Molecular and Cellular Pathobiology

Inactivation of the Dlc1 Gene Cooperates with Downregulation of p15INK4b and p16INK4a, Leading to Neoplastic Transformation and Poor Prognosis in Human Cancer

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Abstract

The tumor suppressor gene deleted in liver cancer-1 (DLC1), which encodes a protein with strong RhoGAP (GTPase activating protein) activity and weak Cdc42GAP activity, is inactivated in various human malignancies. Following Dlc1 inactivation, mouse embryo fibroblasts (MEF) with a conditional Dlc1 knockout allele reproducibly underwent neoplastic transformation. In addition to inactivation of Dlc1 and increased activity of Rho and Cdc42, transformation depended on the subsequent decreased expression of the Cdk4/6 inhibitors p15INK4b and p16INK4a together with increased expression and activation of Cdk4/6. The level of expression of these cell-cycle regulatory genes was relevant to human tumors with low DLC1 expression. Analysis of publicly available annotated datasets of lung and colon cancer with gene expression microarray profiles indicated that, in pairwise comparisons, low DLC1 expression occurred frequently together (\(P < 0.01\)) with downregulation of p15INK4b or p16INK4a or upregulation of Cdk4 or Cdk6. In addition, an unfavorable prognosis (\(P < 0.05\)) was associated with low DLC1 and low p15INK4b in lung cancer and colon cancer, low DLC1 and low p16INK4a in lung cancer, low DLC1 and high Cdk4 in lung cancer, and low DLC1 and high Cdk6 in colon cancer. Thus, several genes and biochemical activities collaborate with the inactivation of DLC1 to give rise to cell transformation in MEFs, and the identified genes are relevant to human tumors with low DLC1 expression. Cancer Res; 72(22); 5900–11. ©2012 AACR.

Introduction

Carcinogenesis is a multistep process that includes the activation of genes, which promote cancer together with inactivation of those that restrict its development (1, 2). Deleted in liver cancer-1 (DLC1) is a tumor suppressor gene that is inactivated, via genetic and epigenetic mechanisms, in a variety of human malignancies, including cancers of the lung, breast, colorectum, and prostate (2–4). DLC1 is the prototypic member of a multigene family that includes 2 closely related genes, DLC2 and DLC3, which have been studied less extensively but are reported to be frequently downregulated in human cancer and have growth suppressive effects.

The principal protein encoded by human DLC1 is a RhoGAP (GTPase activating protein) whose mouse Dlc1 counterpart is highly homologous (Fig. 1A; ref. 5). In addition to the RhoGAP domain, located in the C-terminal half of the protein, the human DLC1 and mouse Dlc1 proteins have a sterile alpha motif (SAM) domain at their N-terminus and a StAR-related lipid transfer (START) domain in the C-terminal half of the protein, the

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than from the simultaneous inactivation of a tumor suppressor gene and activation of an oncogene.

It has not been determined experimentally whether inactivation of Dlc1 by itself can lead to neoplasia or what genes might collaborate with loss of Dlc1 to produce oncogenic transformation. To this end, we here describe the development of mouse embryo fibroblasts (MEF) whose 2 endogenous Dlc1 alleles can be conditionally disrupted by the Cre recombinase because a Dlc1 exon has been engineered to have flanking loxP sites. Using this system, we have evaluated the short-term and long-term phenotypic consequences of disrupting both Dlc1 alleles in the cultured MEFs. The results indicate that while disruption of Dlc1 does not immediately induce oncogenic transformation, long-term culturing of MEFs with disrupted Dlc1 reproducibly leads to neoplastic transformation. This process is associated with several transformation-dependent changes, including loss of the Cdk4/6 inhibitors p15INK4b (p15) and p16INK4a (p16; ref. 14) together with increased RhoGTP and Cdc42GTP (15). The genetic findings in the MEFs are relevant to human malignancies, as DLC1 is frequently downregulated together with the cyclin-dependent kinase (CDK) inhibitors or with upregulated CDK4 or CDK6, and their level of expression may be associated with prognosis.

Materials and Methods

**Generation of a conditional knockout allele of the mouse Dlc1 gene**

The strategy for generating a conditional knockout allele of the mouse Dlc1 gene involved using homologous recombination in embryonic stem cells to insert loxP sites on either side of exon 4. The methods used for gene targeting and for genotyping embryonic stem cells and mice will be described...
in detail elsewhere (Durkin and colleagues, unpublished data). Briefly, a targeting vector was constructed in which a single LoxP site was inserted 0.97 kb upstream of exon 4, and a LoxP- and frt-flanked neomycin-resistance cassette (neo) was inserted 0.61 kb downstream of exon 4. The vector was introduced into embryonic stem cells, and correctly targeted clones were identified and injected into blastocysts to generate chimeric mice that transmitted the conditional knockout allele (Dlc1fl"fl") to their progeny. The neo cassette was removed by breeding Dlc1neo/wt mice with transgenic mice that constitutively express the Flpe recombinase. The "floxed" allele (Dlc1F) present in the offspring retains a LoxP site in intron 4 after excision of frt-flanked segment (Fig. 1B). The Dlc1F"Wt" mice were mated to obtain Dlc1flfl homoyzogotes, which were viable and apparently normal as expected. The genetic background of the mice used in this study was approximately 93% C57BL/6 and 7% 129/Sv.

**Isolation of mouse embryo fibroblasts, adenovirus infection, and RT-PCR analysis**

Embryos from timed matings of Dlc1flfl males and females were dissected out on day 12 to 13 of gestation. After decapsulation and removal of the liver and other visceral, the embryos were minced, incubated in trypsin/EDTA solution at 37°C, and triturated several times. The dissociated cells were seeded in tissue culture flasks and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS.

The high titer adenoviruses (6.8 × 10^9 pfu/mL), with or without Cre recombinase [designated Adeno-Cre and Adeno-Vector (Adeno-Vt), respectively], were produced and purified by the Adneovirus production Group, Gene Expression Laboratory, Science Applications International Corporation-Frederick (SAIC-Frederick). The adenoviruses, at multiplicity of infection of 5 in 2 mL of serum-free DMEM containing 4% polybrene, were added to cultures of the Dlc1flfl MEFs (3.5 × 10^6 cells/100-mm dish) for 30 minutes at 37°C and rocking every 5 minutes. The dishes were then supplemented with complete medium (DMEM with 10% FBS) and incubated for 48 hours.

After 2 passages, the adenovirus-infected MEFs were analyzed by reverse transcription PCR (RT-PCR) to verify deletion of exon 4 in the Dlc1 mRNA. Briefly, total RNA was isolated from MEF-Cre and MEF-Vt cells using TRIzol reagent as recommended by the manufacturer (Invitrogen). First strand cDNA was synthesized from 1 g of the total, using a SuperScript III kit (Invitrogen). PCR was conducted with a forward primer in exon 3 (5’-gct ggt caa gag aga aca tgg) and a reverse primer in exon 5 (5’-gct tgt cag cat gct ttc ggt), which yield products of 307 bp and 243 bp from transcripts with and without exon 4, respectively.

**Retroviral v-RasH infection of MEF-Vt and MEF-Cre cells, cell proliferation assay, soft agar colony growth assay, and isolation of single colonies from agar**

The replication-defective v-RasH1 (1423) expressing retroviral genome pseudotyped by the Moloney murine leukemia virus (Mo-MULV) and control virus Mo-MULV have been described previously (16, 17). The infected MEFs were grown for several days so that the vast majority of the cells in the 1423 v-RasH1 infected cells had become morphologically transformed.

For the monolayer proliferation assay, MEF-Vt and MEF-Cre cells (1 × 10^5/well) were plated in a 12-well dish, and grown for 3 days in DMEM containing high (10%) or low (1%) FBS. Cell numbers were counted daily in triplicate, using a Cellmeter Auto T4 counter (Nexelom Bioscience). The cells were also analyzed for growth in soft agar for 3 to 4 weeks, and colonies were photographed microscopically and quantified with a colony counter as described (10). In some experiments, the ROCK inhibitor Y27362 (5 nmol/L), c-jun-NH2-kinase (JNK) inhibitor VIII (10 nmol/L), or CDK4/6 inhibitor IV (10 nmol/L; all from EMD Millipore) was added to the agar plates every 5 to 6 days to test the contribution of the respective kinase to cell growth in agar.

To isolate clonally derived cells with disrupted Dlc1, several single colonies, from spontaneously transformed long-term passaged MEF-Cre cells (without v-RasH3 infection), were isolated from soft agar plates and dispersed for monolayer growth after trypsinization. They were named MEF-Cre anchorage-independent growth (AIG) clones, and their homozygosity for Dlc1flfl disruption to Dlc1flfl verified by RT-PCR.

**Mouse tumorigenesis studies**

The mouse studies were approved by the National Cancer Institute (NCI, Bethesda, MD) Animal Care and Use Committee and conducted in compliance with the approved protocols. For tumor xenografts, longer-passaged MEF-Vt and MEF-Cre cells were washed with cold PBS, diluted to 10^5/mL with serum-free medium/Matrigel basement membrane matrix (Becton Dickinson Labware) at a ratio of 3:1, and injected subcutaneously into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (10^6 cells per injection). The animals were monitored for tumor growth, and 5 weeks postinjection tumors were excised and weighed.

**Transfection and transwell migration assay**

The isolated homozygous Dlc1flfl knockout clone, AIG-5, was used in stable transfections to express GFP and GFP-tagged human DLC1 (wt or R718A, a RhoGAP-deficient mutant; ref. 10), as well as PEGFP-C1 (clonotech), pcDNA3-p16ink4a (from Beverly Mock, NCI), and pcDNA3-p15ink4b (from Linda Wolff, NCI). Cells were observed for cell proliferation and morphologic changes. The transwell cell migration assay was previously described (18).

MEF-Cre cells were transfected with HA-tagged rat ROKa (ROCK2, from Thomas Leung, National University of Singapore, Singapore) followed by treatment with or without inhibitor Y27362 in the culture. MEF-Cre cells treated with JNK inhibitor VIII or Cdk 4/6 inhibitor IV (EMD Chemical) were verified by immunoblotting to detect phosphorylation of c-jun (S68), phospho-cyclin D1 (T286), and phospho-RB (S780; Cell Signaling Technology), respectively.

**Immunofluorescent staining and confocal microscopy**

MEF-Vt and MEF-Cre AIG-5 cells expressing GFP or GFP-tagged human DLC1 were seeded on glass coverslips and incubated for 24 hours. Cells were fixed with 4% paraformal-
dehyd, permeabilized with 0.25% Triton X-100 in PBS, and then blocked with 5% goat serum in PBS. The cells were incubated with a 1:100 dilution (in PBS) of DLC1 (428) or Ras (Millipore) primary antibodies at 4°C overnight. After thorough washing in PBS, cells were incubated 1:250 with the appropriate Alexa-conjugated secondary antibodies for 1 hour. To visualize actin or nuclei, cells were incubated with phalloidin (1:50; Invitrogen) for 1 hour. After staining, cells were thoroughly washed with PBS and mounted with gel mounting solution (Biomedica Corporation). Fluorescence-labeled cells were viewed in a Zeiss 510 UV confocal microscope, using a ×63 objective.

**Immunoprecipitation, immunoblotting, Raf-RBD, Rhotekin-RBD, and Pak-1-RBD pull-down assays**

Cells were lysed with Golden Lysis Buffer (12). Equal amounts of protein from cell extracts were used for immunoprecipitation by anti-DLC1 (428; ref. 12) or commercial antibody indicated in the figures. RasGTP, RhoGTP, and Cdc42GTP were assayed, respectively, by Raf-RBD, Rhotekin-RBD, and Pak-1-RBD (Millipore), followed, respectively, by anti-Ras (Millipore), anti-RhoA (Cytoskeleton), and anti-Cdc42 (Santa Cruz) blotting, based on the manufacturers’ instructions. For each blot, horseradish peroxidase–conjugated anti-rabbit or anti-mouse immunoglobulin G (GE Healthcare) was used for the second reaction at 1:10,000 dilution. Immunocomplexes were visualized by enhanced chemiluminescence (ECL), using an ECL kit (GE Healthcare).

**siRNA transfection**

Control siRNA and validated siRNAs for mouse or human p15 and p16 and mouse Cdk4 and Cdk6 were all from Qiagen. Target sequences are listed in Supplementary Table S1. MEF cells or human non–small cell lung cancer (NSCLC) A549 cells (from Curt Harris, NCI) were transfected with siRNAs for 18 to 24 hours using Lipofectamine 2000, followed by a change of media. After 3 to 4 days, cell extracts were prepared for various protein assays. For biologic assays, MEFs were seeded for colony growth in soft agar the day after siRNA transfection, or for cell migration 3 to 4 days after transfection.

**Bioinformatic analysis**

The gene expression microarray analyses in this study use data from ArrayExpress of the European Institute of Bioinformatics (EBI), which is publicly available at http://www.ebi.ac.uk/arrayexpress. The analyses include 3 independent experiments with NSCLC and 3 independent experiments with colon cancer for Affymetrix U133 plus 2 chips. The Affymetrix CEL data with raw data from each experiment were directly downloaded from the EBI website and normalized with CEL file quality control evaluation using 3′ Expression Arrays Robust Multi-array Analysis (RMA) from the Affymetrix software Expression Console (http://www.affymetrix.com). The normalized expression values are the probe set intensity on a log-2 scale. Because data from E-GEOD-3629 has no raw data available, processed data from the original author was used for the analysis. The specific probe sets for DLC1, CDKN2A (p16), CDKN2B (p15), CDK4, and CDK6 are 224822_s_at, 209644_x_at, 236313_at, 202246_s_at, and 243000_at, respectively. Probe 209644_x_at is used to detect p16INK4A. Because this gene generates several transcript variants, which differ in their first exon, expression levels measured using 209644_x_at cannot exclude the detection of p14ARF.

**Statistical analysis**

The gene regulation was analyzed to compare different expression values between cancers and controls. For individual genes from each experiment, the median expression level (50% quartile) is considered as a cut-off. Values higher than the cut-off were categorized as “high”; other values were categorized as “low.” The Kaplan–Meier survival and χ² analyses were conducted using statistical computing and graphics software R (www.r-project.org). P < 0.05 was considered statistically significant. For survival analysis, the values higher or lower than the median in each gene group were placed in “high,” “low,” or 2 different combinations for the analysis. All survival times were adjusted to months.

**Results**

**Conditional Dlc1 disruption induces increased RhoGTP and increased susceptibility to ras transformation**

Constitutive disruption of mouse Dlc1 leads to embryonic lethality (5, 19). To develop an endogenous Dlc1 allele that can be conditionally disrupted, we used gene targeting to introduce LoxP sites on both sides of exon 4 of the Dlc1 gene (Fig. 1B). MEFs were then derived from 12- to 13-day-old embryos that had been bred to homozygosity for this allele (Dlc1/LoxP). Exposure of the MEFs to Cre recombinase should induce deletion of the 1.65 kb LoxP-flanked genomic DNA segment. This deletion results in loss of the 64-nt exon 4 sequence in the Dlc1 transcript (Fig. 1B), which produces a reading frame shift after codon 64, leading to premature translation termination and loss of full-length Dlc1 protein expression.

MEFs homozygous for the conditional allele were infected with a Cre-encoding adenovirus (Adeno-Cre), which induced disruption of the Dlc1/LoxP alleles and produced inactive Dlc1<sup>Δ4</sup> alleles, as determined by RT-PCR and loss of detectable full-length Dlc1 protein for 2 independently derived MEF lines, MEF-A, which was selected for further analysis, and MEF-B (Fig. 1C). These changes were not induced by the control adenovirus vector (Adeno-Vt).

Consistent with the strong RhoGAP activity of DLC1, disruption of Dlc1 was associated with increased RhoGTP, as determined by a Rhotekin pull-down assay conducted following Adeno-Cre infection (Fig. 1D, top). However, there was little difference between the level of Cdc42GTP in MEF-Cre and control MEF-Vt (Fig. 1D, bottom), perhaps because the Cdc42GAP activity of DLC1 is less efficient than its RhoGAP activity. MEFs with constitutively disrupted p190B RhoGAP are also reported to have increased RhoGTP (20), whereas there does not seem to be a change for MEFs with constitutively disrupted p190A RhoGAP (21). Despite their increased RhoGTP, the MEF-Cre cells, studied within 2 passages of Adeno-Cre infection, did not grow in soft agar, indicating that loss of Dlc1 does not lead directly to transformation (Fig. 1E).

To examine whether the early-passaged MEF-Cre cells might be more susceptible to transformation by an oncogene, they
were transduced with mutant Ras [v-RasH (v-Ras)]
which induced a greater degree of transformation in MEF-Cre than in MEF-Vt transductants, as determined by the AIG of large colonies in agar (Fig. 1E). The MEF-Vt and MEF-Cre cells displayed a similar increase in RasGTP (Fig. 1F), indicating that the stronger transformation of the MEF-Cre cells was not attributable to differences in RasGTP levels. After submission of this article, a study reported that the incidence of metastatic thymic tumors was increased in mice heterozygous for a gene-trapped Dlc1 allele after Cre-mediated induction of oncogenic K-ras, compared with mice with 2 wild-type Dlc1 alleles (22). These results are analogous to our finding that reduced Dlc1 activity enhances the transforming ability of v-Ras.

**Long-term cultured MEFs with disrupted Dlc1 become spontaneously transformed in association with p15ink4b and p16ink4a inactivation**

Although early-passaged MEF-Cre cells did not display a transformed phenotype, they did undergo neoplastic transformation after long-term cultivation of 15 to 20 passages. Transformation depended on the loss of Dlc1 in the MEF-Cre cells, as identically cultured MEF-Vt cells did not become transformed. Unlike the MEF-Vt cells, the MEF-Cre cells grew to high density in monolayer culture in high serum (10%, Fig. 2A, top) or low serum (1%; Fig. 2A, bottom), underwent AIG in soft agar (Fig. 2B), and formed tumor xenografts in immunocompromised mice (Fig. 2C). The spontaneously transformed MEF-Cre cells were less transformed than the MEF-Cre v-Ras transductants at the same passage level, and were similar to MEF-Vt v-Ras transductants, as determined by monolayer growth and soft agar growth (Fig. 2A and B).

The cells were analyzed for RasGTP, RhoGTP, and Cdc42GTP. The levels of RasGTP remained low in the later-passaged MEF-Vt and MEF-Cre, and remained elevated to a similar degree in both v-Ras lines (Fig. 2D, top). RhoGTP and Cdc42GTP were higher in the later passaged spontaneously transformed MEF-Cre cells than in the later-passaged MEF-Vt cells (Fig. 2D, middle and bottom). The RhoGTP and Cdc42GTP levels were even higher, and similar to each other, in the Ras-transformed MEF-Vt and MEF-Cre cells, as Ras-
transformation of MEFs depends on the activity of both Rho and Cdc42 (23, 24). Consistent with their transformed phenotype and elevated RhoGTP levels, the later-passaged MEF-Cre cells had more stress fibers than MEF-Vt, and the v-Ras expressing MEF-Vt and MEF-Cre also had abundant stress fibers that seemed to be thicker than in MEF-Cre (Supplementary Fig. S1).

The Cdk4/6 inhibitors p15 and p16 have been reported to become downregulated during passage of other MEFs (25, 26). We therefore examined their level of expression in early- and later-passaged cells. p15 and p16 were expressed in early-passaged MEF-Vt and MEF-Cre cells, but were no longer detectable in later-passaged cells of either line, and loss of their expression was associated in both lines with a concomitant increase in Cdk4/6 expression and phosphorylation of cyclin D1 (Fig. 3A). We also verified that the phenomenon seen with the MEF-Cre cells was reproducible, in that adeno-Cre infection of independently derived MEFs resulted in loss of Dlc1 expression and increased RhogGTP after short-term passage and cell transformation after long-term passage (Supplementary Fig. S2).

It is well known that endogenous p15 and p16 negatively regulate the growth of human tumor cells, but it has not been determined whether such regulation is relevant to tumor cells in which DLC1 has been downregulated (14, 27). Therefore, we identified a p15- and p16-expressing human tumor line, the A549 NSCLC line, in which endogenous DLC1 had been downregulated (Fig. 3C). The siRNA-induced reduction of p15 or p16 increased the ability of the A549 cells to grow in agar, indicating that p15 and p16 negatively regulate growth of human tumor cells that do not express DLC1. Furthermore, in earlier passaged nontransformed MEF-Vt cells that expressed p15 and p16, decreased p15 or p16 expression increased their growth potential, as siRNA-mediated reduction of p15 and p16 led to some AIG in soft agar (Fig. 3B).

**Spontaneous transformation of MEFs with disrupted Dlc1 depends on loss of Dlc1, p15, and p16 and activation of Cdk4/6, ROCK, and Jnk**

Before determining whether the identified changes in gene expression and activity were mechanistically involved in transformation of the later-passaged MEF-Cre cells, we first...
developed clonally derived lines to ensure the Dlc1 alleles were disrupted in all cells and characterized the clones. To obtain clonal lines, individual colonies were isolated after growth in soft agar, and their clonality for Dlc1 disruption was analyzed by RT-PCR (Fig. 3D). While the Dlc1 alleles were uniformly disrupted in some colonies (AIG-2 and AIG-5), 1 colony (AIG-7) contained a mixture of intact and disrupted Dlc1 alleles. An interesting correlation was that a later-passaged MEF-Cre clone whose Dlc1 alleles had been disrupted but grew poorly in soft agar (AIG-2) was found to express p16 continuously, but not p15, whereas both p15 and p16 were downregulated in the AIG-5 clone, which grew efficiently in soft agar (Fig. 3E and F; data shown only for p16).

As expected from the RT-PCR results, AIG-5 cells were negative for endogenous Dcl1 protein by microscopy, whereas the long-term passaged MEF-Vt cells were positive (Fig. 4A, top left). The high RhoGTP level in the AIG-5 cells was associated with increased stress fiber formation, a widely studied phenotype that depends on the Rho-associated protein kinases (ROCKs: ROCK1 and ROCK2; refs. 28, 29), which are activated by Rho (Fig. 4A, bottom left). In contrast, the low RhoGTP level in the long-term passaged MEF-Vt cells was associated with less stress fiber formation.

We assessed whether the inactivation of Dlc1, p15, and 16, as well as increased expression of Cdk4 and Cdk6 and increased activity of Rho, Cdc42, and Cdk4/6, which were the identified changes associated with MEF-Cre transformation, contributed mechanistically to the transformed phenotype. We used genetic approaches to evaluate the role of the Dcl1, p15, p16, Cdk4, and Cdk6 genes in MEF-Cre transformation. Pharmacologic inhibitors were used to examine whether the activities of several protein kinase were relevant to the transformation process, these included Cdk4/6, the RhoGTP-dependent kinase ROCK, and JNK, a downstream target of Cdc42GTP (30).

![Figure 4](https://cancerres.aacjournals.org)
Stable transfection of wild-type DLC1 in the AIG-5 clone induced morphologic reversion that was associated with reductions in RhoGTP, Cdc42GTP, stress fiber formation, growth in soft agar, and transwell cell migration (Fig. 4A–D). All of these phenotypes, including morphologic reversion, depended on the GTPase-activating protein (GAP) activity of DLC1, as a DLC1 GAP-dead mutant (R718A) was deficient for inducing these changes, and were not accompanied by reexpression of p15 or p16 (data not shown). These findings confirm that reversion depended on the GAP activity of DLC1 and indicate, in addition, that DLC1 possesses in vivo Cdc42GAP activity in addition to its RhoGAP activity. In vivo Cdc42GAP activity of ectopically expressed DLC1 was also detected in the H1703 human NSCLC line (Fig. 4B, right).

Stable transfection of p15 and p16 reverted the transformed phenotype, as determined by reduced AIG in soft agar (Fig. 5A). Conversely, siRNA-mediated reduction of Cdk4 and Cdk6 inhibited cell migration (Fig. 5B). Pharmacologic studies indicated that transformation also depended on the activities of Cdk4/6, ROCK, and Jnk, as the Cdk4/6 inhibitor, the ROCK inhibitor Y27632, and the Jnk inhibitor VIII reduced their respective enzymatic activity and suppressed growth in soft agar (Fig. 5C–E).

In human cancer, downregulation of DLC1 together with low p15 or p16 or with high CDK4 or CDK6 may have prognostic significance.

To examine whether the genes implicated in MEF-Cre transformation might have relevance to human cancer, we searched publicly available cancer datasets with mRNA gene expression profiling, focusing on DLC1, p15, p16, CDK4, and CDK6, as those were the genes whose changes in expression were associated with MEF transformation. Downregulation of DLC1 occurred together with downregulation of p15 and/or p16 in NSCLC cancer and colon cancer (Table 1), and together with upregulation of CDK4 and CDK6 (Table 2). In addition, we analyzed 2 annotated NSCLC cancer cohorts (62 annotated cases in cohort I and 81 annotated cases in cohort II) and 1 annotated colon cancer cohort (37 annotated cases) for whether the level of DLC1 expression together with that of any one of these genes might have prognostic implications. An unfavorable prognosis was found to be associated with low DLC1 and...
low p15 in one of the NSCLC cohorts (cohort II) and the colon cancer cohort, with low DLC1 and low p16 in both NSCLC cohorts, with low DLC1 and high CDK4 in one of the NSCLC cohorts (cohort I), and low DLC1 and high CDK6 in the colon cancer cohort (Fig. 6 and Supplementary Fig. S2).

**Discussion**

In this report, we found that conditional disruption of both Dlc1 alleles in MEFs reproducibly led to their spontaneous tumorigenic transformation after several passages in culture, a phenotype that was not seen in MEFs with intact Dlc1 that had an identical passage history. This system may mimic some aspects of human tumors, in that the oncogenic transformation occurs over time (1), rather than resulting from the simultaneously forced inactivation of tumor suppressor genes and/or activation of oncogenes. Compared with MEFs that carry a constitutively disrupted growth regulatory gene, conditional disruption means the gene does not need to be inactivated until after the cells have been placed in culture, which makes it easier to rigorously examine the short-term effects of disrupting the gene and compare these effects with those resulting from long-term disruption. This system also has theoretical advantages over the more typical MEFs, which have constitutively disrupted genes, as developmentally related compensatory changes might lead to inappropriate interpretation when these MEFs are compared with wild-type MEFs.

MEFs carrying the conditionally disrupted Dlc1 alleles are well suited to identifying cell autonomous candidate genes and biochemical activities that may have cooperated over time with Dlc1 inactivation to produce the observed oncogenic phenotype. When such modifications are identified in other systems, the findings often remain as correlations (31). With the MEF system, however, the putative contribution of identified candidate genes can readily be evaluated by genetic

**Table 1. Downregulation of DLC1 and p15/p16 gene expression in human cancers**

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>DLC1</th>
<th>DLC1</th>
<th>p16</th>
<th>DLC1</th>
<th>p15</th>
<th>p16</th>
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<tr>
<td>Lung cancer (E-GEOD-18842)</td>
<td>n = 2</td>
<td>n = 2</td>
<td>n = 0</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
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<td>Lung control (n = 45)</td>
<td>n = 44</td>
<td>n = 41</td>
<td>n = 36</td>
<td>n = 34</td>
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<td></td>
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<td>Lung cancer (E-GEOD-19188)</td>
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<td>n = 1</td>
<td>n = 0</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
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<tr>
<td>Normal (n = 50)</td>
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<td>n = 44</td>
<td>n = 44</td>
<td>n = 37</td>
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<tr>
<td>Tumor (n = 68)</td>
<td>n = 1</td>
<td>n = 1</td>
<td>n = 0</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
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<td>n = 4</td>
<td>n = 1</td>
<td>n = 1</td>
<td>P &lt; 0.001</td>
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<td>n = 39</td>
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<td>Colorectal cancer (n = 68)</td>
<td>n = 58</td>
<td>n = 39</td>
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<td>Colon cancer (E-GEOD-23878)</td>
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<td>n = 0</td>
<td>n = 3</td>
<td>P = 0.216</td>
<td>n = 0</td>
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<td>Control (n = 24)</td>
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<td>n = 9</td>
<td>n = 6</td>
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<td></td>
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<tr>
<td>Colorectal cancer (n = 35)</td>
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</tbody>
</table>

NOTE: The data from ArrayExpress of European Bioinformatics Institute are reanalyzed to study the codownregulation of DLC1 and CDK4/6 gene expression from selected cancers. The contents in the parenthesis are the ID number of array experiments. \( \chi^2 \) Analysis is conducted to study whether the downregulation is statistically significant.

**Table 2. The reverse expression pattern of DLC1 and CDK4/6 in human cancers**

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>DLC1</th>
<th>DLC1</th>
<th>CDK4</th>
<th>DLC1</th>
<th>CDK6</th>
<th>DLC1</th>
<th>CDK4/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer (E-GEOD-18842)</td>
<td>n = 2</td>
<td>n = 1</td>
<td>P &lt; 0.001</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Lung control (n = 45)</td>
<td>n = 44</td>
<td>n = 41</td>
<td>n = 36</td>
<td>n = 34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung cancer (E-GEOD-19188)</td>
<td>n = 1</td>
<td>n = 1</td>
<td>P &lt; 0.001</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Normal (n = 50)</td>
<td>n = 58</td>
<td>n = 48</td>
<td>n = 44</td>
<td>n = 37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor (n = 68)</td>
<td>n = 43</td>
<td>n = 39</td>
<td>n = 39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon cancer (E-GEOD-3629)</td>
<td>n = 8</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
<td>n = 0</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Control (n = 24)</td>
<td>n = 21</td>
<td>n = 15</td>
<td>n = 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer (n = 35)</td>
<td>n = 21</td>
<td>n = 19</td>
<td>n = 11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: The data from ArrayExpress of European Bioinformatics Institute are reanalyzed to study the coregulation of DLC1 and CDK4/6 gene expression from selected cancers. The contents in the parenthesis are the ID number of array experiments. \( \chi^2 \) Analysis is conducted to study whether the downregulation is statistically significant.
Cooperative Regulation of Rho and CDK4/6 Pathways

Figure 6. Prognostic significance in NSCLC and colon cancer of codownregulation of DLC1 and p15 or p16 or downregulation of DLC1 with upregulation of CDK4 or CDK6. A–C, Kaplan–Meier analysis of DLC1, p15, p16, CDK4, and CDK6 gene expression in NSCLC (A and B) and colon cancer (C). Low versus high expression of each gene by itself does not have prognostic value in these cohorts ($P > 0.05$), but there is prognostic significance when low DLC1 is combined with low p15 (B and C), with low p16 (A and B), with high CDK4 (A, DL4H), and with high CDK6 (C, DL6H). The $P$ value for each comparison is shown.

manipulation, whereas that of their biochemical activities can be assessed pharmacologically. Using this approach, we identified several genes whose expression and/or activities are altered in conjunction with the transformed phenotype, and confirmed their mechanistic role in maintaining the phenotype. Despite this being an in vitro mouse fibroblast system, we were able to verify that our genetic findings have relevance to human epithelial tumors.

In the MEFs, 1 set of results identified reduced expression of the CDK 4/6 inhibitors p15 and p16 (14, 27) as passage-dependent changes, whether Dlc1 was disrupted or intact [Zindy and colleagues (26)]. The lack of dependence on Dlc1 disruption increased the theoretical possibility that loss of p15 and p16 expression might not have contributed to the transformed state in the MEFs. However, reexpression of p16 or p15 induced reversion of transformation, verifying their role in its maintenance, whereas siRNA-mediated reduction of either gene in nontransformed early-passaged MEF-Cre cells increased their AIG. This role was also reinforced by identification and analysis of an unusual MEF clone that still expressed p16 and grew poorly in agar, although it did not express Dlc1 or p15; siRNA-mediated reduction of its p16 expression enhanced its ability to grow in soft agar.

The reduced p15 and p16 in the transformed MEFs was associated with an increase in Cdk4/6 expression and activity, which contributed to their transformation. There were also transformation-associated biochemical changes not accompanied by alterations in steady-state protein levels, including increases in RhoGTP and Cdc42GTP and their respective targets, ROCK and Jnk (28–30). Using pharmacologic inhibitors, we established a role in transformation for the enzymatic activities of ROCK and Jnk.

Given the apparently sequential nature of the observed changes in gene expression and activity, it was theoretically possible that later changes rendered the fully transformed phenotype independent of some earlier changes. However, that was found not to be the case, which suggests that the process of oncogenic transformation in this system occurs primarily by a series of cooperative changes that continue to be mechanistically important even after development of the neoplastic state. The later-passaged MEF-Vt cells, which have high Cdk4/6 activity but are not morphologically transformed because they have intact Dlc1 alleles, may have phenotypic similarities to the HBEC human bronchial epithelial cells, which constitutively express CDK4 and telomerase (human telomerase reverse transcriptase) but are not transformed (32).
Our findings also verify that DLC1 can regulate Cdc42 in cells. Until now, a role for DLC1 in Cdc42 regulation has been confined to the weak in vitro Cdc42GAP activity mediated by the RhoGAP domain of DLC1, which contrasts with its strong, well-documented in vitro and in vivo RhoGAP activity (6). Here, the transformed MEFs were found to contain high levels of RhoGTP and Cdc42GTP, which could be reduced by reexpression of DLC1. This reduction of active Rho and Cdc42 depends on the GAP activity of DLC1, as the GAP-dead DLC1 mutant did not alter the level of RhoGTP or Cdc42GTP.

Taken together, our observations suggest a multifactorial model for the genes and functions identified here as contributing to neoplastic transformation of the MEFs initiated by inactivation of Dlcl. Key features of the model include the inactivation of Dlcl, p15, and p16, and their consequences, which include activation of Cdk4/6, Rho, ROCK, and Cdc42 and Jnk. We recognize that additional changes, not identified here, probably also contribute to this process.

It is noteworthy that the 5 genes whose expression in the MEFs was altered in a transformation-dependent manner—Dlc1, p16, p15, Cdk4, and Cdk6—are altered in human cancer. Indeed, we found in publicly available datasets with mRNA gene expression profiling that downregulation of Dlcl expression occurs frequently in conjunction with reductions in expression of p15 and/or p16 or upregulation of Cdk4 and/or Cdk6 in NSCLC and colon cancer. Furthermore, downregulation of Dlcl and p15 or p16 was associated with an unfavorable prognosis in annotated NSCLC and colon cancer datasets, despite a relatively small number of annotated tumors in each dataset. Analogous findings were seen in the same datasets for low Dlcl expression together with high Cdk4 or Cdk6. Such associations are not limited to NSCLC and colorectal cancer. In lymphocytic leukemia, promoter methylation of Dlcl, p15, and p16 is reported to be associated with a more advanced stage (33). Their association with a poorer prognosis or a more advanced tumor stage implies that these changes make important contributions to disease outcome.

These results validate the clinical relevance of the genes identified in the transformed MEFs. The clinical findings contribute to the growing list of tumors in which reduced Dlcl expression, either by itself or as part of a multigene signature, is associated with poor prognosis and/or advanced stage (34–36).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: X. Qian, D.R. Lowy

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References
Inactivation of the $p_{lc1}$ Gene Cooperates with Downregulation of $p15^{INK4b}$ and $p16^{INK4a}$, Leading to Neoplastic Transformation and Poor Prognosis in Human Cancer

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