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Selective Blockade of DCAMKL-1 Results in Tumor Growth Arrest by a Let-7a MicroRNA-Dependent Mechanism

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Abstract

Background & Aims—MicroRNAs (miRNAs) are non-protein coding RNAs that regulate gene expression. The tumor suppressor miRNA *let-7a* has been reported to be inhibited post-transcriptionally in embryonic stem cells and in human cancers. The microtubule-associated kinase DCAMKL-1 is a putative intestinal stem cell marker that is expressed in *ApcMin*+/+ adenomas. We investigated the role of DCAMKL-1 on expression of *let-7a* miRNA and the oncogene c-Myc and in tumorigenesis.

Methods—Immunostaining for DCAMKL-1 was performed on human tissue microarray slides. HCT116 and SW480 cells were transfected with DCAMKL-1 small interfering (si)RNA and analyzed for DCAMKL-1, c-Myc (using immunoblot and real-time RT-PCR) and *pri-let-7a* miRNA (using real-time RT-PCR) levels. A liposomal preparation of DCAMKL-1 siRNA was administered into HCT116 xenografts in nude mice and tumor volumes were measured. A luciferase reporter assay, with a plasmid containing a *let-7a* binding site at the 3' UTR, was utilized to measure *let-7a* in cell lines. Cells were isolated from normal mouse intestine using DCAMKL-1 and fluorescence-activated cell sorting (FACS) and subjected to *pri-let-7a* miRNA analysis.

Results—Expression of DCAMKL-1 was increased in human colorectal cancers. siRNA-mediated blockade of DCAMKL-1 resulted in HCT116 tumor xenograft growth arrest, increased levels of *pri-let-7a* miRNA, a corresponding decrease in luciferase activity, as well as decreased expression of the oncogene c-Myc. DCAMKL-1+ cells isolated by FACS demonstrated a significant decrease in *pri-let-7a* miRNA, compared to more-differentiated cells.

Conclusion—DCAMKL-1 is a negative regulator of *let-7a* miRNA biogenesis in intestinal stem and colorectal cancer cells; it could represent a novel target for anti-cancer stem cell-based strategies.

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Keywords

DCAMKL-1; *Let-7a*; microRNA; siRNA; c-Myc; HCT116; SW480; tumor xenograft; stem cell

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression in animal and plant systems^{1, 2}. miRNAs have emerged as important developmental regulators and control critical processes such as cell fate determination and cell death³. There is increasing evidence that several miRNAs are mutated or poorly expressed in human cancers and may act as tumor suppressors or oncogenes^{4, 5}. Gene expression is regulated by miRNAs through complementary elements in the 3' untranslated regions (3'UTRs) of their target messenger RNAs (mRNAs)⁶. *lethal-7 (let-7)*, a founding member of the miRNA family, is required for timing of cell fate determination in *C. elegans*⁷. In humans, various *let-7* genes have been reported to map to regions that are deleted in human cancers⁸. In addition, *let-7* is poorly expressed in lung cancers⁹, suggesting that *let-7* miRNAs may be tumor suppressors. In support of this, overexpression of *let-7* inhibited cell growth of a lung cancer cell line *in vitro*⁹.

Mature miRNAs are produced from primary miRNA transcripts (pri-miRNAs) through sequential cleavages by the Microprocessor complex, comprising the ribonuclease III Drosha component and the double-stranded RNA (dsRNA) binding protein DGCR8¹⁰ and Dicer¹¹. This coordinated enzyme complex results in the release of pri-miRNA and mature miRNA species. Posttranscriptional control of miRNA expression has been reported to occur in a tissue-specific¹² and developmentally regulated fashion^{13, 14}. In mouse embryonic stem (ES) cells and in mouse embryonal carcinoma (EC) cells, the magnitude of the Microprocessor processing block is greatest for members of the *let-7* family of miRNAs; although it is quite possible that the processing of all miRNAs may be regulated at the Microprocessor step^{13, 14}. It has been recently discovered that in many cancers, the miRNA profile is altered when compared to normal tissue¹⁵. It is becoming increasingly recognized that most cancers have a stem-cell-like compartment that is responsible for inciting and sustaining tumorigenesis^{15, 16}. One might hypothesize that miRNA profiles are altered in cancer stem cells (CSCs) within a particular tumor. Moreover, it is quite possible that such alterations are key factors in the initiation of the CSC. Recent evidence suggests that several miRNAs may be responsible for maintaining stem-cell-like characteristics^{17, 18}.

Furthermore, miRNA profiling of human and mouse ES cells reveals high levels of miRNAs expression, previously associated with oncogenesis and cell-cycle control^{19, 20}. Moreover, lack of *let-7* miRNA expression was observed as an indicator for "stemness" in epithelial progenitor cells. Recent studies have also demonstrated that *let-7* expression is absent in certain tumor cell lines, and that re-introduction of *let-7* into these cells causes differentiation and reduction in proliferation and tumor-forming ability^{22–24}. The regulatory mechanisms that control the maturation process of miRNA are unclear and the regulatory factors that control *let-7* miRNA levels, particularly in epithelial stem/progenitor cells, are completely unknown. The study of epithelial stem cell biology has been hampered by the lack of reliable stem cell markers that distinctly define and distinguish between stem and progenitor cell populations. There has been an accelerated interest however, in defining these populations as it is becoming increasingly clear that many important diseases including cancers are likely driven by effects on stem and/or progenitor cells.

We have recently demonstrated that the novel putative intestinal stem cell marker DCAMKL-1, a microtubule associated kinase expressed in post mitotic neurons²¹ and in the stomach²², is

expressed in the intestine, colon and *Apc*^{Min/+} adenomas²³. Given the importance of stem cells in mucosal regeneration and neoplasia, we sought to determine whether DCAMKL-1 played a functional role in tumorigenesis and whether these effects were mediated through regulation of *let-7a* miRNA.

Materials and Methods

Cell culture

HCT116, HCT116 p21^{-/-} and SW480 human colon adenocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin in a humidified chamber at 37°C with 5% CO₂.

Silencer RNA

DCAMKL-1 siRNA (si-DCAMKL-1) sequence targeting the coding region of DCAMKL-1 (Accession # NM_004734) (GGGAGUGAGAACAAUCUACtt) and scrambled control siRNAs (si-Scr) not matching any of the human genes were obtained (Ambion Inc., Austin, TX) and transfected using TransfectolTM (Ambion Inc.).

Real-time reverse transcription-PCR analyses

Total RNA isolated either from cells or from human colon cancer cell tumor xenograft samples was subjected to reverse transcription with SuperscriptTM II RNase H - Reverse Transcriptase and random hexanucleotide primers (Invitrogen, Carlsbad, CA). The cDNA was subsequently used to perform Real-time PCR by SYBR chemistry (SYBR[®] Green I; Molecular Probes) for specific transcripts using gene specific primers and Jumpstart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO). The crossing threshold value assessed by Real-time PCR was noted for the transcripts and normalized with β -actin mRNA. The changes in mRNA were expressed as fold change relative to control with $\pm$ SEM value.

Human primers used are:

β -actin:	Forward: 5'-GGTGATCCACATCTGCTGGAA-3' Reverse: 5'-ATCATTGCTCCTCCTCAGGG-3'
DCAMKL-1:	Forward: 5'-AGTCTTCCGATTCGAGTTGAG-3' Reverse: 5'-CAGCAACCAGGAATGTATTGGA-3'
c-Myc:	Forward: 5'-CACACATCAGCACAACACTACGCA-3' Reverse: 5'-TTGACCCTCTTGGCAGCAG-3'
Mouse primers used are:	
DCAMKL-1:	Forward: 5'-CAGCCTGGACGAGCTGGTGG-3' Reverse: 5'-TGACCAGTTGGGGTTCACAT-3'

miRNA analysis

Total miRNA was isolated using mirVanaTM miRNA isolation kit (Ambion Inc.). Total miRNA isolated either from cells or from human colon cancer cell tumor xenograft samples were subjected to reverse transcription with SuperscriptTM II RNase H - Reverse Transcriptase and random hexanucleotide primers (Invitrogen, Carlsbad, CA). The cDNA was subsequently used to perform Real-time PCR by SYBR chemistry (SYBR[®] Green I; Molecular Probes) for *pri-let-7a* transcript using specific primers and Jumpstart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO). The crossing threshold value assessed by Realtime PCR was noted for *pri-let-7a* miRNA and normalized with *U6* *pri*-miRNA.

The changes in pri-miRNA were expressed as fold change relative to control with $\pm$ SEM value. Primers used are:

<i>pri-U6:</i>	Forward: 5'-CTCGCTTCGGCAGCAC-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
<i>pri-let-7a:</i>	Forward: 5'-GAGGTAGTAGGTTGTATAGTTAGAA-3' Reverse: 5'-AAAGCTAGGAGGCTGTACA-3'

Western Blot analysis

HCT116 and SW480 cells were cultured in a 6 well plates to 40% confluency and were transfected with si-DCAMKL-1 or si-Scr for 72 h. Cells or the tumor xenograft samples were lysed and the concentration of protein was determined by BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). Forty μ g of the protein was size separated in a 7.5–15% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane with a semidry transfer apparatus (Amersham-Pharmacia, Piscataway, NJ). The membrane was blocked in 5% non-fat dry milk for 1 h and probed overnight with a rabbit anti-DCAMKL-1 antibody (Abcam Inc., Cambridge, MA) or with rabbit anti-c-Myc antibody (Santa Cruz). Subsequently, the membrane was incubated with anti-rabbit or anti-goat IgG horseradish peroxidase-conjugated antibodies (Amersham-Pharmacia) for 1 h at room temperature. The 82 kDa DCAMKL-1 and 49 kDa c-Myc proteins were detected using ECLTM Western Blotting detection reagents (Amersham-Pharmacia). Actin (43 kDa), used as loading control was identified using a goat polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Immunohistochemistry

Heat Induced Epitope Retrieval was performed on 4 μ m formalin-fixed paraffin-embedded sections utilizing a pressurized Decloaking Chamber (Biocare Medical) in citrate buffer (pH 6.0) at 99°C for 18 min. **(a) Brightfield:** Slides were incubated in 3% hydrogen peroxide, then normal serum and BSA at room temperature for 20 min. After incubation with primary antibody [DCAMKL-1 C-terminal (Abcam), anti-c-Myc (Santa Cruz Biotechnologies), L-FABP (Santa Cruz Biotechnologies)], the slides were incubated in peroxidase-conjugated EnVisionTM+ polymer detection kit (DAKO). Slides were developed with Diaminobenzidine (Sigma). **(b) Fluorescence:** Slides were first incubated in Image-iT FX signal enhancer (Invitrogen), followed by normal serum and BSA at room temperature for 20 min. After incubation with primary antibody [L-FABP (Santa Cruz Biotechnologies)], slides were incubated in appropriate Alexa Fluor[®] conjugated secondary [488 (green)].

Microscopic Examination

Slides were examined utilizing the Nikon 80i microscope and DXM1200C camera for brightfield. Fluorescent images were taken with PlanFluoro objectives, utilizing CoolSnap ES2 camera (Photometrics). Images were captured utilizing NIS-Elements software (Nikon).

Stem cell isolation

Based on protocols developed in intestinal stem cell biology^{24, 25}, we isolated stem cells from mouse intestine. The intestine was chopped into small strips, washed and incubated with 1 mM DTT (Sigma) for 30 min at room temperature. It was further incubated with 30 mmol/L EDTA (Sigma) for 10 min at 37° C. The strips were shaken vigorously in fresh HBSS (Cellgro) and filtered through 400 μ m mesh (Spectrum Labs) to separate the detached intestinal crypt epithelial cells from the tissue. The filtrate was passed through 80 μ m mesh (BD Falcon) to retain the crypts and washed. The crypts were digested at 37°C to create a single cell suspension.

FACS

The cells isolated from mouse intestine were incubated with 1:100 dilution of Alexa Fluor® 568 (Invitrogen) conjugated DCAMKL-1 antibody (Abcam) for 30 min. The cells were washed twice with HBSS containing 10% serum and sorted using Influx-V cell sorter (Cytospeia). DCAMKL-1 positively and negatively sorted cells were collected and subjected to total mRNA and miRNA isolation. mRNA was reverse transcribed and subjected to real-time RT-PCR for DCAMKL-1. Total miRNA was subjected to real-time RT-PCR for *pri-let-7a* miRNA.

Xenograft tumor model. (a) Liposomal preparation

siRNA was administered into the xenografts after incorporation into 1, 2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) (Avanti Polar Lipids, Alabaster, AL). DOPC and siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) (siRNA/DOPC). Tween 20 (Sigma-Aldrich) was added to the mixture at a ratio of 1:19 (Tween 20 to siRNA/DOPC). The mixture was vortexed and frozen in an acetone/dry ice bath and lyophilized. Before administration, the siRNA preparation was reconstituted in 0.9% sterile saline and injected at a dose of 50 μ l (5 μ M) per injection. **(b) Tumor therapy:** Female athymic nude mice (NCr-*nu*) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. They were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the USPHS “Policy on Human Care and Use of Laboratory Animals,” and all studies were approved and supervised by the Institutional Animal Care and Use Committee. HCT116 cells (6×10^6) were injected subcutaneously into the flanks of 4–6 week-old female athymic nude mice (5 mice per group). Tumors were measured with calipers and the volume was calculated as (length $\times$ width²) $\times$ 0.5. The tumors reached 1000 mm³ after 15 days of injection of cells. These tumors were injected with 50 μ l (5 μ M) of siRNA preparation on every third day from day 15 for a total of 5 doses.

Luciferase reporter gene assay

p*Let7a*-Luc reporter vector contains a *let-7a* miRNA specific binding site at the 3'UTR of the firefly (*Photinus pyralis*) luciferase gene obtained from Signosis Inc (Supplementary Figure 2). HCT116 and SW480 cells were transfected with the p*Let7a*-Luc reporter vector, Renilla luciferase expressing plasmid pRL-TK (Promega) along with DCAMKL-1 or scrambled siRNA using Transfectol™ (Ambion Inc.). Luciferase activity was determined as per the manufacturer's instructions (Dual-Luciferase Reporter Assay System; Promega) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) as described earlier²⁷. The activity, normalized to Renilla Luciferase activity, is presented as relative luciferase units relative to control with $\pm$ SEM values. Assays were performed in triplicate wells and experiments were repeated 3 times.

Statistical analysis

All the experiments were performed in triplicate. The data was analyzed by Student's t-test. Where indicated, the data is presented as mean $\pm$ SEM. A *p* value of <0.01 was considered statistically significant.

Results

DCAMKL-1 is overexpressed in cancer

To determine whether DCAMKL-1 was expressed in human colorectal cancers, we performed immunohistochemical analysis on human cancer tissue microarrays (Tissue Array Network and National Cancer Institute – Tissue Array Research Program). Staining revealed increased DCAMKL-1 protein (Figure 1A; brown – indicated by black arrows) in human colorectal

cancers specimens, compared to normal colonic mucosa. In tumors, the staining pattern was particularly impressive in the stroma surrounding malignant crypts (brown – indicated by blue arrow heads). Representative images of normal mucosa and two different human colorectal cancer specimens are shown in figure 1A. Similarly, we observed DCAMKL-1 expression in a variety of human colon cancer cell lines (Figure 1B). HCT116 and SW480 cells were transfected with DCAMKL-1 and scrambled siRNA; then total RNA was isolated and subjected to real-time RT-PCR. We noted a >70% reduction in DCAMKL-1 mRNA expression in DCAMKL-1 siRNA (si-DCAMKL-1) treated cells (Figure 1C and D). A reduction in DCAMKL-1 protein was also observed following si-DCAMKL-1 transfection (Figure 1C and D). Scrambled siRNA (si-Scr) did not affect the expression of DCAMKL-1 mRNA or protein (Figure 1C and D).

siRNA mediated knockdown of DCAMKL-1 leads to tumor growth arrest

Given the increased DCAMKL-1 expression in human colorectal tumors (Figure 1A) and in *Apc^{Min/+}* adenomas²³, we wanted to determine its role in tumor progression. Tumor xenografts were generated by injecting HCT116 cells (6×10^6) subcutaneously into the flanks of athymic nude mice. After 15 days, si-DCAMKL-1 and si-Scr were injected into the xenografts. Tumor volumes were measured using calipers at various time points before sacrifice and weights were determined after sacrifice^{26, 27}. Administration of si-DCAMKL-1 resulted in a statistically significant reduction ($p < 0.01$) in tumor size compared to the control or the si-Scr treated tumors (Figure 2A and B). Thus inhibition of DCAMKL-1 arrested HCT116 tumor xenograft growth. Total RNA isolated from these tumors was subjected to real-time RT-PCR and demonstrated a significant downregulation (55%) ($p < 0.01$) of DCAMKL-1 mRNA expression in the si-DCAMKL-1-treated tumors compared to control and si-Scr treated tumors (Figure 2C). This downregulation was associated with reduced expression of DCAMKL-1 protein in those tumors by Western blot analyses (Figure 2D).

Knockdown of DCAMKL-1 induces *pri-let-7a* miRNA

To determine the role of DCAMKL-1 mediated regulation of *pri-let-7a* miRNA, control and siRNA treated HCT116 tumor xenografts were analyzed for *pri*-miRNA expression by real-time RT-PCR. Compared to control and si-Scr treated tumors, there was a >3-fold increase in *pri-let-7a* miRNA expression in DCAMKL-1 siRNA treated tumors (Figure 3A). We next analyzed the effects of siRNA-mediated knockdown of DCAMKL-1 on *pri-let-7a* miRNA expression in HCT116 and SW480 cells. Real-time RT-PCR analysis revealed a 4-fold increase in *pri-let-7a* miRNA, compared to controls (Figure 3B and C). These data suggest that DCAMKL-1 negatively regulates *pri-let-7a* miRNA in human colon cancer cells.

DCAMKL-1 negatively regulates *let-7a* miRNA

As stated earlier, lack of *let-7* miRNA is an indicator of “stemness” in epithelial progenitor cells^{28–30}. To determine whether *pri-let-7a* miRNA was expressed in stem cells, we utilized FACS based sorting to isolate DCAMKL-1 positive and negative cells, which were analyzed for *pri-let-7a* miRNA. The antibody used for FACS was directed against the c-terminal extracellular domain of DCAMKL-1^{31 32} and conjugated to the Alexa Fluor® 568 fluorochrome. Following FACS, both sorted cell populations were examined by fluorescence microscopy. The positively sorted cells demonstrated the presence of DCAMKL-1 antibody staining, whereas the negatively sorted cells did not (Supplementary Figure 1A and B). Furthermore, DCAMKL-1 positive cells did not express L-type fatty acid binding protein (L-FABP), a marker of enterocyte lineage known to be expressed in differentiated intestinal epithelia^{35, 36}, indicating a less differentiated state (Supplementary Figure 1C and E). In contrast, L-FABP was found to be expressed in DCAMKL-1 negative cells (Supplementary

Figure 1D and F), indicating that these cells are more differentiated compared to DCAMKL-1 positive cells.

Total miRNA isolated from DCAMKL-1 positive and DCAMKL-1 negative cells were subjected to *pri-let-7a* miRNA expression by real-time RT-PCR and normalized using *pri-U6* miRNA. A 65% reduction in *pri-let-7a* miRNA was observed in DCAMKL-1 positive sorted “stem” cells relative to DCAMKL-1 negative cells (Figure 4A). To confirm sorting specificity, total RNA isolated from the cells was subjected to real-time RT-PCR for DCAMKL-1 mRNA expression (Figure 4B). These data suggests that DCAMKL-1 negatively regulates *pri-let-7a* miRNA in putative intestinal stem/progenitor cells.

To determine quantitatively the effect of siRNA-mediated knockdown of DCAMKL-1 on *let-7a* miRNA, we performed a luciferase reporter gene assay. HCT116 and SW480 cells were transfected with a plasmid containing firefly luciferase gene with a complementary *let-7a* binding site at the 3'UTR (Supplementary Figure 2). A dose dependent reduction in luciferase activity was observed following the knockdown of DCAMKL-1 (Figure 4C and D). This suggests that DCAMKL-1 may be a posttranscriptional regulator of *let-7a* miRNA downstream targets. However we cannot rule out other alternative mechanisms for DCAMKL-1 such as acting as a transcriptional regulator of *let-7a* or as a posttranscriptional regulator of *let-7a* maturation.

Knockdown of DCAMKL-1 inhibits c-Myc

HCT116 tumor xenografts were evaluated for expression of the *let-7a* miRNA downstream oncogenic target c-Myc, following siRNA-mediated knockdown of DCAMKL-1 as described earlier. We observed a 45% reduction in c-Myc mRNA in si-DCAMKL-1 treated tumors compared to controls (Figure 5A). An even more striking reduction of c-Myc protein was seen by Western blot and immunohistochemical analyses (Figure 5B and C) of siDCAMKL-1 treated tumors. A significant reduction in c-Myc mRNA and protein was also observed in siDCAMKL-1 treated HCT116 (Figure 5D and E) and SW480 cells (Figure 5D and F). These data suggest that knockdown of DCAMKL-1 results in a reduced expression of c-Myc by a *let-7a* dependent mechanism.

Discussion

miRNAs play important gene-regulatory roles by pairing to the mRNAs of protein-coding genes to direct their posttranscriptional repression³⁷. The involvement of miRNAs in human cancer has been recently described¹⁵; with several reports indicating that miRNAs might be used as future diagnostic and therapeutic targets³³. Furthermore, characteristic miRNA expression signatures in various cancers that can profoundly affect cancer cell behavior has been reported¹⁵. miRNAs have been shown to play an important role in regulating stem cell self-renewal and differentiation by repressing the translation of selected mRNAs in stem cells and differentiating daughter cells. *Let-7a* is a tumor suppressor miRNA that is blocked posttranscriptionally in ES cells and in several human cancers^{14, 15, 19}. The regulatory factors that control miRNA expression, maturation and function in adult stem cells and cancers are just beginning to be explored.

This study demonstrates that the novel putative intestinal stem cell marker DCAMKL-1 is a negative regulator of *let-7a* miRNA expression/function. Here we demonstrate that DCAMKL-1 expression is increased in human colorectal cancers compared to normal uninvolved tissues. While we have previously shown DCAMKL-1 expression in *Apc^{Min/+}* adenomas, to our knowledge, this is the first demonstration of DCAMKL-1 in human colorectal cancer. In addition to the increased epithelial expression of DCAMKL-1 seen within the colorectal tumors examined, strong staining was also observed in the stroma surrounding

malignant crypts. Given the importance of epithelial–mesenchymal cell interactions in cancer³⁴ and the role of the niche in epithelial stem cell fate³⁵, we speculate that stromal DCAMKL-1 may participate in tumor progression.

Using a tumor xenograft model generated from HCT116 human colorectal cancer cells, we demonstrate near complete tumor growth arrest following siRNA-mediated knockdown of DCAMKL-1. These data strongly implicate a functional role for DCAMKL-1 in the regulation of tumor growth. Given the potential roles of *let-7a* miRNA in the regulation of gene expression in stem cells and cancer, we next assayed the tumor xenografts for *pri-let-7a* miRNA expression. We found a significant increase in *pri-let-7a* miRNA in the tumors following siRNA-mediated inhibition of DCAMKL-1. These data confirm that *pri-let-7a* miRNA is indeed a tumor suppressor miRNA, which is regulated by DCAMKL-1 in colorectal cancer cells.

Cellular transformation and tumorigenesis are driven by activation of oncogenes and/or inactivation of tumor suppressors. Oncogenic c-Myc overexpression is observed in many cancers along with enhanced cell proliferation³⁶. Furthermore, transcripts encoding both c-Myc and Kras are known to contain target sites for the *let-7* miRNA in their 3'UTR³⁷. Such findings led us to speculate that DCAMKL-1 may affect c-Myc expression in colon cancer via a *let-7a* dependent mechanism. Indeed, we found a 45% reduction in c-Myc mRNA and significant decrease in protein levels in the tumors following the inhibition of DCAMKL-1. These findings were confirmed *in vitro* in human colorectal cancer cell lines where knockdown of DCAMKL-1 resulted in increased *pri-let-7a* miRNA, which corresponded with a significant reduction of c-Myc. These data taken together strongly suggests that DCAMKL-1 negatively regulates the tumor suppressor miRNA *let-7a* resulting in reduced expression of its downstream target oncogene c-Myc.

In order to determine the effects of DCAMKL-1 knockdown on *let-7a* miRNA-dependent gene silencing of *let-7a* downstream targets, we performed a luciferase gene reporter assay containing a specific *let-7a* miRNA binding site at its 3'UTR. We found a significant dose-dependent reduction in luciferase activity following knockdown of DCAMKL-1. This provides a potential explanation and mechanism where inhibition of DCAMKL-1 results in decreased c-Myc and possibly other *let-7a* downstream targets.

In this report, we have demonstrated that DCAMKL-1, a protein expressed in both normal stem cells and in cancer, likely promotes tumorigenesis through the regulation of *pri-let-7a* miRNA and c-Myc. The presence of *let-7a* binding sites in the c-Myc 3'UTR leads us to speculate that DCAMKL-1 is regulating c-Myc posttranscriptionally. However, we cannot rule out other alternatives, such as direct transcriptional regulation. Nevertheless, the knockdown of DCAMKL-1 resulted in a marked reduction in c-Myc mRNA and protein *in vitro* and *in vivo*. Moreover, several other oncogenes contain *let-7a* binding sites in their 3'UTRs, thus it is quite possible that DCAMKL-1 may have similar effects on other oncogenic targets including Kras.

miRNAs are known to contribute to the preservation of 'stemness' and associated with self-renewal and differentiation in ES cells³⁸. Previous studies have also shown an overall reduction in miRNA expression in embryonic and tissue stem cells³⁹. We analyzed intestinal epithelial cells following FACS based sorting using DCAMKL-1 for *pri-let-7a* miRNA. A marked reduction in *pri-let-7a* miRNA was observed in DCAMKL-1 positively sorted "stem" cells relative to DCAMKL-1 negative cells. These data suggests that intestinal stem cells, like ES cells, express low levels of *let-7a*.

We cannot rule out the possibility that DCAMKL-1, alone or in concert with other factors, may block other pri-miRNAs in different physiological contexts. Furthermore, additional factors

may be discovered that regulate miRNA processing. Future studies targeting the putative stem cell marker DCAMKL-1 may reveal the mechanisms by which miRNA processing contributes to oncogenesis. The findings presented in this report suggest that regulation of miRNAs may represent an exciting new strategy to combat tumorigenesis, particularly in cancers originating from cancer stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

DCAMKL-1 is overexpressed in colorectal cancer. (A) Immunohistochemistry for DCAMKL-1 (brown) in normal (left panel) and two different colon cancer tissues (middle and right panels). Black arrow indicates representative epithelial cells positive for DCAMKL-1. Blue arrow head indicates the presence of DCAMKL-1 in the stromal compartment. (B) Western blot demonstrating the expression of DCAMKL-1 in three different colon cancer cell lines. Actin serves as control. (C) DCAMKL-1 specific siRNA (si-DCAMKL-1) decreases DCAMKL-1 mRNA (left panel) and protein expression (right panel) in HCT116 colon cancer cells compared to controls. (D) Similar decrease in DCAMKL-1 mRNA (left panel) and protein (right panel) observed following si-DCAMKL-1 transfection in SW480 colon cancer cells. For

C and *D*, values in the bar graphs are given as average $\pm$ SEM and * denote statistically significant differences (* $p < 0.01$) compared to control. All the experiments were performed in triplicates and were repeated 3 times.

Figure 2.

DCAMKL-1 is essential for tumor growth. (A) HCT116 cells were injected into the flanks of athymic nude mice (n=5 per group) to generate tumors. At day 15 siRNAs (si-DCAMKL-1 and si-Scr) were injected directly into the tumors and followed by injections every third day (inset). After 5 injections, tumors were excised at day 28 and are represented above. Tumor sizes with standard error are shown from data collected at the time of every injection. (B) si-DCAMKL-1 treatment resulted in significantly decreased tumor weight when compared to controls. (C) The expression of DCAMKL-1 mRNA in the tumors quantitated by real-time RT-PCR. (D) Western blot analysis for DCAMKL-1 was performed on tumors samples as

indicated. For A – C, values are given as average $\pm$ SEM and * denote statistically significant differences (* $p < 0.01$) compared to control.

Figure 3.

Knockdown of DCAMKL-1 induces *pri-let-7a* miRNA. (A) Quantitative real-time RT-PCR analysis for *pri-let-7a* miRNA in tumor xenografts. siRNA mediated knockdown of DCAMKL-1 results in increased expression of *pri-let-7a* miRNA. (B) si-DCAMKL-1 treated HCT116 cells demonstrate increased expression of *pri-let-7a* miRNA. (C) Similar induction of *pri-let-7a* miRNA was observed in SW480 cells. For A – C, values are given as average $\pm$ SEM and * denote statistically significant differences ($p < 0.01$) compared to control.

Figure 4.

DCAMKL-1 inhibits *let-7a* miRNA. (A) Intestinal stem cells (DCAMKL-1 +) isolated from normal mouse intestine demonstrate decreased *pri-let-7a* compared to more differentiated cells (DCAMKL-1 -). (B) Real-time RT-PCR data demonstrate an increased expression of DCAMKL-1 mRNA in DCAMKL-1+ sorted stem cells compared to more differentiated (DCAMKL-1 -) cells. siRNA mediated knockdown of DCAMKL-1 decreases luciferase activity (Relative Luciferase Units – RLU) following transfection with plasmid encoding luciferase containing *let-7a* binding site in HCT116 (C) and SW480 cells (D). For A – D, values are given as average $\pm$ SEM and * denote statistically significant differences (* p <0.01) compared to control.

Figure 5. Downregulation of DCAMKL-1 results in decreased expression of a *let-7a* downstream target. A decreased expression of c-Myc mRNA (A) and protein (B) was observed in HCT116 tumor xenografts following the knockdown of DCAMKL-1. (C) Decreased c-Myc expression (brown) in si-DCAMKL-1 treated tumors compared to controls by immunohistochemical analysis. siRNA mediated knockdown of DCAMKL-1 results in decreased c-Myc mRNA (D) and protein (E) in HCT116 cells. (D and F) Similar decrease was observed in SW480 cells. For bar graph in A and D, values are given as average $\pm$ SEM and * denote statistically significant differences (* p <0.01) compared to control.