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FOXD3 Regulates CSC Marker, DCLK1-S, and Invasive Potential: Prognostic Implications in Colon Cancer

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Abstract

The 5′ (α)-promoter of the human doublecortin-like kinase 1 (*DCLK1*) gene becomes epigenetically silenced during colon carcinogenesis, resulting in loss of expression of the canonical long(L)-isoform1 (DCLK1-L) in human colon adenocarcinomas (hCRCs). Instead, hCRCs express a short(S)-isoform2 (DCLK1-S) from an alternate (β)-promoter of DCLK1. The current study, examined if the transcriptional activity of the (β)-promoter is suppressed in normal versus cancerous cells. On the basis of *in silico* and molecular approaches, it was discovered that FOXD3 potently inhibits the transcriptional activity of the (β)-promoter. FOXD3 becomes methylated in human colon cancer cells (hCCC), with loss of FOXD3 expression, allowing expression of the DCLK1(S) variant in hCCCs/hCRCs. Relative levels of FOXD3/DCLK1(S/L)

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were measured in a cohort of CRC patient specimens (n = 92), in relation to overall survival (OS). Patients expressing high DCLK1(S), with or without low FOXD3, had significantly worse OS compared with patients expressing low DCLK1(S). The relative levels of DCLK1-L did not correlate with OS. In a pilot retrospective study, colon adenomas from high-risk patients (who developed CRCs in <15 years) demonstrated significantly higher staining for DCLK1(S) + significantly lower staining for FOXD3, compared with adenomas from low-risk patients (who remained free of CRCs). Latter results strongly suggest a prognostic value of measuring DCLK1(S)/FOXD3 in adenomas. Overexpression of DCLK1(S), but not DCLK1(L), caused a significant increase in the invasive potential of hCCCs, which may explain worse outcomes for patients with high DCLK1-S–expressing tumors. On the basis of these data, FOXD3 is a potent repressor of DCLK1-S expression in normal cells; loss of FOXD3 in hCCCs/hCRCs allows upregulation of DCLK1-S, imparting a potent invasive potential to the cells.

Introduction

Colorectal cancers (CRCs) remain one of the most prevalent cancers in the United States and western world (1). Cancer stem cells (CSC) are resistant to currently used chemotherapy/radio-therapy treatments, and are believed to mediate metastatic spread of the disease (2–4). To identify CSCs, several stem cell markers are used including CD44, CD133, Lgr5, and DCLK1 (2–8). Many of the CSC markers are also expressed by normal epithelial cells, and other cell types in the stroma of epithelial tumors (2, 5, 8, 9), and are known to impact the biology of CSCs. Our laboratory is focused on investigating the role of DCLK1 in hCRCs (3, 5, 9–13). DCLK1, a putative kinase, has two doublecortin domains at the N-terminal end and has a Ca^{2}+/calmodulin-independent kinase domain at the C-terminal end. DCLK1 plays a critical role in neurogenesis, cortical development, and migration of neurons, especially during fetal development (14, 15), and is required for maintaining growth of neuroblastomas (16, 17). DCLK1 is also postulated as an epithelial stem cell marker (8, 10, 18, 19). A critical role of DCLK1 was reported in mouse pancreatic/colon carcinogenesis (discussed in ref. 20), and believed to specifically mark CSCs, but not normal stem cells (6). Several reports, however, suggest that DCLK1 probably marks both normal and cancer stem cells (5, 12, 202), including specialized tuft cells (21–23).

Experiments with human colon cancer cells (hCCC) and CRCs have similarly confirmed a critical role of DCLK1 in maintaining spheroidal/tumorous growths of hCCCs, *in vitro* and *in vivo* (3, 5, 12, 20, 23–25). A subset of DCLK1+CSCs was reported to overcome inhibitory effects of chemopreventive/chemotherapeutic agents via autophagic survival; loss of DCLK1 combined with chemopreventive agents was required for eliminating CSCs to avoid relapse of the disease (3). Thus, literature to-date strongly supports a critical role of DCLK1 in tumorigenesis (mouse studies) and in maintaining tumorigenic/metastatic potential of hCCCs.

We now know that 5′(α)-promoter of *DCLK1* gene is hyper-methylated in human epithelial tumors, including CRCs, resulting in loss of expression of the long (L) canonical isoform of DCLK1 (termed isoform-1 in NCBI database; refs. 12, 26, 27). CRCs/hCCCs are, however, positive for significant levels of DCLK1 (3, 5, 9, 28). Discrepancy between epigenetic

silencing of 5′ promoter of *DCLK1* gene, and reported expression of DCLK1 by hCRCs, is due to the novel expression of a short(S)-isoform (isoform-2) of DCLK1 (DCLK1-S), from an alternate(β)-promoter, located in IntronV of h*DCLK1* gene (12); normal colons mainly express the canonical long (L)-isoform1, from 5′(α)-promoter (12). In here, we tested the hypothesis that differential expression of DCLK1-S in normal colons versus hCRCs is due to differences in transcriptional activity of IntronV(β) promoter in normal/cancer cells. Several potential binding sites for FOXD3 were discovered within 3 kb of the transcriptional start site of IntronV(β) promoter (Supplementary Fig. S1). FOXD3, a potent transcription factor, inhibits many cancers (29–38). We therefore examined if FOXD3 dictates transcriptional activity of IntronV (β)-promoter in h*DCLK1* gene.

FOXD3 (Forkhead-Box-D3) is a member of the forkhead box (FOX) family of transcription factors, which is characterized by a distinct FH (forkhead) domain (39). FOXD3 primarily functions as a transcriptional repressor (40), but also activates genes required for suppressing differentiation of stem cells, such as ERBB3 (Erb-B2 Receptor Tyrosine Kinase 3; ref. 41), OCT4, and NANOG (42). Majority of the evidence points towards inhibitory effects of FOXD3 on the growth/metastasis of several epithelial cancers and neuroblastomas (29–38, 43). Inhibitory effects of FOXD3 are likely mediated via transcriptional regulation of specific miRNAs/genes. Upregulation of p21 and/or loss of TWIST1 was reported to mediate loss of proliferative/invasive potential of melanoma cells in response to ectopic expression of FOXD3 (32, 38). However, in majority of the studies, target genes mediating inhibitory effects of FOXD3 have not been identified. Here we report that FOXD3 is a potent repressor of IntronV(β) promoter in h*DCLK1* gene.

Loss of FOXD3 expression has been reported as a prognostic marker for patients with neuroblastomas/gliomas and breast/lung/gastric cancers (30, 31, 34, 44). We previously reported that CRC patients overexpressing DCLK1-S had worse overall survival and disease-free interval, compared with patients expressing low levels of DCLK1-S (12). Here, we evaluated pathophysiologic relevance of FOXD3 ± DCLK1(S) expression in CRC samples from the same patient cohort. Our findings suggest that additional analysis of FOXD3 in the CRC samples did not significantly alter the prognostic value of measuring DCLK1-S alone, as relative expression levels of FOXD3 were very low in CRCs, to start with. DCLK1-L expression levels also did not correlate with overall survival of the patients, providing further evidence that the S-isoform plays a significant role in the progression of hCRCs. Isogenic clones of a colon cancer cell line, COLO-205, overexpressing S-isoform, but not L-isoform, of DCLK1 demonstrated a significant increase in the invasive potential of the cells in an *in vitro* assay, which may explain the worse outcomes for patients expressing the S-isoform in their colonic tumors.

We recently reported that staining of adenomatous polyps from screening colonoscopy, with DCLK1(S)-antibody, helped to identify patients at high risk of developing CRCs (9). In this study, we examined whether FOXD3 staining of adenomas will augment the findings with the novel DCLK1(S)-Antibody, generated by us (9). We discovered that polyps from high-risk patients, stained for FOXD3 at significantly lower levels than polyps from low-risk patients. The latter results suggest that measuring FOXD3 along with DCLK1-S may provide more accurate results for assessing risk of the patients for developing CRCs, at the

early adenoma stage, itself. Thus, our studies so far strongly suggest that loss of expression of DCLK1(L)/FOXD3, associated with increased expression of DCLK1-S, likely confers a highly aggressive phenotype to epithelial cells, at all stages of colon carcinogenesis in humans.

Materials and Methods

Reagents used

Antibodies used in these studies included: anti-β-actin (total; Sigma); anti-DCLK1 antibody (Abcam); anti-FOXD3 antibody (Abcam). Anti-DCLK1-S antibody was generated in our laboratory as described previously (9). The anti-DCLK1-S-Ab was specific for the S-isoform and did not cross-react with the L-isoform (9). Sepharose beads and all other chemical reagents were purchased from Sigma. cDNA Synthesis Master Mix was purchased from GeneDEPOT. SYBR green qRT-PCR kit was purchased from Bio-Rad. Promega GoTaq green Master Mix was used for PCR amplification, using a thermal cycler from Eppendorf. FOXD3 expression plasmid and vector controls were purchased from GeneCopoeia. Smart Pool of target-specific siRNA and nontargeting (control) siRNA were purchased from Dharmacon. Transfection reagent FuGENE6 was bought from Roche, and all primers used were synthesized by Sigma.

Cell culture

HEK293 and HCT116 cell lines were obtained from ATCC, and have been maintained in the laboratory for several years. All other hCCCs used in the current studies, including COLO-205, RKO, COLO-320, and SW1417 cell lines were purchased from ATCC in 2015 and confirmed by ATCC. CCD841 cells were purchased from ATCC in 2015 by Dr. Carla Kantara (UTMB, Galveston, TX) and confirmed by ATCC and were kindly gifted to us. All cell lines were monitored regularly for absence of mycoplasma, and HEK293 and HCT116 cell lines were confirmed to represent human epithelial cell lines with the help of Biosynthesis, Inc., as described previously (12).

Procurement of human patient samples for RT-PCR or IHC analysis

For RT-PCR analysis, samples of normal colonic mucosa and primary colonic adenocarcinomas (AdCA) were obtained as discarded samples (as per our approved UTMB IRB protocol #91-310) from the Tissue Core Facility at Cancer Center, University of Alabama (Tuscaloosa, AL), as part of CHTN Program funded by NIH. All samples were collected and flash-frozen and stored in liquid nitrogen or at −80°C until analyzed. Pathology of all samples was confirmed. For IHC analysis, all samples were obtained as discarded specimens from UTMB Pathology department and pathology of all samples was confirmed as described previously (9).

Analysis of cell lines/tissue samples by RT-PCR/qRT-PCR

Total RNA was isolated from cell lines in monolayer cultures at 60%–70% confluency, or from human patient tissues using TRIzol reagent (Invitrogen), as described previously (12). For qRT-PCR, the iTaq Universal SYBR Green Supermix (Bio-Rad) was used as per the manufacturer's instructions, as described previously (12). The primer sequences used for

PCR amplification of cDNA for both RT-PCR/qRT-PCR analyses are provided in Supplementary Table SI. Electrophoresis gels presented were cropped to present all the bands observed within the range covered by the molecular markers used (between 100 bp and 1,000 bp for RT-PCR data), to avoid primer dimers seen toward the end of the run. Processing of the electrophoresis blots was applied equally across the entire image. Touch-up tools were not used to manipulate data.

Western immunoblot analysis

Cell lines growing as monolayer cultures were harvested and processed for preparing cellular lysates, followed by electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes as described previously (3, 10, 12). Samples containing 30–50 μg of proteins were subjected to electrophoresis and transferred to PVDF membranes as described previously (3, 10, 12). Blots were cut into horizontal strips containing target or loading-control proteins (β-actin), and processed for Western immunoblot analysis, as described previously (3, 10, 12). Antigen–antibody complexes were detected with a Chemiluminescence Reagent kit (Thermo Scientific or GE Healthcare). Membrane strips containing either target or loading control proteins were simultaneously exposed for equal time to autoradiographic films. Western blots presented were cropped to exclude bands beyond the range of the molecular markers at the running ends. Processing of films was applied equally across the entire image. Touch-up tools were not used to manipulate data.

Treatment of cells with 5-azacytidine

Cells were treated with 5-azacytidine as described previously (12). In brief, hCCC cell lines, including HCT116 cells, were seeded in 100-mm dishes at a density of 5×10^6 cells/dish, one day prior to drug treatment. The cells were treated with 10 μmol/L 5aza2′deoxycytidine (5-Azacytidine) on days 2 and 5 of culture. The cells were harvested on day 6 of culture and total RNA isolated. RNA was processed for measuring relative levels of DCLK1-S and FOXD3 by RT-PCR.

IHC

For IHC staining of patient samples, slides containing tissue sections were deparaffinized and hydrated using xylene and ethanol, as described previously (9). Sections were permeabilized by incubating in 0.2% Triton-X-100 for 10 minutes at room temperature followed by unmasking of the antigen by boiling in sodium citrate buffer, pH 6.0, for 20 minutes, with intermittent boiling and cooling cycles. Normal 5% goat serum was used to block nonspecific binding for 2 hours at room temperature. For DCLK1-S staining of samples, the mono-specific DCLK1-S-Ab was used as described previously (9). Rabbit polyclonal primary antibody against FOXD3 (GeneTex catalog no. GTX87777) was used at 1:100 dilution at 4°C overnight. Incubated sections were washed and incubated at room temperature for 2 hours with HRP-conjugated anti-rabbit antibody at 1:200 dilution, as per ABC Kit (Vecta Stain Elite ABC HRB kit, catalog no. PK6101, Vector Laboratories). Sections were stained with hematoxylin, washed, dehydrated, and mounted with coverslips for microscopy. Images from the tissue sections were captured by a Nikon microscope (TS100) equipped with a digital camera at 20× and 40× magnification. The images were

analyzed by ImageJ and intensity of staining was quantified in at least five different areas of the section, as described previously (9).

Transient transfection of cells with oligonucleotides and expression plasmids

Cell lines were transfected with either target specific FOXD3/control siRNA, or expression/control plasmids as indicated, using LipofectamineTM 2000 (Invitrogen) according to manufacturer's instructions, as described previously (10, 12). Transfected cells were propagated in normal growth medium containing 10% FCS, and processed for RT-PCR analysis after 48 hours of transfection for confirming downregulation of the target gene (FOXD3) or expression of indicated expression plasmids.

Promoter–reporter assays

IntronV (β) promoter–reporter constructs were generated as described previously (12). In brief, promoter fragments within IntronV (−2503/−771) were amplified using genomic DNA and cloned into PGL2 basic vector at *Xho*I and *Hin*dIII sites. The purified IntronV promoter–reporter constructs, were confirmed by DNA sequencing. Primer sequences used for PCR amplification of the promoter segments are listed in Supplementary Table SI. Mutant promoter–reporter constructs were designed in which the −2159 FOXD3 binding site was mutated and the promoter–reporter construct was truncated so that the −787 site was no longer included in the construct, as further described in the results section. Mutant constructs and corresponding vector controls were generated by GeneCopoeia. Promoter–reporter assays were performed as described previously (12). Cells were transiently transfected with the indicated promoter–reporter constructs using FuGENE6 for 24 to 48 hours, as per manufacturer's instructions. Promoter–reporter assays were conducted according to instructions of the manufacturer (Pro-mega). Luciferase activity was measured using a luminometer (Dynex Technologies) after 10 seconds of addition of substrate, as described previously (10, 12).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (12). In brief, cells were fixed in 1% formaldehyde to crosslink DNA to bound proteins, and reaction stopped by adding 0.125 mol/L glycine. Cells were washed with cold PBS, pelleted, and resuspended in ChIP sonication buffer, followed by sonication and centrifugation of fragments (600–700 bp long). The crosslinked chromatin supernatant was immunoprecipitated using target specific antibody (2–5 μg purified IgG) at 4°C, overnight. Control samples contained no antibody. For obtaining input levels of the corresponding proteins, equivalent numbers of cells were also processed for Western Immunoblot analysis. Protein A/G Sepharose beads, preabsorbed by Herring sperm DNA (100 μg/mL) was added to the chromatin–antibody complex and centrifuged to sediment the beads. DNA was eluted from the beads with elution buffer and DNA was precipitated using a high salt method, as described previously (12). The extracted DNA was purified and purified DNA was used for PCR amplification of the immunoprecipitated DNA with specific primers designed around the FOXD3-binding sites. The primer sequences used for this purpose are listed in Supplementary Table S1.

Procurement of colorectal tumor samples from patients for Kaplan–Meier survival curves

Ninety-two colorectal carcinoma tissues from patients with stage I–IV colorectal cancer were used for clinical validation of DCLK1-L, DCLK1-S, and/or FOXD3 expression from an independent cohort of 92 patients, as described previously (12). These specimens were preserved immediately after surgical resection in RNA later (Qiagen) and stored at −80°C until RNA extraction. The surgical samples were obtained from the Mie University Hospital, Japan, from patients enrolled from 2005 to 2011. Written informed consent was obtained from each patient (as per approved BCM IRB protocol #005-134). All tissues were collected in accordance with the approved guidelines set forth by UTMB and BCM for the IRB protocols.

Generation of isogenic clones of COLO205 cells (205 clones), stably overexpressing full-length GFP-tagged DCLK1-L/S proteins

The isogenic clones of the human colon cancer cell line, COLO205, were generated to overexpress GFP-tagged S or L isoform of DCLK1, as described previously (9). Briefly, eukaryotic expression plasmids expressing N-terminally GFP-tagged full-length coding sequences of either DCLK1-L or DCLK1-S were purchased (GeneCopoeia). 205 clones stably expressing full-length DCLK1-L (205-L) or DCLK1-S (205-S) were generated as described previously (9). Vector-transfected clones (205-C) expressing only GFP served as controls. GFP and DCLK1 expression by the clones was confirmed by Western blot analysis, as reported previously (9).

In vitro invasion assay

Transwell chambers, precoated with Matrigel, were purchased from Corning Inc, and the invasion assay was conducted essentially as per the protocol provided by Corning life sciences (Corning). Isogenic COLO205 clones (205 clones), as described above, were seeded in 10-cm tissue culture plates. After 24 hours, the isogenic clones were serum-starved for 24 hours. After an additional 24 hours, cells were scraped from the plates and washed three times with serum-free media. Cells were resuspended in 0.5-mL of serum-free media and transferred to the top of Transwell chambers (5×10^4 cells/well), as described below. The Transwell chambers were placed in a 24-well plate with 0.5-mL media containing 5% FCS. Each isogenic clone (205-L, 205-S, 205-C) was transferred to the top of eight Transwell chambers per clone. After 12 hours, cells that had invaded through the Matrigel to the bottom surface of the chamber were visualized with crystal violet and allowed to dry. The cells that had invaded through the Transwell membranes were counted using the ImageJ (Particle counter) program. Total number of cells, which had invaded the control wells, was arbitrarily assigned 100% value, and cell numbers from all 205-L and 205-S wells were calculated as a percent of control values, for purposes of comparing percent invasion, after normalizing the data to controls.

Design of the pilot retrospective study

The design of the study was the same as described previously (9), for analyzing predictive value of staining the polyps for DCLK1-S. Briefly, to assess the predictive value of FOXD3 staining in resected patient polyps at the time of baseline colonoscopy, a pilot retrospective

study was designed. Archived FFPE samples were obtained from Department of Pathology at UTMB. We have an approved IRB Protocol (91–310) for discarded specimens of colonic growths (Hps, hyperproliferative growths; Ads, adenomas; and AdCAs, adenocarcionomas). Two databases were available to us for selecting FFPE blocks that can be potentially assigned to either the low-risk group or the high-risk group. The two databases were arbitrarily termed the polyp database and the CRC database, as described previously (9).

The UH numbers of the identified patients were used to print PDI (previous diagnostic inquiry) reports, which contains pathology reports from all colonoscopies conducted at UTMB. Patients positive for adenomas at index and follow-up colonoscopies, who remained free of advanced adenomas (A-Ads) or CRCs at all subsequent colonoscopies for >15 years were labeled low-risk patients. We selected 6 patients from this group, as representative of low-risk group, as none of them developed A-Ads or AdCAs during the follow-up period. The CRC database was used for identifying patient samples for the high-risk arm of the retrospective study. We screened the 2014–2015 database of CRC patients, and identified several patients who had prior history of colonoscopies at UTMB, dating back to >8–12 years. Many of these patients were positive for only benign growths at the time of index/follow-up colonoscopies, and were selected as examples of high-risk patients. Patient FFPE blocks were obtained, irrespective of ethnicity, age, and gender. The ethnic mix of patients reflects the population of Galveston County in Texas. The number, size (whenever available), and pathology of all polyps that were collected at each colonoscopy for the low- and high-risk arms of the study, was tabulated and is further described in the Results section.

On an average, slides with consecutive tissue sections were prepared from approximately 7 FFPE blocks from each patient (to include at least 2 adenoma specimens, each, from initial and follow-up colonoscopies). At least one slide/polyp was stained with hematoxylin and eosin (H&E) to confirm morphology/pathology as described in the reports. The remaining slides were processed for IHC by our published procedures (9). Sections were stained with FOXD3-Ab in duplicate. In a few experiments, the consecutive sections were also stained for DCLK1-S. The ImageJ method, with conversion of positive staining to red scale, was used for quantifying the relative staining of the specimens, in terms of either percent area or percent epithelial cells stained. The highest stained polyp from an index and follow-up colonoscopy was used for purposes of comparison.

Statistical analysis

Data are presented as mean ± SEM of values obtained from indicated number of patient samples or experiments. To test for significant differences between means, nonparametric Mann–Whitney test was employed using STAT view 4.1 (Abacus Concepts, Inc). χ^2 tests were used to analyze the relationship between DCLK1-S and/or FOXD3 expression, with clinicopathologic factors. Overall survival curves were analyzed using Kaplan–Meier method, and comparisons were made using the log-rank test. The cut-off threshold between high and low expression group for DCLK1-S/L and FOXD3 transcripts was defined by the median values of the gene's expression in cancerous tissue. All P values were two-sided and differences were considered to be statistically significant if <0.05. Similarly, correlation, if any, between expression levels of the three proteins and disease-free survival were also

analyzed in 67 patients with stage I–III CRC disease, and data are presented in the Results section.

Results

FOXD3 expression inversely correlates with DCLK1-S expression in hCCCs/hCRCs

For reasons described in Introduction, FOXD3 expression was evaluated in relation to the expression of DCLK1-S in normal epithelial cells and hCCCs. We previously reported that all 15 hCCCs examined were negative for DCLK1-L, while 13 of 15 were positive for DCLK1-S (12). We now report that all cell lines positive for DCLK1-S were negative for FOXD3, while two cell lines negative for DCLK1-S (COLO205, SW1417), were positive for FOXD3 (Supplementary Table S2). Representative data from four hCCCs, positive or negative for DCLK1-S, are presented in Fig. 1A. HEK293, a normal human (h) embryonic epithelial cell line, and CCD841, a representative normal (h) colonic epithelial cell line, are negative for DCLK1-S, but positive for DCLK1-L (12). We confirmed absence of DCLK1-S in both the normal epithelial cells, and found both to be positive for FOXD3, at RNA (Fig. 1B) and protein (Fig. 1C) levels. Relative expression of DCLK1(S)/FOXD3 was examined in samples of normal colonic mucosa (from patients negative for adenomas/CRCs), and in samples from CRC patients; representative data are presented in Fig. 1D and E. The age/sex/ethnicity of the patients from whom the normal mucosal samples and AdCA samples were obtained is shown in Supplementary Table S3A. All normal mucosal samples were negative for DCLK1-S, but positive for FOXD3 (Fig. 1D), while the opposite was true for hCRC samples (Fig. 1E). Thus, FOXD3 expression inversely correlated with DCLK1-S expression, in all the cell lines/patient samples examined.

FOXD3 promoter is epigenetically silenced in hCCCs

The promoter of FOXD3 is hypermethylated in various cancers including hCRCs (37, 45). We previously reported reexpression of the epigenetically silenced DCLK1-L in HCT116 cells, on treatment with 5′-Azacytidine (Aza). Surprisingly, DCLK1-S levels were reduced on Aza treatment (12), leading us to speculate reexpression of a possible repressive factor of IntronV(β) promoter in Aza-treated hCCCs (12). The latter possibility was confirmed in the current studies. FOXD3 expression was restored in Aza-treated hCCCs, with concomitant loss of DCLK1-S expression (Supplementary Table S4); representative data from HCT116 cells are presented in Fig. 1F. Reexpression of FOXD3 in response to DNA methylase inhibitor confirms that FOXD3 promoter is epigenetically silenced in hCCCs, due to hypermethylation, as reported previously (37, 45). Importantly, the expression profile of DCLK1-L and FOXD3 did not change in the normal HEK293 cells, treated with Aza (Supplementary Table S4).

Analysis of FOXD3 expression by IHC staining of patient samples

Normal colonic mucosa (NRM) and AdCA samples were obtained from patients as described under Materials and Methods. The formalin-fixed paraffin-embedded (FFPE) samples were processed for IHC analysis with a specific FOXD3-Ab, as described in Materials and Methods; representative data from NRM/AdCA samples are presented in Fig. 2A. Five separate areas of each section/slide × 2 slides/sample were analyzed by ImageJ,

and converted to a red scale, as described previously (9). The red scale intensity represents the actual staining of the samples after normalizing the color intensity by filtering the background noise. The percent area stained for each sample was calculated as described in Materials and Methods, and data from all samples are presented as a bar graph in Fig. 2B. The gender/age/ethnicity of patients from whom the samples were obtained are shown in Supplementary Table S3B. On an average, the percent area stained with FOXD3-Ab was significantly higher in normal samples, compared with that in AdCA samples. Majority of the staining (>95%) was in the epithelial component for both NRM/AdCA samples, with <5% in the stroma.

Identification of functional FOXD3 binding sites in the IntronV(β) promoter of *DCLK1* gene in the normal and cancer cells

The consensus DNA binding sequence for FOXD3 (5′-A) [A/T] T (A/G) [TTTGTTT-3′], includes two overlapping forkhead-binding sites (40). Four potential binding sites for FOXD3 were present within 3 kb of the start site of β promoter with the associated nucleotide sequences (as shown in Supplementary Fig. S1). FOXD3 binding, *in situ*, to the potential FOXD3 binding sites was examined using ChIP assays. Only −2159 and −787 FOXD3 binding sites (labeled as 1 and 3 in Supplementary Fig. S1) were determined to be functional (bound to FOXD3) in HEK293 and CCD841 cells, and are diagrammatically shown in Fig. 3A. HEK293 cells were used for the remainder of the studies, as CCD841 cells are difficult to transfect. Similar to CCD841, HEK293 cells were null for DCLK1-S expression, but expressed relatively high levels of FOXD3, at both the transcript and protein levels (Fig. 1B and C). In ChIP assays, FOXD3 demonstrated binding to both −2159 and −787 binding sites in HEK293 cells, while HCT116 cells were negative for binding at both the sites (Fig. 3B and C). To confirm specific binding of the antibody to the indicated FOXD3 binding sites in the IntronV (β) promoter in the two cell lines, heat-inactivated antibody was also used in the ChIP assays as shown in Fig. 3D. As can be seen, the heat-inactivated antibody did not show any binding to the FOXD3 binding sites. Relative binding of FOXD3 to the indicated FOXD3 binding sites in HEK293 cells from several experiments is presented as percent of total FOXD3 (input) in the cells (Fig. 3E). For reasons unknown, binding to −787 site was significantly more pronounced than binding to −2159 site (Fig. 3). These results suggest that FOXD3 *cis*-elements likely play a direct role in transcriptional regulation of IntronV(β) promoter.

Role of FOXD3 and FOXD3 *cis*-elements in regulating transcriptional activity of IntronV(β) promoter

To further evaluate the role of FOXD3 in transcriptional regulation of DCLK1-S, HCT116 cells were transiently transfected with mammalian expression vectors, expressing either FOXD3 cDNA or the empty control vector (Control; as described in Materials and Methods). In cells expressing FOXD3-cDNA, DCLK1-S expression was significantly down-regulated at both the transcript (Fig. 4A) and protein levels (Fig. 4B, i and ii); significant expression of FOXD3 was confirmed in cells transfected with FOXD3-expressing plasmids (Fig. 4B, i and ii). Promoter–reporter studies were conducted to further confirm trans-regulatory effects of FOXD3 on IntronV(β) promoter. An IntronV(β) promoter(−2503/−771) reporter (LUC) construct, labeled DCLK1-S-LUC1 (Fig. 4C) was used. HCT116 cells were

transiently cotransfected with FOXD3-cDNA expression plasmid, along with DCLK1-S-LUC1 construct; control cells were transfected with empty plasmid and/or empty LUC vector, as shown in Fig. 4D. The transcriptional activity of DCLK1-S-LUC1 construct was significantly decreased in cells co transfected with FOXD3-cDNA (Fig. 4D).

In the next set of studies, trans-regulatory effects of FOXD3 were examined in HEK293 cells, using a different strategy. HEK293 cells were transiently transfected with either control siRNA (Con) or FOXD3 siRNA. In cells transfected with FOXD3-siRNA, DCLK1-S expression levels were significantly upregulated at both the transcript (Fig. 5A) and protein levels (Fig. 5B, i and ii) in HEK293 cells, confirming an important inhibitory role of FOXD3 on the expression of DCLK1-S in normal cells. In HEK293 cells, transfected with FOXD3-siRNA, significant downregulation of FOXD3 was confirmed (Fig. 5B, i and ii). Unlike the β promoter, the 5′(α) promoter of *DCLK1* gene lacks FOXD3 binding sites (12). Expression levels of the L-isoform remained unchanged in HEK293 cells on treatment with FOXD3-siRNA (Fig. 5C and D), strongly supporting specific effects of FOXD3 on trans-regulating promoters containing functional FOXD3 binding sites. In promoter–reporter studies, HEK293 cells were transiently cotransfected with control/FOXD3-siRNA, along with DCLK1-S-LUC1 vector (containing wild-type FOXD3 sites). The transcriptional activity of the WT promoter–reporter construct was significantly increased in HEK293 cells, transfected with FOXD3-siRNA, compared with that of cells transfected with the control siRNA (Fig. 5E). The DCLK1-S-LUC1-vector was mutated/truncated (MUT) for the FOXD3 binding sites as shown in Supplementary Fig. S2. Briefly, the MUT promoter–reporter construct was designed so that the −2159 FOXD3 binding site was mutated, while the −787 site was removed by truncating the fragment to −807, rather than −771. HEK293 cells were transiently transfected with either the WT DCLK1-S-LUC1 construct or the MUT/truncated construct. The transcriptional activity of MUT construct in the transfected HEK293 cells was significantly increased compared with that of cells transfected with the WT construct (Fig. 5F). The results with the MUT construct (Fig. 5F) resembled the results with FOXD3-siRNA (Fig. 5E), confirming an important role of FOXD3 and its binding sites in negatively trans-regulating the β promoter of h*DCLK1* gene in normal cells. Interestingly, even after downregulating FOXD3 expression in HEK293 cells or mutating/truncating FOXD3 binding sites (Fig. 5E and F), transcriptional activity of IntronV(β) promoter–reporter constructs remained significantly lower than that in HCT116 cells (Fig. 4D), suggesting that besides loss of FOXD3, upregulation of oncogenic pathways, such as activated NFκB (12), are required for optimally activating the expression of IntronV(β) promoter in cells.

High expression of DCLK1-S, with or without low expression of FOXD3, in AdCA samples is associated with poor overall survival of CRC patients

The expression patterns of DCLK1-S and FOXD3 transcripts, in relation to clinicopathologic parameters, were analyzed using an independent cohort of 92 patient specimens, as described in Materials and Methods. As previously described (12), high expression of DCLK1-S significantly correlated with overall poor patient survival in patients with stage I–IV disease (Fig. 6A). Low or high expression of FOXD3, on the other hand, did not correlate with overall patient survival (Fig. 6B). The combined expression of both

DCLK1-S and FOXD3 also correlated significantly with overall survival of CRC patients, but the correlation was not any more significant that with DCLK1-S alone (Fig. 6A and C). The clinicopathologic variables and FOXD3/DCLK1(S) expression in the 92 curatively resected colorectal cancer patients at stage I–IV are presented in Supplementary Table S5. The prognostic value of DCLK1-S/L and FOXD3 was further examined using more stringent criterion (Supplementary Fig. S3). The disease-free survival of 67 patients with stage I–III CRC once again correlated significantly with DCLK1-S levels, with or without FOXD3 (Supplementary Fig. S3A–S3C). The clinicopathologic variables and FOXD3/DCLK1(S) expression in the 67 curatively resected colorectal cancer patients, at stage I–III are presented in Supplementary Table S6A. The multi-variant analysis for predictors of disease-free survival are presented in Supplementary Table S6B; the data suggest that high expression of DCLK1-S along with low expression of FOXD3 may represent an independent prognostic indicator for worse disease-free survival of the patients. We recently reported that while DCLK1-S expression is increasingly elevated in colonic tumors during colon carcinogenesis (9, 12), the relative expression of DCLK1-L is significantly reduced/lost in the epithelial component of the tumors during colon carcinogenesis (9, 12). However, significant levels of DCLK1-L were expressed in the stroma of AdCAs, with unknown significance (9). We therefore additionally examined possible correlation of DCLK1-L expression in the CRC specimens with overall survival and disease-free survival. No significant correlation was obtained between the relative expression levels of DCLK1-L with overall survival of the 92 CRC patients (Fig. 6D), or between expression levels of DCLK1-L and disease-free survival in 67 patients with stage I–III disease (Supplementary Fig. S3D).

The benign/premalignant growths (Hps/Ads) at initial and follow-up colonoscopies, from high-risk patients, express significantly lower levels of FOXD3, compared with polyps from low-risk patients: inverse correlation with DCLK1-S levels

We recently reported that DCLK1-S staining of polyps at index colonoscopy was significantly higher in patients at high-risk of developing CRCs compared with that in patients at low-risk, and that the DCLK1-S staining of the polyps remained significantly lower in the low-risk patients at subsequent colonoscopies (9). As loss of FOXD3 allows DCLK1-S expression, we used the polyp specimens from the high- and low-risk patients, as described previously (9), and examined whether FOXD3 staining could be used as an additional biomarker for predicting risk of the patients for developing CRCs. Criteria used for assigning high/low-risk to patients, for developing CRCs, is described in Materials and Methods. The age, gender, and ethnicity of the patients whose samples were obtained from the Department of Pathology as FFPE blocks are presented in Supplementary Tables S7A and S7B. The type of polyps and size (whenever available) at the time of initial and subsequent follow-up colonoscopies are also presented in the Supplementary Table S7A and S7B. The FFPE blocks were processed for making slides with 2/3 sections per slide by routine histologic methods, as published previously (9), and stained with FOXD3-Ab. IHC data from polyps of representative patients, at initial and follow-up colonoscopies, from the low- and high-risk groups are presented in Fig. 7A; numbers 1, 2, 3 in the figure legend represent initial and follow-up colonoscopies. The staining data from all the patients were quantified by ImageJ and red scale method, and presented as percent area stained, as shown in Fig. 7B. The total number (n) of polyps analyzed in the high- and low-risk patients per

colonoscopy is shown above each bar graph (Supplementary Fig. S7B). At least two or more polyps were analyzed per colonoscopy from each patient for data presented in Fig. 7B. The percent staining of the polyps for FOXD3 in the high-risk patients was significantly lower than that in polyps from low-risk patients at all time points and resembled the level of staining measured in AdCA samples, shown in Fig. 2.

We next examined whether the inverse relationship between DCLK1-S and FOXD3 expression was maintained in the polyps at subsequent colonoscopies. Staining from representative polyps for both DCLK1-S/FOXD3, from adjacent sections of adenomas, removed at three consecutive colonoscopies, are presented in Supplementary Fig. S4A and S4B (low-risk patients) and Supplementary Fig. S4C and S4D (high-risk patients). Data from all the polyps examined from the two groups of patients is presented as either percent area stained (Supplementary Fig. S5A and S5B), or as percent epithelial cells stained (Supplementary Fig. S5C and S5D). Data presented in Supplementary Figs. S4 and S5 clearly demonstrate the inverse relationship between staining for DCLK1-S and FOXD3 in low-and high-risk patients. FOXD3 staining was significantly higher than that of DCLK1-S in polyps of low-risk patients, at all three colonoscopies, while the pattern was reversed in the case of high-risk patients (Supplementary Figs. S4 and S5). All polyps expressed significantly higher levels of DCLK1-S (4–10 fold higher than FOXD3) in high-risk patients (Supplementary Figs. S4 and S5). DCLK1-S staining remained high or increased in polyps from high-risk patients at subsequent colonoscopies, but the relative expression levels of FOXD3 continued to decrease in the polyps of high-risk patients from subsequent colonoscopies (Supplementary Figs. S4 and S5). Thus, combining the staining data for both markers (DCLK1(S)/FOXD3) may allow for identification of patients at high-risk for developing CRCs, with greater confidence at early stages of the disease, and give an opportunity for developing targeted preventative strategies for these patients.

Overexpression of DCLK1-S imparts a significant increase in the invasive potential of colon cancer cells

The colon cancer cell line, COLO205 (205), does not express either isoform of DCLK1(12) (Fig. 1A), and was used for developing isogenic clones which either overexpressed S-(205-S) or L-isoform (205-L), as described in Materials and Methods; control clones (205-C) only expressed GFP. The invasive potential of the isogenic clones was examined in an *in vitro* invasion assay, as described in Materials and Methods. Representative data from 1/8 transmembranes, containing cells that invaded through the transmembranes are presented in Fig. 7C(i). Data from all eight transwells/clone are shown in Fig. 7C(ii), as bar graphs. The 205-S clones were significantly more invasive (by >~500%) than 205-L/205-C clones. The significant difference in the invasive potential of S-expressing clones may explain the worse outcomes for patients with high S-expressing tumors (Figs. 6 and 7; Supplementary Figs. S4 and S5).

Discussion

In this study, we have described for the first time an important role of FOXD3 in suppressing the expression of isoform-2 of DCLK1 (DCLK1-S), from the alternate β-promoter in

normal, nontumorigenic cells (Figs. 4 and 5). Our findings strongly suggest that loss of FOXD3 expression in hCCCs/hCRCs, due to epigenetic silencing of FOXD3 promoter (Fig. 1F; Supplementary Table S4), allows the expression of isoform-2 from the β promoter in cancer cells, and that FOXD3 expression inversely correlates with that of DCLK1-S in normal/cancer epithelial cells (Figs. 1, 4, 5, 7; Supplementary Fig. S4 and S5; Supplementary Table S2). FOXD3 expression in hCCCs caused loss of DCLK1-S expression in the cells (Fig. 4), supporting a repressive effect of FOXD3 on the β promoter. Our findings strongly suggest that FOXD3 directly binds the β promoter, as confirmed in ChIP assays, and that at least two functional FOXD3 binding sites are present within 3 kb of the core β promoter in h*DCLK1* gene (Fig. 3).

Our results also suggest that loss of FOXD3 expression in normal cells can result in the expression of DCLK1-S, as seen in HEK293-cells (Fig. 5). But the expression levels in nontumorigenic, HEK293 cells remained significantly lower than that in hCCCs (such as HCT116), that may reflect the relative absence of activated NFκBp65 in HEK293 versus HCT116 cells, as reported previously (12). Besides FOXD3 and NFκBp65, it is expected that several other inhibitory/stimulatory transcription factors likely play an important role in regulating the expression of IntronV-β promoter in h*DCLK1* gene, as several *cis*-elements are present within the core 3-kb of the β promoter (Supplementary Fig. S1A).

A role of FOXD3 in development is well established (40), and knockdown of FOXD3 is embryonically lethal in mice (46). In cancer cells, FOXD3 has primarily been described as a negative regulator of proliferation and metastasis (30, 31, 34, 35, 44), including hCCCs (43). FOXD3 expression is downregulated in several types of cancers, compared with its levels in corresponding normal tissues (30, 31, 34, 35, 44), as confirmed in hCRCs in here (Figs. 1 and 2). Loss of FOXD3 expression was reported as a useful prognostic biomarker for predicting overall survival of patients with high-grade gliomas (30) and breast cancers (36). In this study, we did not find a statistically significant correlation between relative levels of FOXD3 transcript with overall survival of hCRC patients (Fig. 6B), as FOXD3 expression, to begin with, was very low in almost all hCRCs. Even though combination of FOXD3 with DCLK1-S correlated significantly with overall survival (Fig. 6C) and disease-free-survival (Supplementary Fig. S3C) of hCRC patients, the values were not any more significant than values for DCLK1-S alone (Fig. 6A; Supplementary Fig. S3A). However, high-DCLK1(S)/low-FOXD3 could serve as independent prognostic factors for predicting disease-free survival of patients with CRCs (Supplementary Table S6B). Similarly, combining FOX04 with FOXD3 improved prognostic value of FOXD3 in patients with gastric cancer (44). Thus, DCLK1-S and FOXD3, measured together, may predict overall survival/disease-free survival of hCRC patients more accurately.

In preliminary studies, we reported that DCLK1-S overex-pression in hCCCs imparts a potent invasive potential to the cells (47), which was confirmed in here (Fig. 7C). Thus, derepression of DCLK1-S expression may represent an important mechanism by which loss of FOXD3 in cancer cells imparts an invasive potential to the cells. As FOXD3 also represses expression of TWIST1 (32), several molecular pathways associated with metastasis of cancer cells are likely modified by FOXD3, directly/indirectly, in its role as a negative regulator of the invasive potential of cancer cells (31–35, 43). An important finding

of the current study was that while overexpression of DCLK1-S caused several fold increase in the invasive potential of hCCCs, overexpression of DCLK1-L did not (Fig. 7C, i and ii). We recently reported that the S-isoform primarily localizes to the nuclear and mitochondrial fractions of hCCCs, unlike L-isoform (9), strongly suggesting that the biology of the two isoforms is quite different, likely due to differences in the 3D structure of the two proteins, and the absence of microtubule-binding domain in S- versus L-isoform (12). In preliminary studies, we recently reported significant differences in the downstream targets of S/L isoforms (48), which may explain worse outcomes for patients with high S-expressing tumors.

Using integrative genome-wide scans, *FOXD3* promoter was reported to be hypermethylated in gastric cancers of *Helicobacter pylori*–infected patients, and in gastric cancer patients, with short-term survival (37). FOXD3 expression is thus lost during tumorigenesis, mainly due to methylation and epigenetic silencing of *FOXD3* promoter (37, 45), as further supported by our current findings (Fig. 1F; Supplementary Table S4). To confirm methylation of *FOXD3* promoter, one needs to conduct methylation analysis of the promoter. However, *FOXD3* promoter is highly GC-rich, which makes the methylation analysis of *FOXD3* promoter/introns challenging, as discussed previously (37, 45). It remains to be determined whether methylation status of the *FOXD3* promoter can be used for prognostic/diagnostic purposes, as currently suggested for 5′(α) promoter of *DCLK1* gene (26, 27).

Besides a possible use of FOXD3/DCLK1-S as prognostic markers for predicting patient outcomes for hCRC patients, an additional novel finding of our current studies is the possible use of FOXD3/DCLK1-S as biomarkers for predicting the risk of the patient for developing CRCs, at the time of screening colonoscopy (Fig. 7A and B; Supplementary Figs. S4 and S5). We recently reported that adenomas/polyps from initial and follow-up colonoscopies of high-risk patients (who developed CRCs within 10–15 years) stained for DCLK1-S at significantly higher levels (3–5 fold) than adenomas/polyps from low-risk patients (who did not develop hCRCs for >20 years; ref. 9). In this study, polyps from the same patients were additionally stained for FOXD3, and our results demonstrated that while polyps from low-risk patients stained strongly for FOXD3, polyps from high-risk patients stained poorly, and there was an inverse relationship between DCLK1-S and FOXD3 staining (Supplementary Figs. S4 and S5), just as we had observed with expression levels of DCLK1(S)/FOXD3 in patient adenocarcinomas (Fig. 1). Thus, based on results presented in here (Fig. 7A and B; Supplementary Figs. S4 and S5) we can deduce that measuring both DCLK1-S and its negative regulator, FOXD3, may allow more accurate prediction of patient outcomes for patients positive for colonic adenomas.

Colonoscopy is a commonly performed procedure (in many millions; ref. 49). However, of the millions who receive colonoscopies, 1%–2% develop CRCs (49). Molecular biomarkers of CRC risk are required, to accurately identify patients at the time of screening colonoscopy itself. Available molecular markers do not identify high-risk patients at the early adenomatous stage (50, 51). On the basis of our pilot studies so far, it is proposed that relative staining pattern of polyps for DCLK1(S)/FOXD3 may allow for accurate identification of high-risk patients, at the relatively early timepoint of screening colonoscopy

itself, which could be used for triggering additional preventative/interventional strategies, besides follow-up colonoscopies.

In summary, we report for the first time that FOXD3 is a potent repressor of IntronV(β) promoter of h*DCLK1* gene in normal cells. Loss of FOXD3 expression during colon carcinogenesis likely occurs due to hypermethylation and silencing of *FOXD3* gene, resulting in the expression of DCLK1-S in hCRCs. Our findings also suggest a prognostic/diagnostic value of measuring relative expression levels of DCLK1(S)/FOXD3 in colonic tumors of patients. It is speculated that loss of expression of both DCLK1(L) (Fig. 6; Supplementary Fig. S3) and FOXD3 (Figs. 1, 4–7; Supplementary Figs. S4 and S5), associated with increased expression of DCLK1-S (Figs. 1, 5, 6; Supplementary Figs. S3–S5), can be used as early diagnostic markers of epigenetic changes associated with colon carcinogenesis in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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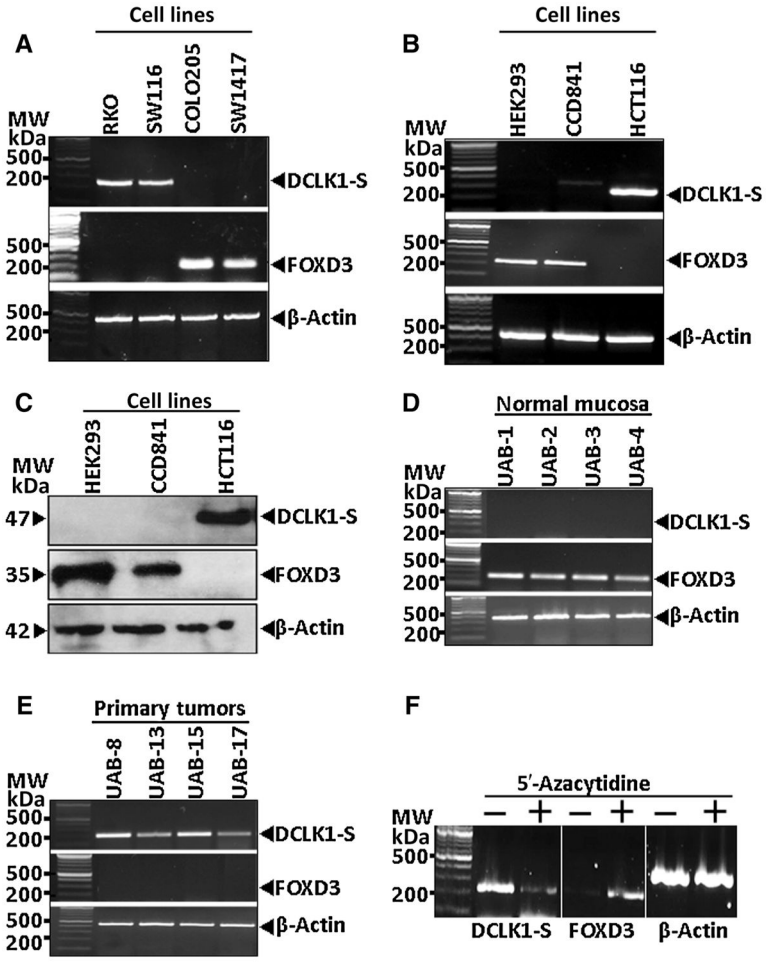

Figure 1.

Inverse relationship of DCLK1-S expression with FOXD3 expression in human cell lines/patient samples: effect of 5-azacytidine. Relative expression of DCLK1-S/FOXD3 was measured in normal human epithelial cells (HEK293/CCD841) and hCCCs (HCT116/RKO/SW116/COLO205/SW1417); representative RT-PCR (**A** and **B**) and Western blot data (**C**) are presented. Data from all cell lines are shown in Supplementary Table S2. Representative RT-PCR data from normal colonic mucosa (**D**) and hCRCs (**E**) is presented. Patients from whom samples were obtained are shown in Supplementary Table S3A. Effect of 5-Azacytidine was examined on relative expression of DCLK1-S/FOXD3 in hCCCs; representative RT-PCR data from control (−) and treated (+) HCT116 cells is shown (**F**). For data presented in **A–F**, β-actin was used as an internal control. All data presented are representative of data from 2–3 experiments. The molecular weight/mass in terms of bps or kDa are shown on left side of each panel.

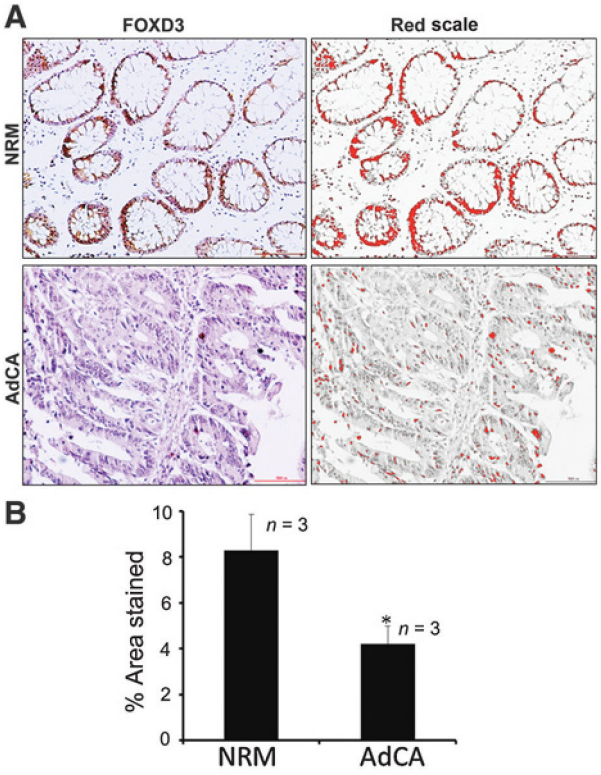

Figure 2.

Immunostaining of patient samples with anti-FOXD3-Ab. Formalin-fixed paraffin-embedded (FFPE) samples from normal colonic mucosa (NRM) or colonic adenocarcinomas (AdCA) were obtained as described in Materials and Methods, and the patient information is shown in Supplementary Table S3B. Staining of representative sections is presented in **A**. The staining data was quantified by converting to red scale (right), as described in Materials and Methods. The percent area stained from sections of all patient samples are presented as bar graphs in **B**. n = patient samples analyzed. *, $P < 0.05$ versus. normal values.

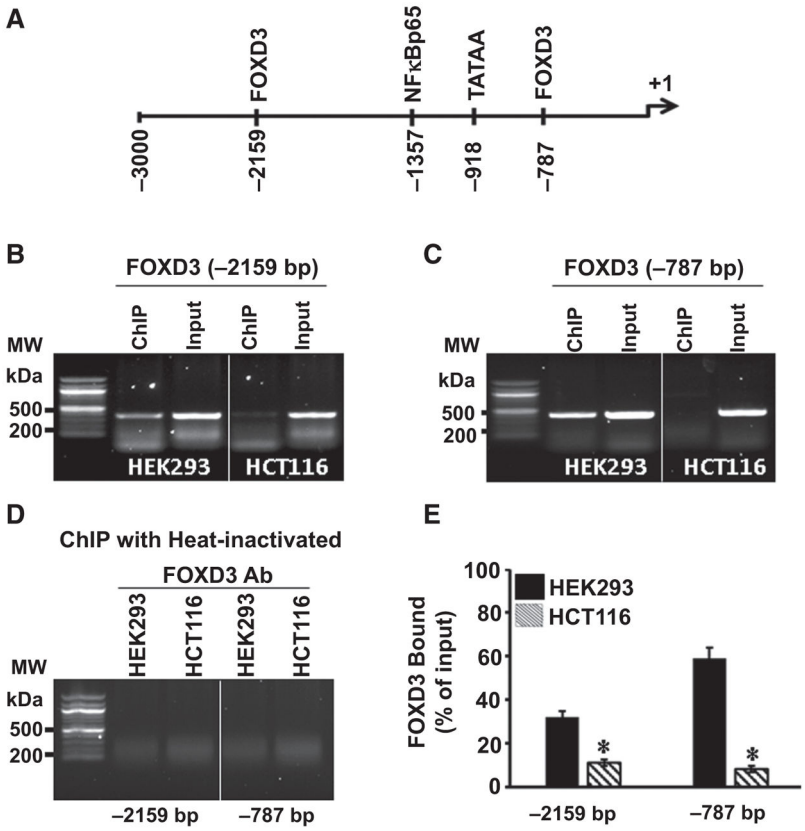

Figure 3.

FOXD3 binding to functional FOXD3 *cis*-elements in β-promoter of h*DCLK1* gene, in normal versus cancer cells. Diagrammatic representation of the two functional FOXD3 binding sites, in relation to TATA box and RelA binding site, in the β promoter is presented (**A**). Relative binding of FOXD3, to the indicated FOXD3 binding sites, in ChIP assays with HEK293/HCT116 cells, in relation to total FOXD3 (input) in the cells is shown (**B** and **C**). ChIP assay for binding of FOXD3 to the indicated FOXD3 binding site with heat inactivated antibody (**D**). Relative binding of FOXD3, *in situ*, to the two FOXD3 binding sites, is presented as percent input, from several experiments (**E**). Each bar graph = mean ± SEM of duplicates from three experiments.*, $P < 0.05$ versus HEK293 values.

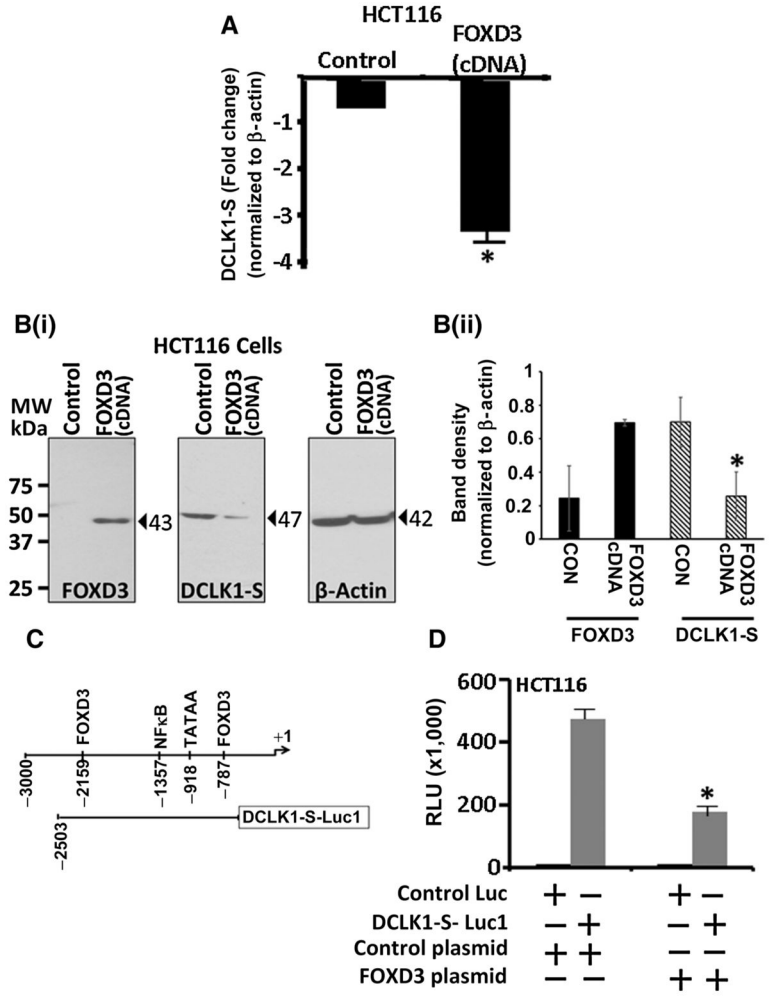

Figure 4.

Overexpression of FOXD3-cDNA, inhibits expression of DCLK1-S, at the transcriptional level. HCT116 cells, transiently transfected with either the EMPTY vector (control) or FOXD3-cDNA plasmids for 48 hours, were processed for measuring relative levels of DCLK1-S/FOXD3 by either qRT-PCR (**A**) or Western blot (**B**,**i** and **ii**). Representative Western blot data are shown in **B**(**i**), and data from all three experiments is presented as bar graphs **B**(**ii**), after normalizing the data to corresponding β-actin levels in the samples. β-Actin was measured as an internal control. The promoter–reporter construct (DCLK1-S-Luc1), containing both the FOXD3 binding sites, is diagrammatically presented, in relation to the β-promoter map (**C**). HCT116 cells were transiently transfected with either the control or DCLK1-S-Luc1 vector in the presence of either control or FOXD3-cDNA plasmid, as shown in **D**, for 48 hours. Relative transcriptional/luciferase activity (RLU) of cells transfected with the indicated plasmids is presented in **D**. Each bar graph in **A**, **B**(**ii**), **D** = mean ± SEM of duplicates from three experiments.*, $P < 0.05$ versus corresponding control plasmid (no FOXD3 expression).

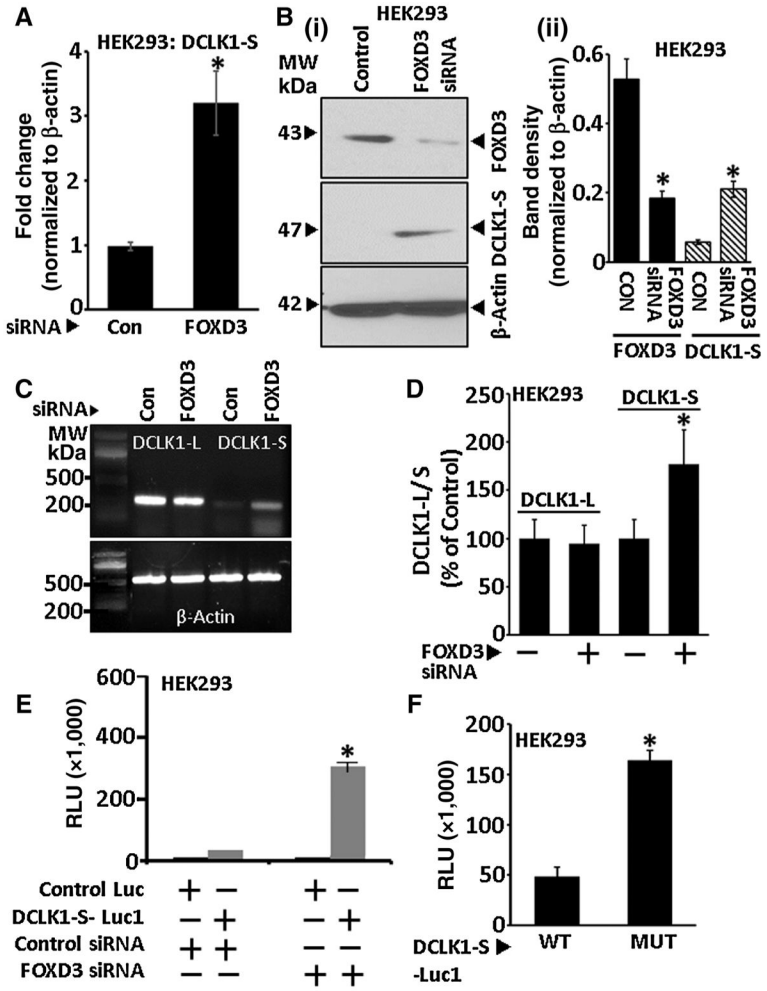

Figure 5.

Downregulation of FOXD3 in normal HEK293 cells results in upregulation of DCLK1-S (but not DCLK1-L) expression in the cells. HEK293 cells were treated with either control (con) or FOXD3-siRNA for 48 hours and processed for measuring relative levels of DCLK1-S (**A–D**), FOXD3 (**B**) and DCLK1-L (**C** and **D**), by either qRT-PCR (**A**), Western blot (**B**,**i** and **ii**), or RT-PCR (**C** and **D**). Representative Western blot data are presented in **B**(**i**), and data from all three experiments are presented as bar graphs in **B**(**ii**), after normalizing the data to corresponding β-actin levels in the samples. HEK293 cells were also transiently transfected with either control of DCLK1-S-Luc1 vectors in the presence or absence of control or FOXD3-siRNA, as indicated in **E**. Relative transcriptional/luciferase activity (RLU) of HEK293 cells, thus treated for 48 hours, is presented in **E**. Each bar graph in **A**, **B**(**ii**), **D–F** = mean ± SEM of duplicates from three experiments.*, $P < 0.05$ versus control cells treated with control siRNA. HEK293 cells were also transfected with vectors, expressing either wild-type (WT) or a mutated/truncated (MUT) promoter–reporter construct (DCLK1-S-Luc1), for 48 hours, and luciferase activity (RLU) measured, as shown in **F**. The mutant construct used is diagrammatically presented in Supplementary Fig. S2, and described in the text.

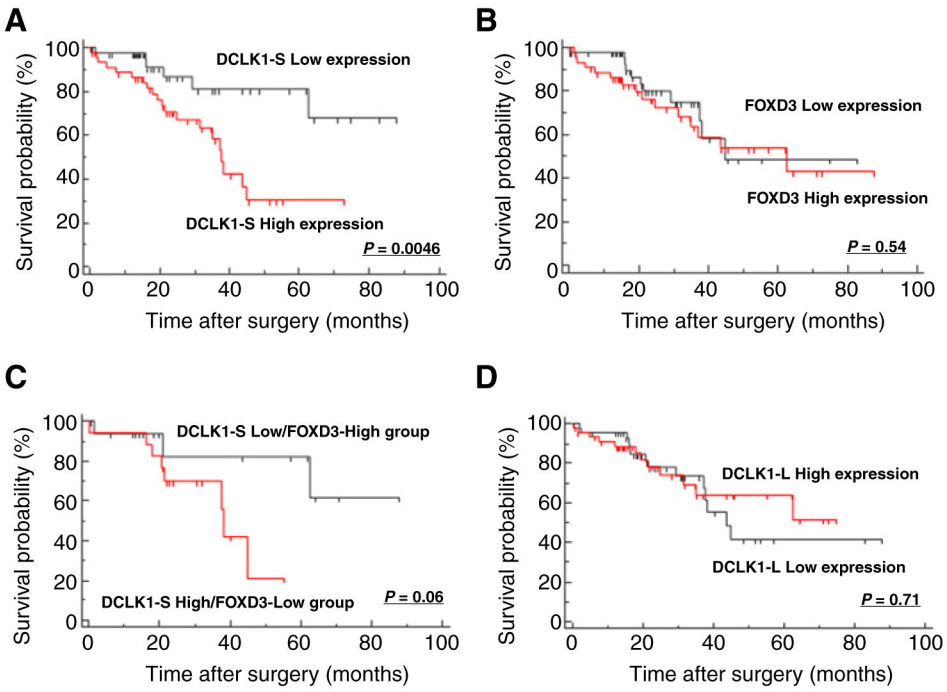

Figure 6.

Overall survival of patients with CRC in relation to low or high expression of DCLK1-S/L and FOXD3. **A,** Kaplan–Meier overall survival curves of CRC patients with stage I–IV disease, in relation to relative expression levels of DCLK1-S, measured by qRT-PCR; $n = 92$ patients. **B,** Kaplan–Meier overall survival curves of CRC patients, in relation to relative expression levels of FOXD3. **C,** Kaplan–Meier overall survival curves of CRC patients, in relation to relative expression levels of DCLK1-S and FOXD3 expression. **D,** Kaplan–Meier overall survival curves of CRC patients, in relation to relative expression levels of DCLK1-L. The cutoff threshold values were defined by using the median values in each case.

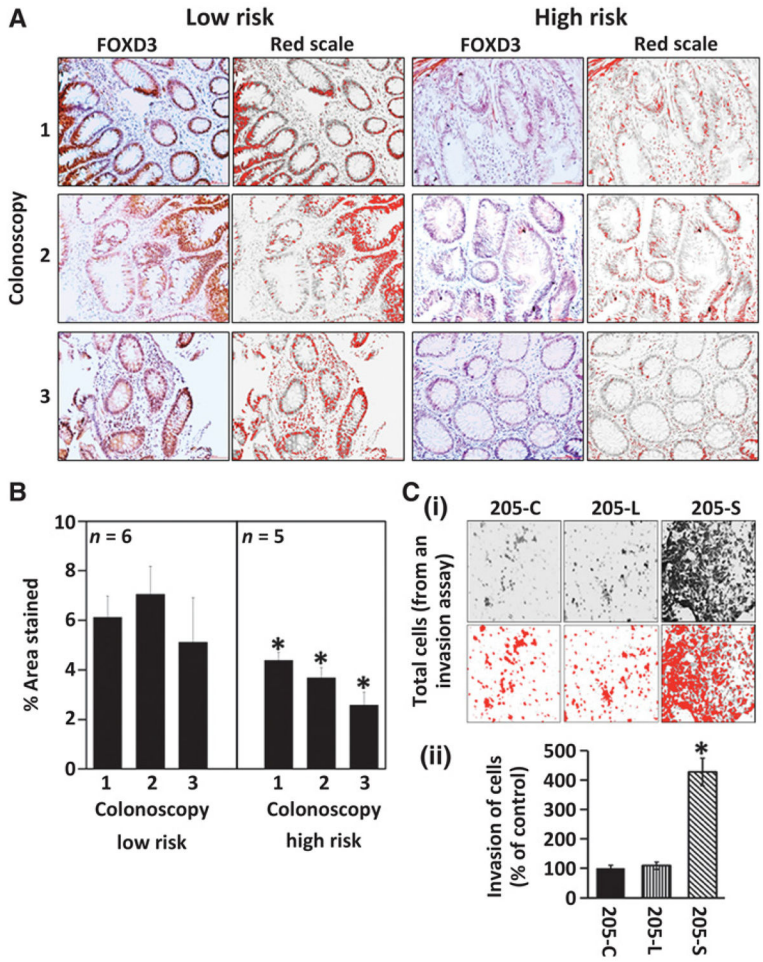

Figure 7.

A and **B,** Relative levels of FOXD3 staining in the adenomas from low- versus high-risk patients in a pilot retrospective study. Patients who had multiple colonoscopies at UTMB were separated in the two groups of low versus high risk, based on the absence/presence of AdCAs in the colon, within 10–15 years of initial colonoscopy, as described in Materials and Methods. Available FFPE samples of adenomas, from the identified patients (Supplementary Table S7A and S7B), from the initial and follow-up colonoscopies were obtained as described in Materials and Methods and processed for IHC staining with anti-FOXD3-Ab. IHC staining of representative polyps, removed at initial colonoscopy from a patient (colonoscopy #1) and subsequent follow-up colonoscopies (colonoscopies #2 and #3) are presented on the left (representing low-risk patients) and right side (representing high-risk patients) in **A**. In each case, the red-scale conversion of specific staining is presented. Staining from the total number (*n*) of polyps from the indicated patients in Supplementary Tables S7A and S7B are presented as percent area stained, in bar graphs presented in **B**. The data in each bar-graph are the mean ± SEM of indicated number (*n*) of samples. *, $P > 0.05$ between corresponding data for low-risk patients in **B**. The FOXD3 staining data, in relation to corresponding DCLK1-S staining data, for low-risk versus high-risk patients, is also presented in Supplementary Fig. S4. **C,** *In vitro* invasive potential of COLO205 cells

overexpressing L/S isoforms of DCLK1. Isogenic clones of COLO205 cells were generated to overexpress either the short (205-S) or long (205-L) isoforms, as described and confirmed in ref. 9; *in vitro* invasive potential of cells was examined as described in Materials and Methods. Clones that did not express either isoform served as controls (205-C). Representative images of the transmembranes, stained with crystal violet, are shown in top panels of **C**(**i**); bottom, ImageJ analysis of staining using particle counter. Data in **C**(**ii**), invasion of cells in all wells/clone, expressed as percent of that in control wells (as described in Materials and Methods)*, $P > 0.05$ between the corresponding clones.