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Promoter hypermethylation of *DLC-1*, a candidate tumor suppressor gene, in several common human cancers

Bao-Zhu Yuan[a], Marian E. Durkin[b], Nicholas C. Popescu[b,*]

[a]*Health Effects Laboratory Division, Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA*

[b]*Laboratory of Experimental Carcinogenesis, Building 37 Room 3C05, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

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Abstract Aberrant methylation of CpG islands within the promoter regions of tumor suppressor or cancer-related genes is a common mechanism leading to the silencing of gene expression. To determine whether aberrant methylation is a contributing factor to transcriptional inactivation of *DLC-1* (deleted in liver cancer-1), a candidate tumor suppressor gene, we examined its methylation status in twelve hepatocellular carcinoma, breast, colon, and prostate tumor cell lines with low or undetectable expression of *DLC-1*. By Southern blot analysis of DNA digested with the methylation sensitive enzyme HpaII, we found a different degree of promoter hypermethylation in all cell lines with aberrant *DLC-1* expression. The hypermethylation status was reversed by the addition of 5-aza-2′-deoxycytidine, a demethylating agent, in one human hepatocellular carcinoma line. These observations suggest that hypermethylation is responsible for abrogating the function of the *DLC-1* gene in a subset of liver, breast, colon, and prostate cancers.

1. Introduction

Chromosomal regions that are frequently deleted in cancer are thought to be the loci of tumor suppressor genes, whose loss allows unrestricted cell proliferation. The human *DLC-1* (deleted in liver cancer-1) was isolated from a primary human hepatocellular carcinoma (HCC). The *DLC-1* cDNA encodes a 1083− amino acid protein that is 92.5% identical to the rat *p122-RhoGAP* [1,2]. The *Rho* proteins, members of the *ras* superfamily, regulate cell morphology, motility, and proliferation, and the *RhoGAP* (GTPase activating proteins) convert the active, GTP-bound form of *Rho* to the inactive, GDP-bound form. *RhoGAP* may function as tumor suppressors by down-regulating *Rho* activity, which is increased in transformed cells [3,4]. The *DLC-1* gene was localized to chromosome 8p21∼p22, a region of loss of heterozygosity (LOH) in a variety of human cancers, including prostate, colon, breast, ovarian, liver, lung, bladder, and head and neck cancer [reviewed in 5,6]. Loss of DNA copy-number was found by comparative genomic hybridization in cancers of the breast, liver, colon, lung, ovary, bladder, and prostate as well as in osteosarcomas, mesotheliomas, B-cell lymphomas, and mantle cell lymphoma [7,8].

Deletion of the *DLC-1* gene has been detected in over 40% of human primary HCC and in 90% of HCC cell lines. Moreover, while the *DLC-1* mRNA is expressed in all normal tissues, 30% of HCC cell lines lack expression [1]. The absence or down-regulation of *DLC-1* was detected in significant number of breast, colon, and prostate tumor cell lines [1,9,10]. In our experiments and those of others, transfection of *DLC-1* into HCC cell lines inhibited in vitro cell growth and colony formation [11]. Furthermore, we recently demonstrated *DLC-1* suppression in vitro cell growth and in vivo tumorigenicity of breast cancer cells [12].

In the past several years, altered promoter DNA methylation has been recognized as an epigenetic mechanism commonly associated with the inactivation of tumor suppressor genes and other genes functionally important in cancer development [13–15]. Characteristic patterns of gene promoter hypermethylation have been found in a number of human tumor types [16,17]. In this study, to see whether aberrant methylation contributes to the transcriptional inactivation of *DLC-1* gene, we isolated the promoter region of

* Corresponding author. Tel.: (301) 496-5688, ext 240; fax: (301) 496-0734.

E-mail address: popescun@mail.nih.gov (N.C. Popescu).

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the human gene. Hypermethylation of the gene was detected in several HCC, breast, colon, and prostate tumor cell lines that lack *DLC-1* expression, demonstrating that aberrant promoter methylation is responsible for *DLC-1* inactivation in some cancer cells.

2. Materials and methods

2.1. Cell lines

The human tumor cell lines used in this study were purchased from the American Type Culture Collection. The cells were cultured in DMEM/F12 or RPMI-1640 media with 10% fetal bovine serum and antibiotics.

2.2. Cloning and characterization of DLC-1 *promoter region*

A genomic clone containing the *DLC-1* gene was identified by PCR-based screening of a human P1 library (Genome Systems, St. Louis, MO, USA). Ten micrograms of P1 DNA were digested to completion with the restriction enzyme BamH1, and the DNA was ligated to the pBluescriptSK(+) vector using a shotgun strategy. Plasmid subclones containing the promoter region of the gene were isolated by colony hybridization using a radiolabeled 0.5-kb probe located at the 5′ end of the *DLC-1* cDNA. The inserts of positive clones were characterized by restriction enzyme mapping and DNA sequencing. The sequence was compiled and computer analysis was performed as previously described [1,18]. To locate potential CpG islands at the 5′ end of the gene, the sequence was analyzed using the program CPGPLOT, available online at http://biowebpasteur.fr/seqanal/interfaces/cpgplot.html.

2.3. Detection of DNA methylation of the promoter region by Southern blot analysis

Aliquots of genomic DNA (10 μg) from tumor cell lines were digested with the methylation-sensitive restriction enzyme HpaII, separated on a 1% agarose gel, transferred to a nylon membrane and hybridized to a radiolabeled 0.33-kb probe encompassing the transcription start site and first exon of the *DLC-1* gene. Hybridization was performed in QuikHyb buffer (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The blot was washed twice at room temperature in 1× SSC/0.1% SDS for 30 minute each, once in 0.1× SSC/0.1% SDS at 62°C for 30 minutes, then exposed to a PhosphorImager plate and the image analyzed by ImageQuant Version 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA). Normal human genomic DNA samples used for controls were purchased from Promega (Madison, WI) or extracted from the peripheral blood cells of healthy volunteers and from normal human liver tissue obtained at autopsy.

2.4. 5-Aza-2′-deoxycytidine (5-aza-dC) demethylation treatment and Northern blot detection

Tumor cells grown to 60% confluence were treated with 0 μM, 2.5 μM, and 25 μM of 5-aza-dC (Sigma). After 48 hours of treatment, total RNA was extracted from the cells using TRIzol reagent (Invitrogen). Aliquots of total RNA (25 μg) were separated on 1% agarose/MOPS/formaldehyde gels, transferred to a nylon membrane and hybridized to a radiolabeled full-length *DLC-1* cDNA probe. Hybridization and washing of the blot were performed as described above.

3. Results

3.1. Characterization of the 5′ end of the human DLC-1 *gene*

The exon/intron organization of the mouse and human *DLC-1* genes has been determined [18]. The human *DLC-1* gene spans 50 kb and consists of 14 exons (Fig. 1). The D8S1754 microsatellite marker is present in the first intron. To study the regulation of *DLC-1* expression, we cloned and characterized the promoter region of the human gene. A 6.9-kb BamH1 fragment carrying the first exon was subcloned from a P1 genomic clone that contains the *DLC-1* gene, and 2.7 kb of DNA upstream of the ATG translation start codon was sequenced (Fig. 2). Based on the results of 5′ RACE, the transcription start point was assigned 0.45 kb upstream of the translation initiation site. The sequence of the first exon and the flanking DNA is GC-rich and typical of a CpG island.

When the sequence was analyzed by the CPGPLOT program, a CpG island was predicted to extend from 0.28 kb upstream of the transcription start site to 0.42 kb into the

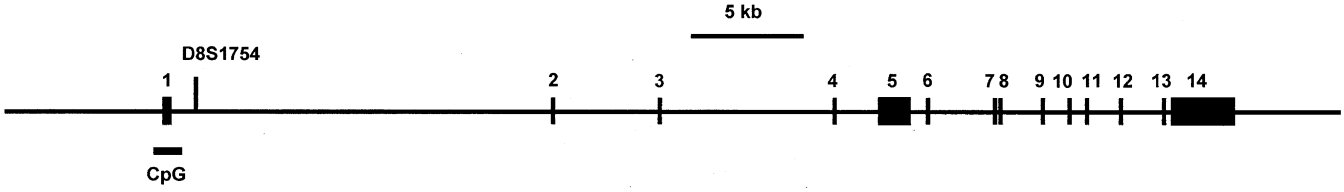

Fig. 1. Organization of the human *DLC-1* gene. Schematic representation of the human *DLC-1* gene, in which the exons are numbered and depicted as solid boxes. Lines denote the introns and the 5′ and 3′ flanking regions. A horizontal bar indicates the location of the CpG island at the 5′ end of the gene. The position of the D8S1754 microsatellite marker in the first intron is shown.

-2913 GGATCCAGGGACAGTGGCATCCATAATCAAGGAGAGAGGAATGGGGCTTGAATAAGAAGAGAAGTTTTGCCTGTTTTACTCTGGAAAGATGAATGCTAAG

-2813 TGGAATAAAAAGACTCCTGGGAACCTAATTAAATTAGAATACAACTTTAACCAGCAAACAAAAAATATTAAGAACTTATTCTGAGAATTGGTGTATTTGG

-2713 AGGGGAGATATTTAGAATATATTTTTAAAGTCTGAATTTTTAATAAATAAGATAAATTAGTATGTTTCGAAGGTGCACTCTTATTTTTTAAGTTTGAATA

-2613 TTACACTGCAGCCTGAGCGATCTTTTCAAAATGCAAATCCAATCACGGGATGCGTTCTTCCATTAAAATGGCTTCTCGACCGGGCGCGGTGGCTCACGCC

-2523 TGTTATCCCAGCACTTTGGGAGGCTGAGGTAGGCGGATCACGAGGTCAGGAGATCGAGACCATCTTGGCTAACACAGTGAAACCCTGTCTCTACTAAAAA

-2413 TACAAAAAAATTAGCTGGGTGTGGTGGCGGGCACCTGTAGTCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATGGCGTGAACCCGAGATCGTGTCACTGC

-2313 ACTCCAGCCTGGGCGACACAGCGAGACTTTGTCTCAAAAAAAAAAAAAAAAAAGGCTTCTCACTGTTTATCACTCCTAAGGACTTTCCTTAACATAGTCA

-2213 CGCAGGGGACTGAAACTTCTTGCTTTTACTTTCCTGGCCTCCCGTTGAATCACACTCCCCCTTGCTGGTGACGGTGGGCTCTTGGGCAGCTCCTCCGGCT

-2113 CCCACACTCATGAGGGCAACTGCATCATCTTGGGCCAGATCAGATCTCCATTTCCTGGGCCCTCACAGCACCGCTTTTTCATACAACCCTGTTGCTTCCT

-2013 CCCACCAGGCTGTAGCCTTTGCACCCTCAGCAGTTACCAGTAGGTAAAAAGCCTGGAAACATCTGTTGAACGGAGGAGTCCCATACCCTTTAGAGCTGAA

-1913 TTCTGCTTTGGGTAAATTAGGGAGCCAGTAGTGGAAGGTAGCTTATTTATTTATTTATTTTTGTACAGATGGAGTCTTGCTATATTGCCAACCCTGGTCT

-1813 CAAGCTCCCGGGATCAAGCAATCCTCCCACCTCGGTTCCCAAAAGCGCTAGGATTACAGGTGTGAGCCACTGCATTCACCCAGCATTTTTGTTTTATCAA

-1713 AATTTATGAGGGAAATGGGAGAAGAGTCTTCTGTATGTAAACTAAGATATCGCTATGAAACCAATAAGTTTTAAACTATAATTAAGAGACTAAATTCTCC

-1613 TATTTGTATTTGCCCCATTTAAAAACAAATTGTGCCCTTCCCCAGCTCCAATATTACCTCCCTGCTTTATGTATCTCCATCAGACATACTCTATATATCT

-1513 CATTTATTTTGTTTGTTATGCATTTTTCCCACGAGAAAGTAACCTCCAGGAGGGAAGGGATATTTGTCTCTTTTGCTCACTTCTGTATCTCCCAGTCTAC

-1413 AGCTGTGTTTGGCACATAGTAAATGCTCTTTCTGTATTTGTTGAATGAATGAGTTTAGTTCAGTAGTCTTCCTTGCCCAGAGATAGCAGGCAAAAACATC

-1313 AATTAACCCCGAAGTAAATTTTCATGGAATCCAGTTCTGGATTTTTTAAAGGCTCTTAATGCAACTATATTTAGCTGTGATCCCTTCACATTAGGCTTCC

-1213 TAATACATCAGGGGTCCTCAAACTACAGGCCATCAGAATCATCTGAGAGCTTGTAAAAGTTGCAGATTCTCATCATCAAAGATTGGAATTATGCAGTCAG

-1113 ACCCCAGCTAAGGGCAGTTCCAGGAGCTGCATTCGGACTGTGGCCCAAGGGCCAGTTTGAGAAACACGGGCTGTGACCCCCAGCACGTGCCGAAGTGAAA

-1013 GCTCAATTCCTCTAAGGCAGTATTTATCACCTTACATTGGCTCCGTACCCATGCGGGTTTTAGCAATAAATAACCAATGTCCAAGGGACAGGTACCCTTC

-913 GCTCGTTTTTACAGATCTGGAAACACTTGACCATCCCTTCCTCTCCGCTTCCCCTCCGTTCCCGCAAATTTACGTGGTTCCCGCCAGGATAAAAGACAGG

-813 TAAAAAGTACGCAGAGCTACCAAGAAAAAGAAGGGACGCTCAAGGCACACTAGGGTCCAGGCCGGACACCTCCGCCAAGTAAATGCCTTGTGACCTTTGC

-713 CTTTGCACCCGGGGCCAATAGTTGGCGACTTTTCGGCGCTTCCCGCTGGGAACGTGGAGGCCCGTGGGGGAAACATTCCAGCCTTCGGCGGAGGAGGCGC

-613 GGGGCGGGGCGGGGAGGAGGAGGGAACCGAGGGAACCGAGGGGGCGGGACAGAGGAGAGGCGGGGCCTGGGCGGGGCGGGGCTAGAGGGCGGCCTGAGGC

-513 TGCGGCCACGAGGAAGAGGGGCGGGGGGCGGAGCCTGGGAATGGGGCGGGGCCGGAGAACAGCCCGTCGCTAAGGCCTGCGACCCAGACAGCGGGGAGGA

-413 GGCGGTGCCGCCCGCTCTGGCCCCGCCCCTCGGGGGGTGGGCCCAGCGGGAGGGCCCGGCTGGCGGAGCGCGCGGGAGACAGTTCGCTCCGACTGCCCGA

-313 GCGAGGGCGCTTCGCTCCCAGCCAGGACATGGCCGCACCTCTCCGCATCAGGAGCGCCGGC

Fig. 2. Sequence of the promoter region of the human *DLC-1* gene. The sequence is numbered relative to the putative ATG translation start codon (nt 349 of the cDNA sequence; AF035119). An Alu repetitive element between nt −2546 and −2279 is underlined. Potential binding sites for transcription factor SP1 (GGGCGG) are shaded. The underlined nucleotides at −612, −546, −473, and −390 match the consensus sequence for transcription factor GCF (BVSCGSSSCB, where B = C, G, or T; V = A, C, or G; and S = C or G [25]). The nucleotides marked with a double underline represent the 5′ ends of the longest products amplified by 5′ RACE and were designated as the transcription start point. The overlap with the previously reported cDNA sequence begins at nt −320 of the genomic DNA. The nucleotide sequence reported here is available in the GenBank database under accession no. AF514295.

first intron. A 264-nt sequence in this region of the first intron is identical to that of a CpG island fragment isolated using a methylated DNA binding column (GenBank accession no. Z59108 [19]) (not shown). The sequence contains a number of potential binding sites for transcription factors, including *SP1* and G*CF*. To determine whether the putative promoter region was able to direct transcription, a 2.7-kb BamHI/NgoM IV fragment was cloned upstream of the green fluorescent protein gene in the pEGFP-1 vector. The genomic DNA was able to drive expression of green fluorescent protein in cells both transiently and stably transfected with the expression plasmid (not shown).

3.2. Methylation of the DLC-1 gene promoter in human cancer cell lines

Twelve HCC, breast, colon, and prostate carcinoma tumor cell lines, which showed no expression or low levels of *DLC-1* expression, were selected for methylation analysis

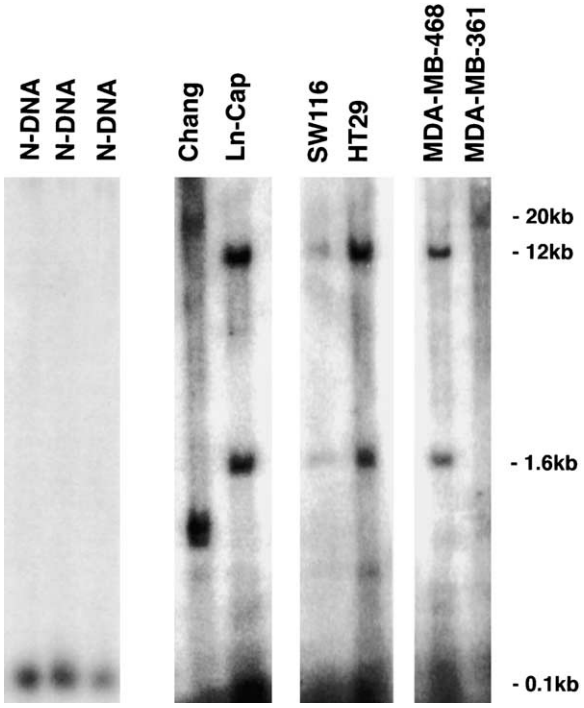

Fig. 3. Methylation status of the *DLC-1* gene promoter region in tumor cells that lack expression of the gene. Autoradiogram of a Southern blot of HpaII-digested genomic DNA from three normal human DNA samples (N-DNA) and from Chang HCC cells, Ln-Cap prostate carcinoma cells, SW1116 and HT29 colon carcinoma cells, and MDA-MB-468 and MDA-MB-361 breast carcinoma cells. The sizes of the bands that hybridized to the 5′ *DLC-1* probe are shown on the right.

of the *DLC-1* promoter. Southern blots of genomic DNA digested with the methylation-sensitive enzyme HpaII were hybridized to a probe from the promoter region. All tumor cell lines showed different degrees of methylation of the promoter region of *DLC-1* gene, whereas no methylation was found in normal genomic DNA extracted from peripheral blood lymphocytes (Fig. 3). Among the tumor cells, two HCC cell lines (7703 and Chang) and the breast tumor line MDA-361 showed heavier hypermethylation, in which an ≈20-kb band but no low molecular weight bands were observed, while other cell lines displayed moderate hypermethylation with 12 kb, 1.6 kb, and small smear-like bands. In addition to the 20-kb band, a 1.0-kb band was found in Chang HCC cells.

3.3. Promoter demethylation induces DLC-1 *expression in 7703 HCC cells*

The 7703 HCC cells, which do not have detectable levels of *DLC-1* mRNA [1] were treated with the demethylating agent 5-aza-dC. Northern blot analysis was performed to examine the expression of *DLC-1* in treated and untreated cells. The 7.5-kb *DLC-1* transcript was found in 7703 cells after exposure to 5-aza-dC, while no signal was detected in untreated cells (Fig. 4A). The methylation status of the promoter region of *DLC-1* gene was examined in 5-aza-dC-treated and untreated 7703 cells. On Southern blots of genomic DNA digested with HpaII, only a 20-kb band hybridized to the *DLC-1* probe in parental 7703 cells, while a signal in the 20–60 bp range was detected in normal human genomic and in drug-treated 7703 cells (Fig. 4B). In addition to the low molecular weight fragments, two bands of 1.6 kb and 12 kb were detected in drug-treated cells, most likely resulting from incomplete digestion of the promoter region DNA due to 5-aza-dC–resistant partial hypermethylation.

4. Discussion

Several lines of evidence suggest that the *DLC-1* gene plays a role in cancer development by acting as a tumor suppressor. *DLC-1* is located on 8p21∼p22, a region of recurrent deletions and LOH in a variety of cancers, and deletion of the gene has been detected in several solid tumors. *DLC-1* mRNA levels are reduced in many cancers and tumor-derived cell lines [1,10,11] and expression of the *DLC-1* cDNA in carcinoma cell lines has an inhibitory effect on growth in vitro and on tumorigenicity in vivo [11,12]. However, somatic mutation of the *DLC-1* gene appears to be rare in colon and ovarian carcinomas and medulloblastomas [10,20]. The results presented in this study demonstrate that inactivation of the *DLC-1* gene may be achieved through hypermethylation of the promoter, a common mechanism for loss of function of tumor suppressor genes [13,14,21].

Analysis of the sequence at the 5′ end of the gene shows that it contains a CpG island that encompasses the transcription start site and harbors several potential transcription fac-

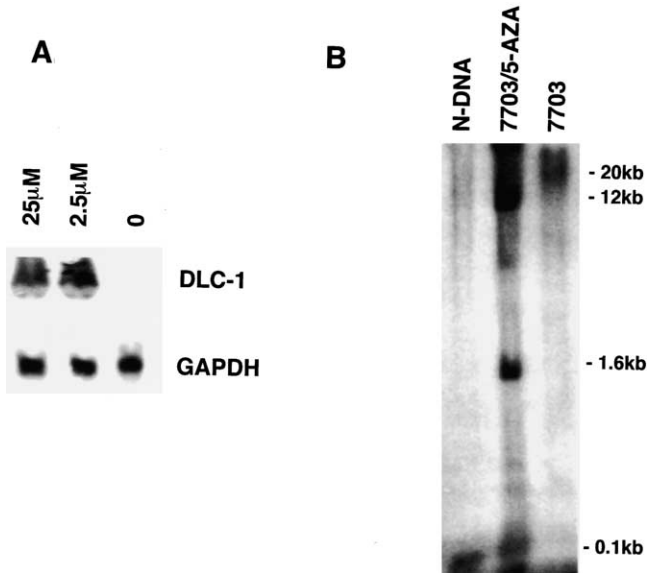

Fig. 4. Effect of 5-Aza-2′- deoxycytidine on the *DLC-1* gene in 7703 HCC cells. (A) Northern blot of total RNA from 7703 cells treated with 0, 2.5, and 25 μM 5-aza-dC and hybridized to a *DLC-1* cDNA probe (top panel). The same blot was re-hybridized to a GAPDH cDNA probe as a control for loading (bottom panel). (B). Southern blot of HpaII-digested genomic DNA from normal human cells (N-DNA), untreated 7703 cells (7703) and cells treated with 5-aza-dC (7703/5-AZA). The sizes of the bands that hybridized to the *DLC-1* probe are indicated.

tor-binding sites. A 2.7-kb genomic DNA fragment that includes the CpG island has promoter activity when transfected into cultured cells. The CpG dinucleotides in CpG islands are normally protected from methylation, but their aberrant methylation can promote the binding of proteins that recognize methyl-CpGs and lead to alterations in chromatin structure that repress transcription. Our results demonstrate that the 5′ CpG island of the *DLC-1* gene is unmethylated in DNA from normal cells but is methylated in DNA from several tumor cell lines that have been shown to lack detectable levels of *DLC-1* mRNA on Northern blots. In the 7703 HCC cell line, treatment with the demethylating agent 5-aza-dC was able to induce expression of *DLC-1* mRNA, and this is associated with a partial demethylation of the *DLC-1* promoter.

These results indicate that *DLC-1* may be one of a growing list of tumor suppressor genes silenced by promoter methylation in human cancers. To provide further support for this, the methylation status of the *DLC-1* CpG island should be analyzed in primary tumors in addition to cell lines. Due to a higher prevalence of tumor suppressor gene promoter methylation in cancer cell lines compared with primary tumors, it has been suspected that CpG island methylation may reflect an artifact acquired during in vitro cultivation [14,21–23]. This suspicion was dispelled by a recent comparative analysis of 150 CpG islands in pancreatic cancer cell lines and primary tumors; most of the methylation of tumor suppressor gene DNA in cell lines was found in the primary tumors from which they derived [23]. Furthermore, promoter hypermethylation of *DLC-1* was recently found in 32 of 110 primary gastric carcinomas and 2 of 5 gastric cancer cell lines [24]. Since altered DNA methylation is one of the most promising markers for early detection, prediction of cancer risk and prognosis of disease, examination of *DLC-1* methylation of primary tumors for a larger spectrum of solid tumors and hematological malignancies is warranted. The role of decreased *DLC-1* expression during neoplastic development is not yet known, and the generation of knockout mice deficient in *DLC-1* will be an important step in documenting the function of the gene.

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