the Wnt pathway, stabilized β-catenin together with T cell factor-4 and lymphoid enhancer factor-1 DNA-binding proteins (1) *trans*-activates target genes (2), causal for growth. Mutation-activated *K-Ras* (3) also promotes tumor growth via the MAPK[2] pathway, whereas the mutation-disabled type II transforming growth factor (TGF-β) receptor gene (*TGF-β RII*) (signaling through the *MADH*-encoded Smad transcription factors (4)) is incapable of restraining proliferation. Targeting of the p53 tumor suppressor also contributes to the pathogenesis of this cancer, as damaged cells are unable to arrest for DNA repair (5), thereby accumulating DNA damage and mutation of key genes crucial to tumor development/progression.

Notwithstanding these landmark observations, recent studies (6–11) suggest that the heterogeneity of this disease likely involves the contribution of multiple other gene products acting in various combinations to promote cancer development and progression (7, 10). For example, several genes in the MKK (mitogen-activated protein kinase kinase)/JNKK1 (c-Jun amino-terminal kinase kinase) and phosphatidyl 3-kinase (PI3K) pathways are altered (8), and mutation of one of the genes (in the catalytic site of PI3K) in the latter module reduces growth factor dependence and facilitates tumor progression (9). Furthermore, inactivation of the UNC5C netrin-1 receptor either epigenetically or by loss of heterozygosity (LOH) in colorectal cancer and loss of XAF1 (X-linked inhibitor of apoptosis-associated factor 1), a candidate tumor suppressor, again by promoter methylation and LOH, both contribute to progression of this malignancy (12, 13). Moreover, genome-wide scans for tag single nucleotide polymorphisms identified a susceptibility variant at chromosome 8q24.21 (7, 10). Indeed, Wood *et al.* (6) 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. These findings emphasize the heterogeneity and complexity of this disease.

In a recent search for other "drivers" of colorectal cancer progression, we identified ZKSCAN3 (related to *bowl* required

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; CAST-ing, cyclic amplification and selection of targets; EMSA, electrophoretic mobility shift assay; KRAB, Kruppel-associated box; MST1R, c-Met-related tyrosine kinase; shRNA, short hairpin RNA; wt, wild type; mt, mutant; kb, kilobase(s); RT, reverse transcription; Q-PCR, quantitative-PCR.

for *Drosophila* hindgut development (14)) as a novel gene product promoting the progression of this malignancy (15). However, the mechanism by which ZKSCAN3 augmented colorectal tumorigenicity and progression was not addressed. Because the predicted ZKSCAN3 protein sequence included tandem zinc fingers, KRAB and SCAN domains typical of proteins that control gene expression (16–18), we hypothesized that this zinc finger protein regulates the expression of one or multiple genes favoring tumor progression. To answer this question, we employed unbiased screening methods to identify a ZKSCAN3 DNA binding site and downstream targets.

EXPERIMENTAL PROCEDURES

Cyclic Amplification and Selection of Targets (CAST-ing)—A random oligonucleotide library was synthesized as 5′-CACGTGAGTTCAGCGGATCCTGTCG**N**GAGGCGAATTCAGTGCAACTGCAGC-3′ (where N represents 26 random nucleotides). Binding reactions included poly(dI·dC), immobilized FLAG-ZKSCAN3, acetylated bovine serum albumin, and 500 ng of the random oligonucleotide library. After DNA-protein complex formation (room temperature, 20 min), complexes were proteinase K-treated, DNA-extracted, precipitated, and dissolved in Tris-EDTA buffer. PCR was used to enrich the ZKSCAN3-bound oligonucleotides. The 76′-mer PCR products were purified and used in subsequent rounds (total of 6) as described above. In the final round PCR products were labeled with [^{32}P]dCTP mixed with purified FLAG-ZKSCAN3 protein (0–100 ng), and protein-DNA complexes were resolved in an polyacrylamide gel. Recovered oligonucleotides in DNA-protein complexes were cloned into the pGEM-T Easy Vector (Promega, #A1360) and sequenced.

Chromatin Immunoprecipitation Assay—These were as described previously (19) using 2.5 μg of antibody and the following PCR primers for the integrin β4 intron 1 (specific 5′, 5′-GGGAAGGACAGCAGGAGGGAC-3′; specific 3′, 5′-CTCTGGGCACACCTGCTCCT-3′; nonspecific 5′, 5′-ATGGCACGGAACAGGGCA-3′; nonspecific 3′, 5′-GCCATCCTCTTCCTCCTGCAG-3′) and VEGF (specific, 5′-GGGCAAAGCCCCAGAGGG-3′; specific 3′-GGAGGCTGGAGGGGTTCC-3′); nonspecific, 5′-GTGTCCCTCTCCCCACCC-3′; 5′-CTGTCCAGAGACACGCGCC-3′) genes.

Real-time quantitation of chromatin immunoprecipitation was performed using the ChIP-IT Enzymatic kit (Active Motif, catalog # 53006) according to the manufacturer's instructions. For primer design, the integrin β4 intron 1 sequence was analyzed by the File Builder v3.1 software (Applied Biosystems) and the Basic Local Alignment Search Tool (BLAST) to exclude repeat elements, low complexity DNA, and regions with sequence similarity. The resulting intronic sequences spanning or distant from the ZSCAN3 binding sites were chosen to design custom TaqMan primers (specific and nonspecific Taqman primers, respectively).

EMSA—Nuclear extract (10 μg) was mixed with 0.6 μg of poly(dI/dC) and (2×10^4 cpm) of a γ-^{32}P-labeled oligonucleotide and, where indicated, anti-ZKSCAN3 or pre-immune IgG (1 and 3 μg). The VEGF oligonucleotide sequence was 5′-TTGGCTTATGGGGGTGGGGGGTGCC-3′. The integrin β4 oligonucleotide sequences used were (wt-5′-GTC CCT GAG GGG AGG AGA TGT GAC A-3′; mt-probe, 5′-GTC CCT GAt ata AGG AGA TGT GAC A-3′) spanning the putative ZKSCAN3 binding motif in the integrin β4 promoter.

Expression Profiling—RNA was extracted from orthotopically established tumors derived from HCT 116 cells harboring the empty vector or expressing the ZKSCAN3 coding sequence and subjected to expression profiling using the U133A Affymetrix chip harboring ~18,000 cDNAs. We chose tumors instead of monolayer cells because the progression effects of ZKSCAN3 are so clearly evident *in vivo*. To increase our chances that we were identifying direct ZKSCAN3 targets, we queried the upstream −2-kb and downstream non-coding sequences including the first intron of the regulated genes for the ZKSCAN3 binding motif (KRDGGGG) identified by CAST-ing. Because this sequence would be expected to occur by chance 3× per 2 kb of nucleotide sequence (both strands), modulated genes harboring <4 copies of this motif in 2 kb of regulatory sequence were excluded.

Immunocytochemistry—RKO cells were transiently transfected with pcDNA3-FLAG-ZKSCAN3 or the empty vector, and cells were fixed 48 h later and incubated with an anti-FLAG-M2 antibody (1:400) and then with a fluorescein isothiocyanate-conjugated secondary antibody. Nuclei were 4,6-diamidino-2-phenylindole-stained.

Immunohistochemistry—After de-waxing 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, a mouse anti-human integrin β4 (Chemicon MAB2058) (1:50), or a polyclonal anti human VEGF (1:100) (Santa Cruz sc-152) and then with a horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was detected with the DAB chromogen (Research Genetics). For negative controls, the anti-ZKSCAN3 antibody was substituted with an equivalent amount of pre-immune IgG.

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 (20). All experiments were approved by the Institutional Animal Care and Use Committees.

Purification of the FLAG-ZKSCAN3 Protein—Lysates of ZKSCAN3-expressing HCT116 cells in a buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA 1% Triton X-100) were incubated with anti-FLAG M2 affinity gel (4 °C overnight). The FLAG-ZKSCAN3 protein was eluted with 3× FLAG peptide.

Reporter Assays—These were performed as described previously (21). For the ZKSCAN3 reporter, duplicate copies of the wt (TGAGGGG) or mutated (TGAatat) ZKSCAN3 binding site derived from the integrin-β4 intron-1 were cloned upstream of a minimal *tk*-regulated luciferase reporter.

Retroviral Transductions—AmphoPack™-293 cells (Clontech, #631505) were transfected with 10 μg of DNA (pSuperior-IGB4-shRNA) (22) using Lipofectamine 2000. After 10–24 h, medium was aspirated, and cells were washed 2× with phosphate-buffered saline and replenished with culture medium. Culture supernatant (containing the retroviral-encoded integrin β4-targeting shRNA) was collected every 12 h thereafter for 48 h, filtered through a 0.45-μm filter, and diluted 2-fold

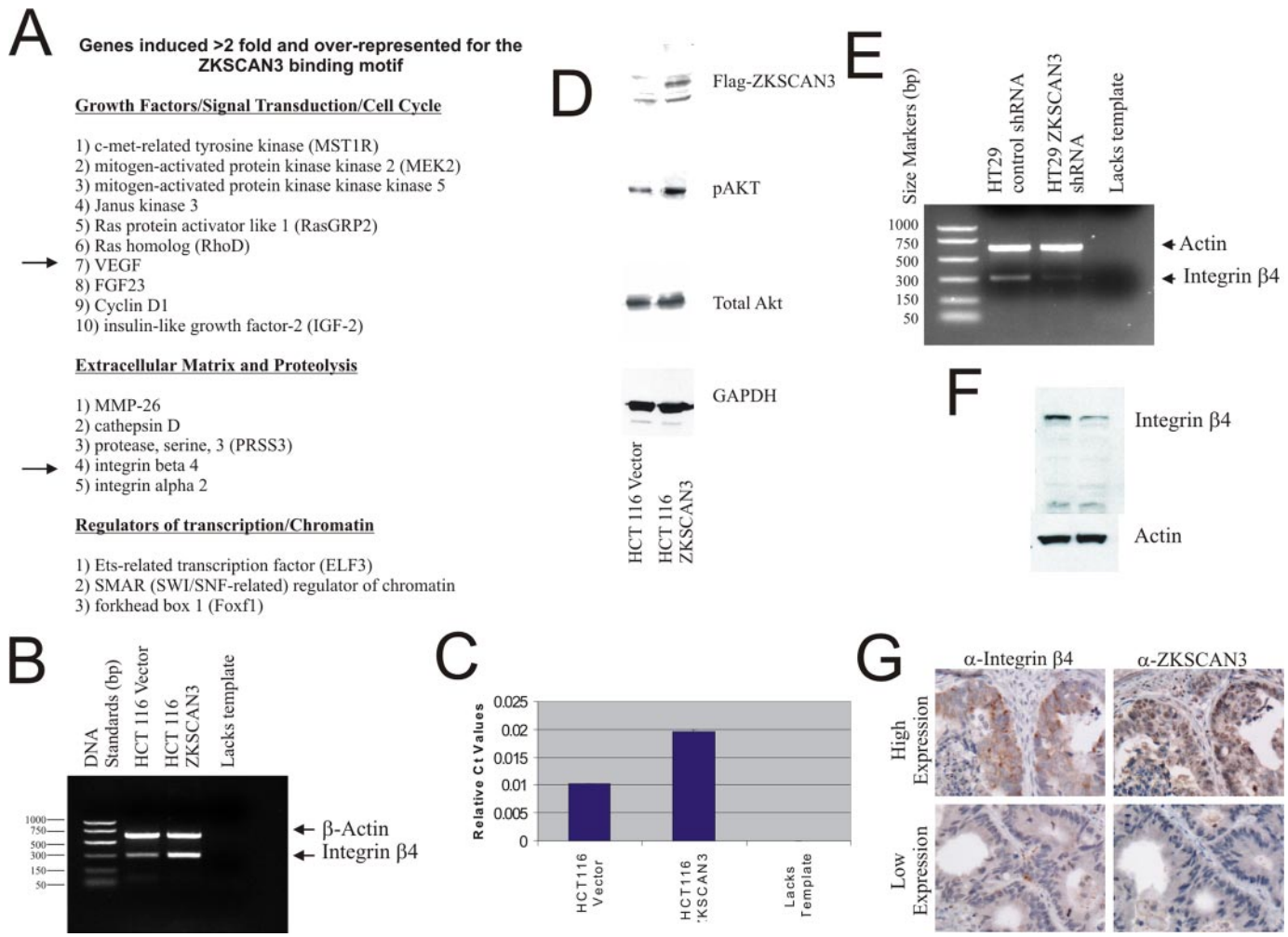

FIGURE 1. **Expression profiling identifies candidate ZKSCAN3 downstream targets including integrin β4 and VEGF.** *Panel A*, differentially expressed transcripts identified using total RNA from orthotopic tumors generated from HCT 116 cells bearing the ZKSCAN3 cDNA or empty vector (15). A U133A 2.0 Affymetrix chip was used. *Panels B* and *C*, integrin β4 mRNA levels were semiquantified by RT-PCR and Q-PCR, respectively, using total RNA from pooled tumor tissue as per *panel A*. *Panel D*, pooled HCT 116 clones were grown in suspension (16 h), and cell lysates were analyzed by Western blotting. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *p-Akt*, phosphorylated Akt. *Panel E* and *F*, HT29 cells were transduced with a retro-ZKSCAN3 shRNA and selected with puromycin for 12 days and analyzed (*panel E*) as per *panel B*, or total cell lysate was subjected to Western blotting (*panel F*) with an anti-integrin β4 antibody (Chemicon, catalog MAB2058). *Panel G*, serial sections of resected colorectal cancers were subjected to immunohistochemistry for integrin β4 and ZKSCAN3. Data are representative of at least two separate experiments.

with fresh medium. Actively dividing target cells were transduced with the virus-containing medium using Polybrene (final concentration = 4 μg/ml), and cells were selected with puromycin.

RT-PCR Semiquantitation of Transcripts—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 of TURBO DNA-*free* DNase enzyme (Ambion, #1907), and after DNase inactivation, cDNA was synthesized with avian myeloblastosis virus reverse transcriptase. PCR was performed using primers for ZKSCAN3 (100 ng each RT-5, 5′-GGC CCT GAC CCT CAC CCC-3′; RT-3, 5′-CAG ATG TGC CGC CTC CCT CC-3′), β-actin (10 ng), 1 unit of Taq polymerase in 30 amplification cycles. ZKSCAN3 primers were located in exons 6 and 7 with a PCR product of 294 bp. To semiquantitate the integrin β4 transcript, cDNA was prepared from 2 μg of RNA, and RT-PCR was performed using forward (5′-CAA GGA GGA GGG CCA GCC-3′) and reverse primers (5′-GGG TCA GCC CAT CCA CTA GG-3′) respectively. The predicted PCR product size was 288 bp. RT-PCR for VEGF employed the following primers: forward (5′-CCT CAC CAA GGC CAG CAC-3′ and reverse (5′-CTC ACC GCC TCG GCT TGT C-3′) with the predicted amplicon sizes: 318 bp, 244 bp, 112 bp, for VEGF isoforms 189, 16S, and 121, respectively.

Q-PCR for Quantitation of Transcript Levels—Total RNA was isolated from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed using the cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). Q-PCR was performed in duplicate (1 μl of cDNA in a total volume of 20 μl) using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Expression of integrin β4 was determined using the Applied Biosystems Taq-Man Gene Expression Assay. Primers and probes were purchased from Applied Biosystems and were labeled with a 6-carboxyfluorescein, major Groove Binder quencher. Normalization was with β-actin.

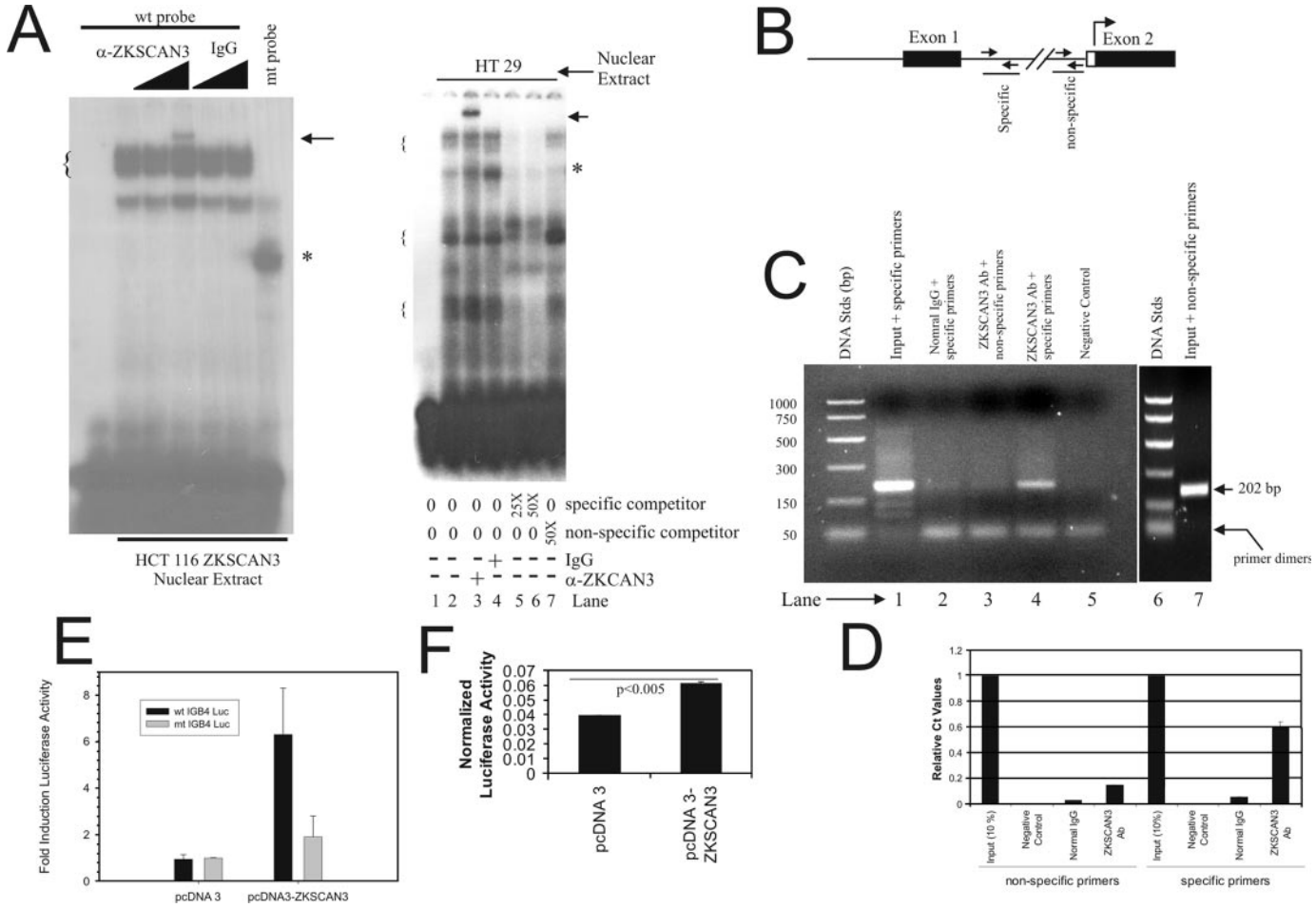

FIGURE 2. **Integrin β4 is a direct ZKSCAN3 target.** *Panel A*, EMSA using the following oligonucleotides: wt (specific, 5′-GTCC**TGAGGGG**AGGAGATGTGACA-3′ or mt (nonspecific) probe (5′-GTCCC**TGAtata**AGGAGATGTGACA-3′). Antibody inputs = 1 and 3 μg. *Panel B*, schematic of the integrin β4 intron 1 indicating amplicons. *Panel C*, chromatin immunoprecipitation assay using chromatin from HCT 116 cells stably expressing ZKSCAN3 and the anti-ZKSCAN3 antibody (2.5 μg) or an equivalent amount of pre-immune IgG and primers indicated in *panel B*. *Input* represents 10% of the total. The negative control lacks chromatin input. *Panel D*, chromatin immunoprecipitation of HT29 cells using primers either spanning (specific primers) the integrin β4 intron 1 ZKSCAN3 binding sites or distant from the motif (nonspecific primers). Ct values are shown relative to that achieved with 10% input for each primer set with data representing the average ± range of duplicate experiments. Negative control lacks chromatin input. *Panel E*, a reporter (100 ng) fused to duplicate wild type or mutated ZKSCAN3 binding motifs (wt IGB4 Luc and mt IGB4 Luc, respectively) was co-transfected into HCT 116 cells with the indicated expression construct, and cells assayed for luciferase activity 24 h later. *Panel F*, same as *panel E* but where the reporter was driven by the integrin β4 intron 1 sequence +1905/+2933. Data are the mean values ± S.D. of triplicate observations.

Soft-agar Assay—Cells were seeded in 0.3% agar and incubated at 37 °C for 14 days.

VEGF Protein Measurements—We used an enzyme-linked immunosorbent assay (ELISA; Quantikine Human VEGF ELISA kit, R&D Systems, catalog# DVE00) as per the manufacturer's protocol.

Statistics—Statistical differences were determined using an unpaired *t* test and GraphPad Prism (Version 3.03) software.

RESULTS

Identification of a DNA Binding Site for ZKSCAN3 by Cyclic Amplification and Selection of Targets—Although we previously implicated ZKSCAN3 as a new driver of colon cancer progression (15), the underlying mechanism was not determined. Because the ZKSCAN3 sequence predicts protein domains typical of transcription factors, we hypothesized that the encoded protein is regulatory for one or multiple genes favoring colorectal cancer progression. Nuclear localization was evident in RKO cells transfected with a FLAG-tagged ZKSCAN3 (supplemental Fig. 1*A*, *arrows*) consistent with our immunohistochemistry on resected human colorectal cancers (15) and as expected for a regulator of gene expression. We then undertook cyclic amplification and selection of targets (CASTing) to identify a consensus DNA binding site. Purified ZKSCAN3 protein (supplemental Fig. 1*B*, *first lane*) was mixed with a random oligonucleotide library (complexity = 4×10^{15}), protein-bound oligonucleotides were PCR-amplified, and the enriched population was subjected to additional rounds of enrichment (supplemental Fig. 1*C*). In the sixth round, enriched oligonucleotides were radiolabeled and subjected to EMSA with FLAG-ZKSCAN3 protein (supplemental Fig. 1*D*). Oligonucleotides in the DNA-protein complex were extracted, subcloned, and sequenced. Using the Align-Ace algorithm (23), a consensus DNA binding site of KRDGGGG (K = G/T, R = A/G, and D = A/G/T) was derived.

Identification of Candidate ZKSCAN3 Downstream Targets—To identify candidate downstream targets, we performed parallel expression profiling experiments using RNA from tumors

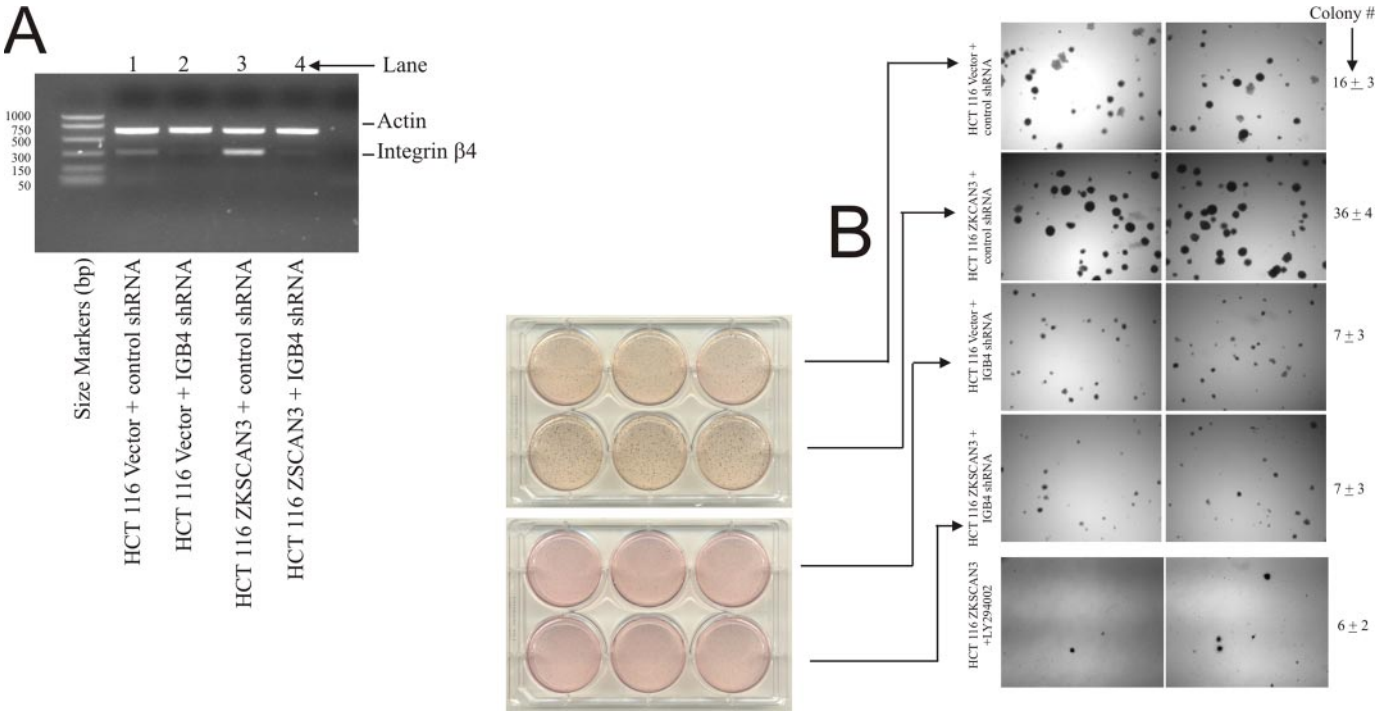

FIGURE 3. **Effect of integrin β4 silencing on ZKSCAN3-dependent growth in soft agar.** Pooled HCT116-vector and HCT116-ZKSCAN3 cells were transduced with an shRNA-targeting integrin β4 or a control sequence. Pooled puromycin-selected cells were analyzed for integrin β4 mRNA by RT-PCR (*panel A*) or grown in soft agar (*panel B*) with or without LY294002 (25 μM). Data represent average values ± S.D. of >7 determinations.

(15) generated from HCT 116 colon cancer cells expressing the ZKSCAN3 cDNA or the empty vector. HCT 116 express modest endogenous ZKSCAN3 levels and form small orthotopic tumors at a low incidence, whereas forced ZKSCAN3 cDNA expression in these cells yields 100% tumor take in nude mice, and tumors are larger (15). Transcription factors bearing zinc-finger SCAN and KRAB domains activate and repress gene expression (24, 25), and we identified 204 genes induced 2–29-fold and 76 genes reduced 2–5-fold by ZKSCAN3 (data not shown). To reduce this large dataset we undertook 2 strategies; (*a*) we eliminated transcripts showing no change in parallel expression profiling using tissue cultured ZKSCAN3/vector-expressing HCT 116 cells, and (*b*) to enrich for direct targets, we eliminated genes under-represented (≤3) for the ZKSCAN3 binding motif (identified by CAST-ing) in 2 kb of regulatory sequence as this motif would by chance occur 3× per 2 kb. Fig. 1*A* shows induced putative downstream targets satisfying these criteria including several genes that contribute to increased growth (c-Met-related tyrosine kinase (MST1R) (26), MEK2, the guanine nucleotide exchanger RasGRP2 (27), insulin-like growth factor-2 (28, 29), integrin β4 (30, 31)), cell migration (MST1R (32)), angiogenesis (VEGF), and proteolysis (MMP26, cathepsin D (33), PRSS3 (34)).

Integrin β4 Is a Direct ZKSCAN3 Target—The induction of integrin β4 mRNA evident in expression profiling (up to 6-fold) and verified by RT-PCR and Q-PCR using RNA from tumors generated from the indicated cells (Fig. 1, *B* and *C*) was intriguing because this protein has been implicated in tumorigenicity (22, 35) and cell migration (36). Moreover, integrin β4 stimulates the phosphatidyl 3-kinase (PI3K) signaling module (37) implicated in colorectal cancer progression (9), and indeed increased phosphorylated Akt levels (Fig. 1*D*) downstream of PI3K was evident in pooled HCT 116 ZKSCAN3 transfectants. Conversely, knockdown of ZKSCAN3 in HT29 cells, which intrinsically expresses this endogenous zinc finger protein (15), reduced integrin β4 mRNA amounts as evident by RT-PCR (Fig. 1*E*) and Q-PCR (data not shown) as well as protein levels (Fig. 1*F*), again supporting the notion that the latter is indeed a target of the former. Moreover, in immunohistochemistry, a strong concordance between integrin β4 expression and nuclear ZKSCAN3 expression was evident in tumor cells in 8/8 colorectal cancer patients (Fig. 1*G*), further supporting the view that the former is regulated by the latter.

If integrin β4 is a direct ZKSCAN3 target, we would predict that the regulatory region bearing the binding motif identified by CAST-ing would be bound with ZKSCAN3. Intron 1 of the integrin β4 gene contains an enhancer regulatory for its expression (38) including a putative ZKSCAN3 binding site (TGAGGGG) conforming to the KRDGGGG consensus site. In EMSAs, nuclear extract from either HCT 116 cells forced to overexpress ZKSCAN3 (Fig. 2*A*, *left panel*) or HT 29 cells, which intrinsically overexpress the DNA-binding protein (15) (Fig. 2*A*, *right panel*), retarded the mobility of an oligonucleotide spanning this binding site (Fig. 2*A*, *parentheses*). Furthermore, our anti-ZKSCAN3 antibody, but not the preimmune IgG, caused a supershift (*arrow*). A substituted radioactive oligonucleotide (mt probe) failed to generate a comparably shifted band with HCT 116 ZKSCAN3 nuclear extract (Fig. 2*A*, *left panel*). Presumably the faster migrating band (*) indicates a nonspecific protein-probe interaction. With HT29 nuclear extract, the addition of an excess of the wild type oligonucleotide (Fig. 2*A*, *right panel*, *lanes 5* and *6*) reduced the intensity of the shifted bands (parenthesis), whereas an excess of the non-specific oligonucleotide had only a minor effect in this regard

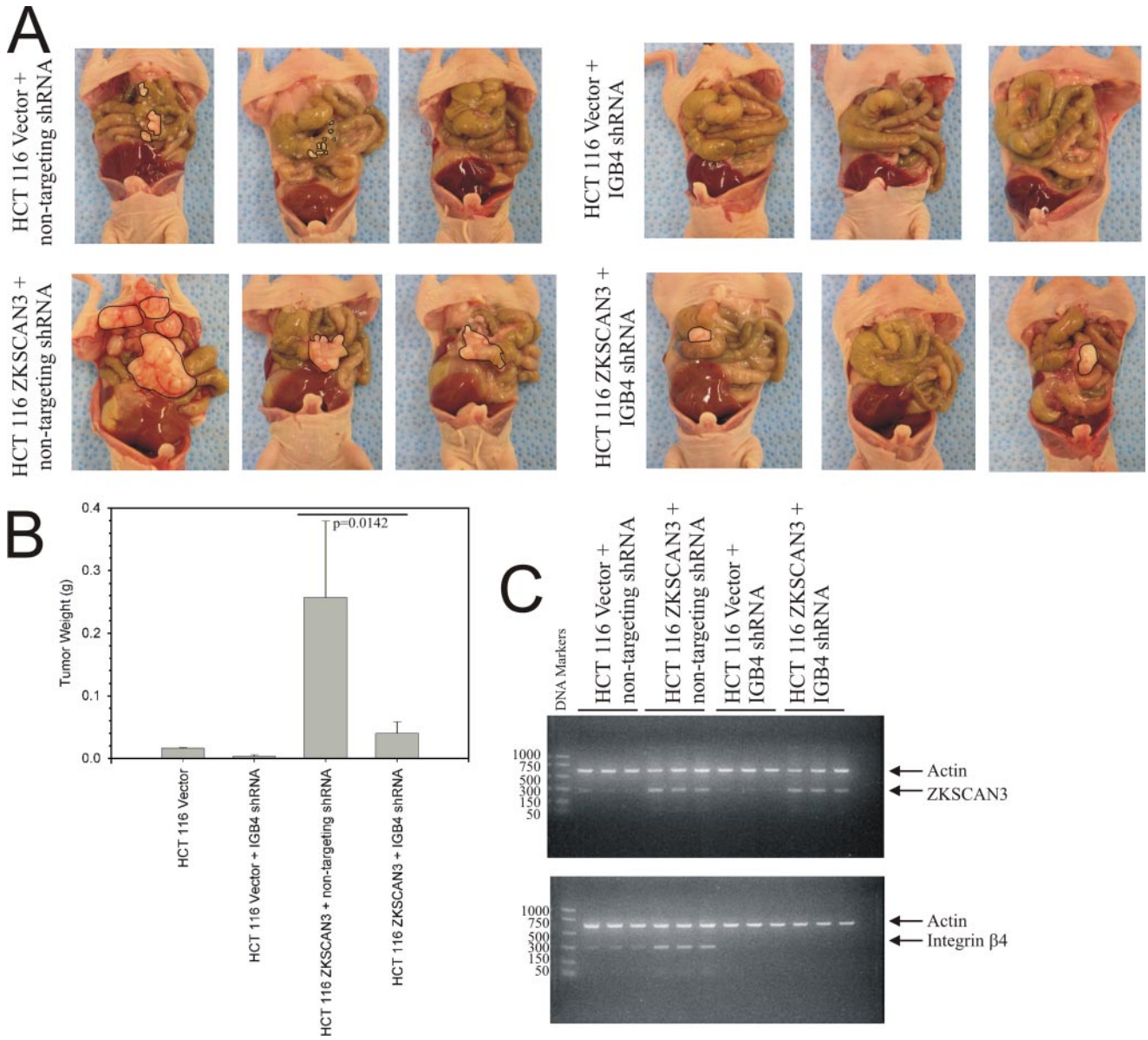

FIGURE 4. **Effect of integrin β4 silencing on ZKSCAN3-dependent orthotopic tumor growth.** The indicated cells (10^6) cells were injected into the cecal wall of nude mice (groups of 10 mice). After 6 weeks mice were sacrificed, and tumors (*panel A* circumscribed areas) were weighed (*panel B*) and analyzed by RT-PCR (*panel C*) for integrin β4 and ZKSCAN3 mRNA levels.

(Fig. 2*A*, *right panel*, *lane 7*). As before, we presume that bands (*) diminished in intensity by inclusion of an excess of nonspecific competitor reflect nonspecific interactions.

Chromatin immunoprecipitation (Fig. 2*C*) using ZKSCAN3 cDNA-expressing HCT 116 cells yielded a band (*lane 4*) with the anti-ZKSCAN3 antibody used in conjunction with primers (specific) flanking the ZKSCAN3 binding motif (Fig. 2*B*) but not with primers (nonspecific) located downstream of the ZKSCAN3 site (*lane 3*). Chromatin immunoprecipitation assays were also performed on HT29 cells that intrinsically express ZKSCAN3 as we showed previously (15). Again, our ZKSCAN3 antibody enriched chromatin, spanning the DNA binding motif more than 11-fold over that achieved with normal IgG (Fig. 2*D*, *specific primers*), whereas the corresponding enrichment with primers distant from the ZKSCAN3 binding sites was modest (~3-fold). These data suggest that the ZKSCAN3-spanning region of the integrin β4 intron 1 is bound with endogenous ZKSCAN3.

Moreover, this ZKSCAN3 binding element was regulatory for expression because a minimal *tk* promoter-luciferase reporter driven by duplicate tandem copies of the integrin β4-derived motif (wt IGB4 Luc), but not the substituted site (mt IGB4), was activated 6-fold by ZKSCAN3 co-expression in HCT 116 cells (Fig. 2*E*). Similarly, ZKSCAN3 activated ($p < 0.005$) a luciferase construct (Fig. 2*F*) driven by the region of the integrin β4 intron 1 (+1905+/2933) inclusive of the enhancer and the ZKSCAN3 binding site (38). Thus, integrin β4 is a direct downstream target of ZKSCAN3.

Effect of Integrin β4 Silencing on the Ability of ZKSCAN3 to Augment in Vitro and in Vivo Tumorigenicity—To determine whether integrin β4 mediates at least in part the pro-tumorigenic effects of ZKSCAN3, ZKSCAN3 cDNA/empty vector-

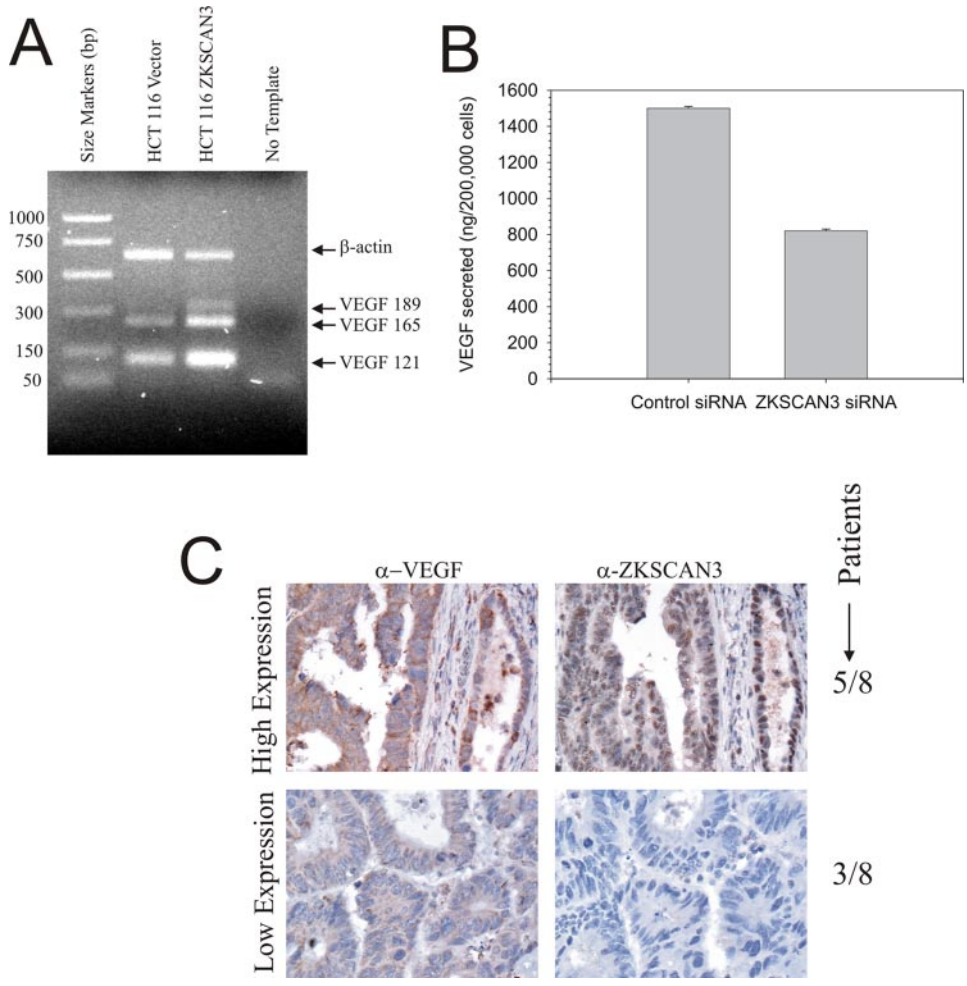

FIGURE 5. **Validation of VEGF as a downstream ZKSCAN3 target gene.** *Panel A*, total RNA was extracted from tumor tissue as per Fig. 1 and analyzed by RT-PCR. *Panel B*, enzyme-linked immunosorbent assay for VEGF using conditioned medium from RKO cells knocked down for ZKSCAN3 (15). *siRNA*, small interfering RNA. *Panel C*, serial sections of resected colorectal cancers subjected to immunohistochemistry for VEGF and ZKSCAN3 expression as per Fig. 1 with the exception that an anti-VEGF antibody (1:500) was used where indicated. The experiments were repeated at least twice.

expressing HCT 116 cells were transduced with a retrovirus bearing an integrin β4-targeting shRNA (22). Expectedly, although ZKSCAN3 induced integrin β4 mRNA levels (Fig. 3*A*, compare *lanes 3* and *1*), the integrin β4-targeting shRNA substantially reduced integrin β4 transcript levels in HCT 116 cells expressing the ZKSCAN3 or the corresponding empty vector (Fig. 3*A*, *lanes 2* and *4*). Strikingly, integrin β4 knockdown countered the ZKSCAN3-dependent augmented anchorage-independent growth ($p < 0.0001$) as did a phosphatidyl 3-kinase inhibitor (LY294002) (Fig. 3*B*). Although integrin β4 silencing in HCT 116 cells lacking the ZKSCAN3 cDNA reduced colony growth, this presumably reflecting repression of the endogenous ZKSCAN3 transcript (compare Fig. 3*A*, *lanes 1* and *2*), the % reduction (58 ± 7) was significantly less ($p < 0.05$) than that evident for knockdown of the ZKSCAN3-overexpressing cells (80 ± 3) (Fig. 3*B*). The integrin β4-targeting shRNA only marginally reduced monolayer growth (data not shown).

To further corroborate these findings, we orthotopically injected the ZKSCAN3/vector-expressing HCT 116 cells transduced with the integrin β4-targeting shRNA or the non-targeting sequence (Fig. 4). As expected, ZKSCAN3 caused a robust induction of tumorigenicity (circumscribed areas Fig. 4*A*) as evidenced by a 10-fold increase in tumor weight (Fig. 4*B*). However, this increased tumor size achieved with ZKSCAN3 cDNA expression was practically abrogated (Fig. 4, *A* and *B*) by concurrent knockdown of integrin β4 (Fig. 4*C*). Taken together, these data implicate integrin β4 as one downstream target of ZKSCAN3 that contributes to the tumorigenic phenotype.

VEGF Represents a Direct ZKSCAN3 Target—Expression profiling indicated several other genes including VEGF (Fig. 1*A*) that were also modulated by ZKSCAN3. VEGF was of particular interest considering its prominent role in angiogenesis, a prerequisite for tumor expansion. RT-PCR with RNA derived from tumors generated with the indicated cells verified the expression profiling data showing a marked induction of the various VEGF transcripts (Fig. 5*A*). Conversely, transient ZKSCAN3 knockdown in RKO cells, which express endogenous ZKSCAN3 (15), reduced VEGF secretion (Fig. 5*B*). To further corroborate VEGF as a ZKSCAN3 target, we stained sections of resected colorectal cancers from eight patients for these two proteins. Concordant high expression of cytoplasmic VEGF and nuclear ZKSCAN3 proteins (Fig. 5*C*, *High Expression*) was evident in five individuals. Sections from the three remaining patients expressing little or no ZKSCAN3 showed weak VEGF immunoreactivity (Fig. 5*C*, *Low Expression*) presumably due to ZKSCAN3-independent regulation (39, 40). Nonetheless, taken together these data suggest that VEGF is regulated directly or indirectly by ZKSCAN3.

We then determined if VEGF is directly targeted by ZKSCAN3. A previously described (41) regulatory region (−2275/−1176) of the VEGF gene bears a motif (GGTGGGG at −2270) conforming to the ZKSCAN3 binding site (KRDGGGG) identified by CAST-ing, and an oligonucleotide spanning this motif was retarded in EMSA (*arrows*) with nuclear extract from ZKSCAN3 cDNA-expressing HCT 116 cells (Fig. 6*A*, *lane 2*). The retarded bands were competed with an excess of like oligonucleotide (*lane 5*) but not with an excess of an unrelated probe (*lane 6*). Furthermore, the anti-ZKSCAN3 antibody, but not IgG (*lanes 3* and *4*), yielded a "supershift" (*). Chromatin immunoprecipitation with RKO cells, intrinsically expressing ZKSCAN3 (15), revealed binding

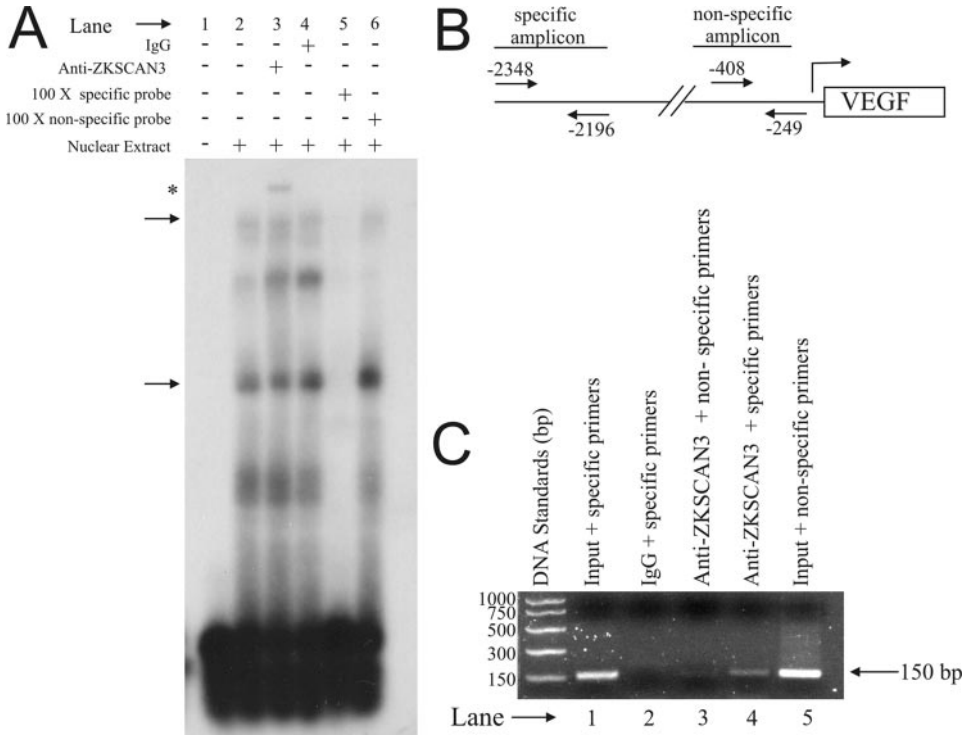

FIGURE 6. **The VEGF gene is a direct ZKSCAN3 target.** *Panel A*, EMSA using nuclear extract from ZKSCAN3 cDNA-expressing HCT 116 cells and an oligonucleotide spanning the putative ZKSCAN3 binding motif in the VEGF promoter. *Panels B* and *C*, schematic (*panel B*) of the VEGF promoter indicating primers employed for chromatin immunoprecipitation (*panel C*) using chromatin from RKO cells. Specific/nonspecific primers amplify DNA spanning/downstream of the putative ZKSCAN3 motif, respectively. *Input* represents 10% of the total amount.

of this protein to the endogenous VEGF promoter region harboring the ZKSCAN3 motif (Fig. 6, *B* and C, *lane 4*) but minimally to a region lacking this site (*lane 3*). Thus, like integrin β4, VEGF also represents a direct ZKSCAN3 target.

DISCUSSION

Recent studies (6–11) strongly suggest 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 reported previously 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 (15). ZKSCAN3 bears structural features strongly resembling a transcriptional regulator, and employing unbiased approaches, we have identified a DNA binding site and downstream targets including integrin β4 and VEGF. That ZKSCAN3 is a transcriptional regulator is not surprising considering that the related, Zfp-38 bearing 43% identity with ZKSCAN3 (16), ZNF 383 and ZNF 436 (51 and 43% similarity index compared with ZKSCAN3, respectively) (16, 17) are also modulatory for gene expression.

Our data invoke integrin β4 as a direct target and possibly one of multiple downstream effectors of ZKSCAN3. Its expression is up-regulated in colorectal cancer (42) presumably due in part to ZKSCAN3 as we have shown herein. Integrin β4 has recently emerged as a mediator of cancer development and tumor progression (22, 43, 44), albeit in skin and breast cancer, and may function as a "servo" oncogene (43) by virtue of its ability to co-opt diverse receptor tyrosine kinases (30, 31) and phosphatidyl 3-kinase signaling downstream (37). In fact, the ability of integrin β4 to intersect with phosphatidyl 3-kinase signaling is notable considering the recent implication of the latter in driving colorectal cancer progression (9, 45). Indeed, elevated activated Akt levels evident with the ZKSCAN3 transfectants and the observation that a phosphatidyl 3-kinase inhibitor abrogated ZKSCAN3-dependent anchorage-independent growth make it likely that this transcription factor funnels into this module.

Notwithstanding these findings, our data point to multiple downstream targets some with well established roles in tumor progression. One such candidate is VEGF with its well recognized role in angiogenesis a prerequisite for tumor expansion beyond 1 mm^3. Indeed, our data showing ZKSCAN3-dependent VEGF expression combined with the observation that the VEGF promoter is bound with this endogenous zinc finger protein are consistent with the view that this gene also represents a direct ZKSCAN3 transcriptional target. Nevertheless, VEGF may also be indirectly regulated by ZKSCAN as we noted in our expression profiling data an induction of mitogen-activated protein kinase kinase kinase 5, a regulator of expression of this angiogenic protein (46). Irrespective of whether VEGF is induced by ZKSCAN3 directly or indirectly, this regulation may have implications with respect to vasculogenesis in a subset of colorectal tumors wild type for K-Ras and/or with a quiescent Wnt pathway. High VEGF levels in colorectal cancer are maintained partly via an oncogenic K-Ras that intersects with the Wnt pathway (47). However, such an angiogenic stimulus would be absent in tumors lacking an activated K-Ras and with a concurrent silent Wnt pathway. Because we have shown that ZKSCAN3 is also expressed in a colorectal tumor subset wild type for this GTP-binding protein and with a concurrent quiescent Wnt module (15), its ability to augment VEGF expression may represent a means for colon cancer cells to maintain high levels of this angiogenic protein in tumors unaltered for the K-Ras/andenomatous polyposis coli/β-catenin genes.

Our data argue against the notion that ZKSCAN3 intersects with the classical Wnt, transforming growth factor-β and p53 pathways. In our previous study (15) we found minimal modulation of reporters for these modules. Moreover, in the current study our expression profiling failed to show

any substantial change in the amount of transcript encoding downstream targets in these modules (c-Myc for Wnt, $p21^{cip1}$ and PAI-1 for transforming growth factor-β, and PUMA/mdm2 for p53). On the other hand, ZKSCAN3 could very well intersect with MAPK signaling downstream of K-Ras, and our findings that the expression of MEK2 and Ras protein activator-like 1 were augmented by ZKSCAN3, resonate with this notion as well as a parallel study reporting ZKSCAN3 as a proliferation inducer (48). If this is the case, in the 60–70% of colorectal cancers wild type for this GTP-binding protein (49, 50), ZKSCAN3 expression could substitute for an activated K-Ras. Indeed, our observation of ZKSCAN3 synthesis in tumors genotyped as unaltered for *K-Ras* (15) is in agreement with this supposition.

One question that remains unanswered is the role of co-activators and co-repressors in modulating expression of the down-stream targets of ZKSCAN3. The transcription factor Evi1 (encoded by the ecotropic viral integration site 1 gene), like ZKSCAN3, both activates and represses gene expression (51) and interacts with the methyl binding domain 3b protein, a member of the Mi-2/NuRD histone deacetylase complex. Induced gene transcription is achieved via its inhibition of the histone deacetylase function in this complex (51). ZNF217, a Kruppel-like zinc finger-containing protein, interacts with CoREST and histone deacetylase 2 to repress E-cadherin expression (52). In addition to effecting post-translational modifications of histones at target genes, some zinc finger transcription factors target the chromatin remodeling machinery as well. One example is hZimp10 (human zinc finger-containing Miz1, PIAS-like protein on chromosome 10), which physically interacts with Brg1 and BAF57, components of the Swi/Snf chromatin-remodeling complex, to augment transcriptional activity of androgen receptor-responsive genes (53). Another possibility is that ZKSCAN3 modulates gene expression through a co-activator function, as does the C2H2 zinc finger protein Zac1. Zac1 stabilizes the interaction of p300 with pCAF, thus favoring histone H4 acetylation and gene transcription (54).

An alternative mechanism for ZKSCAN3 in driving tumor progression that we have not explored may relate to a non-transcriptional role for ZKSCAN3. In some instances tandem zinc fingers function via protein-protein interactions. Indeed, the distant relative, LMO4 (LIM domain only 4 protein, which induces mammary cell invasion) bears an LIM domain comprised of tandem zinc fingers, the latter allowing this protein to act as an adaptor for multiprotein complex assembly (55). Protein-protein interactions also may yield protein sequestration as with XAF1, which renders the proapoptotic XIAP (X-linked inhibitor of apoptosis protein) inaccessible (13).

Identification of transcription factors as modulators of tumor progression have garnered much interest since Twist (56), (and two homeobox transcription factors) Goosecoid (57), and Six homeobox-1 (58) were identified as key drivers of breast cancer and rhabdomyosarcoma dissemination, and recent studies have shown a high mutation rate for zinc finger transcription factors in breast cancer (59). ZKSCAN3 adds to a relatively short list of transcription regulators favoring tumor progression, and we argue that the underlying mechanism may reflect in part the induction of integrin β4 and VEGF, two gene products previously implicated in colon cancer progression.

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