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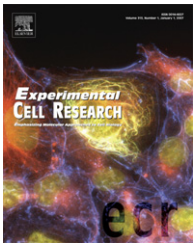

Research Article

Morphological changes and nuclear translocation of DLC1 tumor suppressor protein precede apoptosis in human non-small cell lung carcinoma cells

Bao-Zhu Yuan[a,*], *Amy M. Jefferson*[a], *Lyndell Millecchia*[a], *Nicholas C. Popescu*[b], *Steven H. Reynolds*[a]

[a]*Laboratory of Molecular Genetics, Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, CDC, Morgantown, WV 26505, USA*
[b]*Laboratory of Experimental Carcinogenesis, NCI, NIH, Bethesda, MD 28092, USA*

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ABSTRACT

We have previously shown that reactivation of DLC1, a RhoGAP containing tumor suppressor gene, inhibits tumorigenicity of human non-small cell lung carcinoma cells (NSCLC). After transfection of NSCLC cells with wild type (WT) DLC1, changes in cell morphology were observed. To determine whether such changes have functional implications, we generated several DLC1 mutants and examined their effects on cell morphology, proliferation, migration and apoptosis in a DLC1 deficient NSCLC cell line. We show that WT DLC1 caused actin cytoskeleton-based morphological alterations manifested as cytoplasmic extensions and membrane blebbings in most cells. Subsequently, a fraction of cells exhibiting DLC1 protein nuclear translocation (PNT) underwent caspase 3-dependent apoptosis. We also show that the RhoGAP domain is essential for the occurrence of morphological alterations, PNT and apoptosis, and the inhibition of cell migration. DLC1 PNT is dependent on a bipartite nuclear localizing sequence and most likely is regulated by a serine-rich domain at N-terminal part of the DLC1 protein. Also, we found that DLC1 functions in the cytoplasm as an inhibitor of tumor cell proliferation and migration, but in the nucleus as an inducer of apoptosis. Our analyses provide evidence for a possible link between morphological alterations, PNT and proapoptotic and anti-oncogenic activities of DLC1 in lung cancer.

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Introduction

The DLC1 (Deleted in Liver Cancer) gene that was isolated from a region of recurrent deletions in human hepatocellular carcinoma is transcriptionally silenced by genetic and/or epigenetic mechanisms in several types of human solid tumors and hematological malignancies [1–10]. Ectopic restoration of DLC1 suppresses *in vitro* tumor cell growth and reduces or abolishes *in vivo* tumorigenicity of human breast, liver and lung cancer cells [2,4,6,11–13]. DLC1 is also a metastasis suppressor gene, as

* *Corresponding author*. Laboratory of Molecular Genetics, Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Mailstop L-3014, Morgantown, WV 26505-2888, USA. Fax: +1 304 285 5708.
E-mail address: bby1@cdc.gov (B.-Z. Yuan)

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demonstrated with a model utilizing clones with opposite metastatic potential [14].

NSCLC, the most common form of lung cancer, is the leading cause of cancer death in both men and women in the United States [15]. Although most lung cancers are related to tobacco use, it is also ranked second only to bladder cancer in the proportion cases thought to be associated with occupational exposures [16]. In over 95% of primary human NSCLCs, DLC1 mRNA expression is low or absent. Such a high frequency of DLC1 alteration underlines its importance in the pathogenesis of lung cancer [2,17].

DLC1 is a 122 kDa multidomain protein which contains SAM, RhoGAP and START domains [2,18]. Among these domains, the RhoGAP domain is most likely associated with DLC1 tumor suppression function because it can catalyze GTP hydrolysis for RhoA and Cdc42 proteins [11,19]. Both GTP-RhoA and GTP-Cdc42 proteins are associated with progression of tumorigenicity through their roles in regulating cell morphology, motility, metastasis, proliferation and apoptosis [20–25]. The rat DLC1 homolog induces cell morphological changes via RhoGAP domain-mediated actin cytoskeleton reorganization. Ectopic restoration of DLC1 decreases Bcl-2 but increases caspase 3 protein levels in liver cancer cells [13]. DLC1 inhibits *in vitro* cell migration and invasion in human breast and liver cancer cells [12,14]. All these findings support the notion that DLC1 is an important inhibitor of multi-faceted Rho protein-related tumorigenicity. DLC1 also contains a serine-rich domain which is generally viewed as a regulatory component for a variety of functions, especially inter-compartment movement [26]. However, new functions of these domains and other still uncharacterized domains or regions in DLC1 need to be examined for a better understanding of its tumor suppression function.

During DLC1 transfections of lung cancer cells we observed branched cell extensions in a large number of cells. This observation led us to uncover a feature of DLC1 protein nuclear translocation (PNT) that depends on a bipartite nuclear localizing sequence as well as the integrity of the RhoGAP domain. Here we provide evidence compatible with the notion that DLC1-mediated morphological alterations and PNT are involved in the processes of programmed cell death and suppression of proliferation of lung cancer cells.

Materials and methods

Materials

NCI-H358, a human NSCLC cell line, was from ATCC (Rockville, MD). Rhodamine-conjugated phalloidin was from Molecular Probes (Eugene, OR). A monoclonal antibody against DLC1 and Ac-DEVD-AMC, a caspase 3 substrate, were from BD Biosciences (Palo Alto, CA). Antibodies against Flag tag (M2) and actin, fibronectin and EZ Prep Nuclei Isolation Kit were from Sigma (St. Louis, MI). Lamin B1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Z-VAD-fmk, a wide spectrum caspase inhibitor, was from Calbiochem-EMD Biosciences (San Diego, CA). Immunohistochemical (IHC) staining kit was from VECTOR Laboratories (Burlingame, CA). QuickChange site-directed mutagenesis kit was from STRAGAGENE (La Jolla, CA).

Expression vectors

The DLC1-Δ372 mutant was created by a PCR which deleted a 5′ region encoding the first 372 amino acids from WT DLC1 cDNA and added a new ATG start codon and a Flag tag sequence at the 5′ end of the mutant cDNA. The DLC1-662 mutant was generated by creating a new stop codon after residue 662 in WT DLC1 via site-directed mutagenesis. DLC1-R718E was created by converting an arginine into a glutamic acid at codon 718 in WT DLC1 via site-directed mutagenesis [27]. Both WT DLC1 and its mutant cDNAs were constructed in the pcDNA3.1Zeo(+) vector. Green fluorescent protein (GFP)-tagged DLC1s were created by fusing GFP cDNA with the 3′ end of DLC1 cDNAs in the pEGFP-N1 vector.

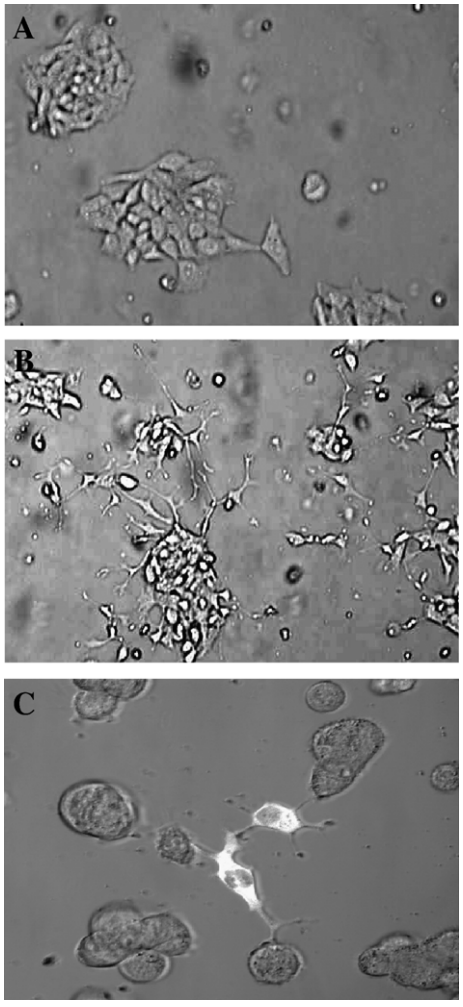


Fig. 1 – DLC1 induces cell extensions in NCI-H358 cells. At 24 h after transfecton, cell extensions were observed after transfection with pcDNA3.1Zeo(+)-DLC1 (B), but not with vector alone (A). Under a fluorescent microscope, only GFP-positive cells (brighter in black/white) exhibited cell extensions at 12 h after transfection with pEGFP-N1-DLC1 (C).

Gene transfection, colony formation and cell proliferation assays

NCI-H358 was chosen as the host cell line in this study. Gene transfection, colony formation and cell proliferation assays were performed following previously described procedures [2].

In vitro cell migration and invasion assays

1.5×10^5 cells were loaded on a 20 ng/ml fibronectin-coated transwell (24 well, 8 μm pore, BD Biosciences). After 24 h of incubation, non-migrated cells were removed and migrated cells were fixed and stained with Diff-Quick solutions (IHC World). Five fields were counted for each chamber, and the average number of migrating cells was calculated from the total number of cells counted per chamber. Results are expressed as the mean±S.D. With Matrigel (BD Labware) coating, the same procedure was also used to determine *in vitro* cell invasion.

Laser scanning fluorescent confocal microscopy

1) Multi-track laser scanning: Tumor cells transiently transfected with different GFP-DLC1s in chamberslides were fixed with 3.7% formaldehyde and stained with rhodamine-conjugated phalloidin and DAPI. A Zeiss LSM 510 laser scanning confocal microscope was utilized to detect green, red and blue signals for DLC1 proteins, actin cytoskeleton and the nucleus, respectively. 2) Time lapse observation: the chamberslide carrying the gene-transfected cells was placed in a stage-mounted microincubator (Zeiss Temperature Insert P, Incubator S, and CTI-controller) which provides a constant culture environment (T=37 °C, CO_2=5%) to enable the continuous observation of the same cells for up to 48 h. The images were scanned in z-stack and projected at a 20–30 min interval, and assembled at the end of observation as a time-lapse file using Zeiss software.

Western blotting and IHC staining

Procedures for conventional Western blotting were followed to detect protein expression of WT DLC1 and its mutants in NCI-H358 cells. RIPA lysis buffer supplemented with proteinase inhibitors was utilized to lyze cells. IHC staining was used to detect WT DLC1 expression in NCI-H358 cells after transfection with pcDNA3.1Zeo(+)-DLC1 for 24 h, and in normal human lung epithelial cells in a normal lung tissue specimen. The staining procedures provided in a VECTASTAIN ABC Kit were followed.

Caspase 3 activity assay

Following transient gene transfection, cell lysates were collected in caspase 3 lysis buffer. Protein concentration was measured using a BCA Protein Assay Kit (PIERCE) in a

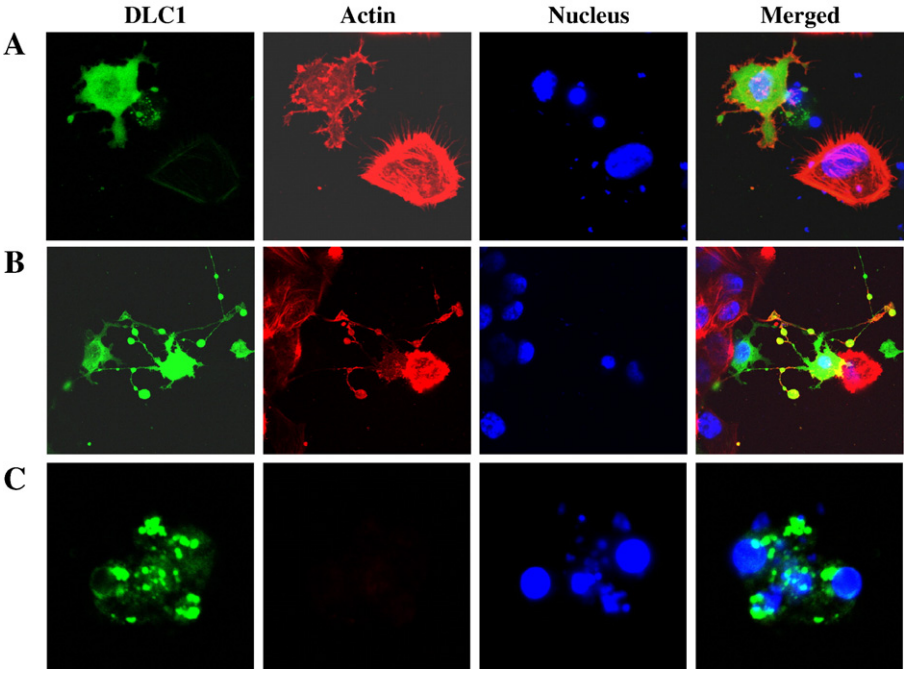


Fig. 2 – DLC1 induces actin cytoskeleton reorganization. Under a confocal microscope, the DLC1 protein (green), actin cytoskeleton (red) and nucleus (blue) were seen in NCI-H358 cells after transfection with pEGFP-N1-DLC1. (A) At 24 h, a significant reduction in stress fibers and filopodia was observed in a GFP-positive cell as compared with a neighboring GFP-negative cell. (B) Membrane blebbings exhibiting a stronger actin staining along the cytoplasmic extensions were observed at 24 h in two GFP-positive cells, but not in GFP-negative cells. (C) Apoptotic morphologies manifested as cell decomposition with scattered green granules and nuclear fragmentation with no actin cytoskeleton staining were observed at 48 h in a GFP-positive cell.

SpectraMax 2.6 microwell reader. Fluorogenic activity was measured in a CytoFluor Multiwell Plate Reader (PerSeptive Biosystems) with excitation at 380 nm and emission at 420–460 nm. The results are expressed as the mean ± S.D. of three individual experiments.

Results

DLC1 induces cytoplasmic extensions in NCI-H358 cells

We selected NCI-H358 cells because of their high sensitivity to DLC1 tumor suppression functions [2]. Transfection of these cells with WT DLC1, but not vector alone, induced a morphological change manifested as branched cell extensions (Figs. 1A and B). Such a change was not produced by insufficient growth stimulation due to low quality serum, or by LPA and other reagents affecting actin polymerization. Furthermore, fluorescent microscopy revealed that only WT DLC1 (GFP-tagged) positive cells exhibited cytoplasmic extensions; none of the DLC1-negative cells underwent any type of morphological changes (Fig. 1C), suggesting that the induction of cytoplasmic extensions is specific for DLC1.

DLC1 induces actin cytoskeleton alteration, membrane blebbing and apoptosis in NSCLC cells

To further characterize morphological changes, we examined the actin cytoskeleton in the cells transfected with GFP-tagged DLC1 utilizing a multi-track laser scanning confocal micro-

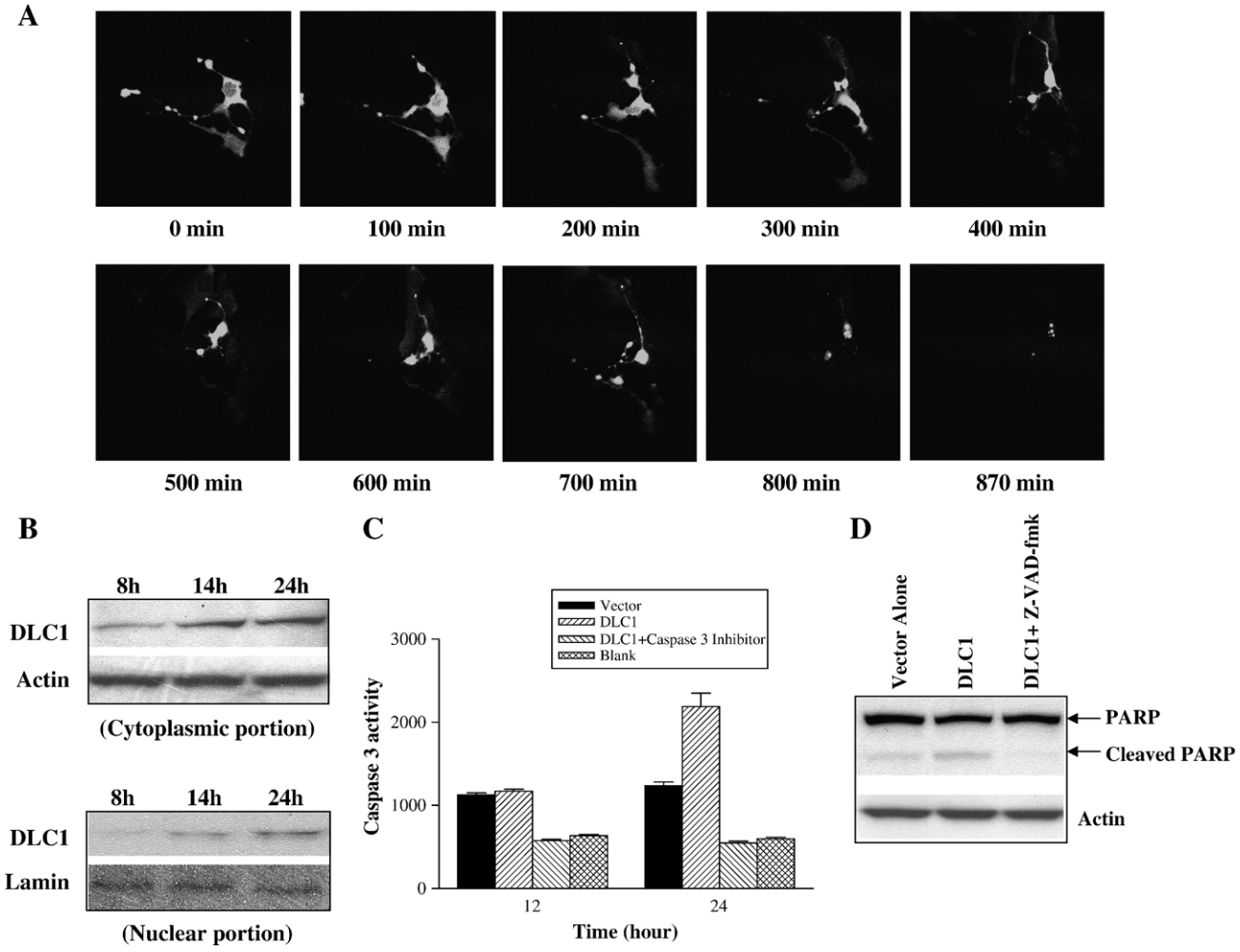


Fig. 3 – Morphological changes and DLC1 PNT precedes apoptosis induction. (A) Time lapse observation. A WT DLC1 positive cell with cytoplasmic extensions, membrane blebbings and cytoplasmic location of the DLC1 protein at 24 h after transfection was subjected to a time lapse observation. It underwent a progressive shrinkage with gradual appearance of GFP signals in the nucleus. At the end, it became completely decomposed with only cell fragments remaining. (B) Western blotting. The DLC1 protein was examined in both the cytoplasm and nucleus at different time points after transfection with pcDNA3.1Zeo(+)-DLC1. (C) Caspase 3 cleavage activity assay. It was performed at 12 h and 24 h after transfection with pcDNA3.1Zeo(+)-DLC1. The caspase activity was completely inhibited by 10 μM Z-VAD-fmk. (D) Western blotting. Transfection with pcDNA3.1Zeo(+)-DLC1 caused an increase in PARP protein cleavage, as compared with vector alone, which was inhibited by 10 μM Z-VAD-fmk. Cleaved and non-cleaved PARP proteins are indicated.

scope. A significant reduction of stress fibers and filopodia was observed at 24 h after transfection in most DLC1 positive cells (Fig. 2A). In addition to the cytoplamic extensions, small membrane swellings, or blebs, along the cellular extensions were also observed in over half of DLC1 positive cells. The periphery of each bleb exhibited stronger DLC1 and actin staining than the remaining cytoplasm (Fig. 2B). Approximately 10% of DLC1 positive cells exhibited a complete cell decomposition manifested as scattered granular green color with a condensed or fragmented nucleus and a dramatic reduction of or absence of actin staining (Fig. 2C). Cell decomposition, nuclear condensation or fragmentation and reduced actin staining, which probably reflects actin cytoskeleton collapsing, are characteristics of apoptosis that are frequently observed at end-stage of the process [28].

DLC1-induced apoptosis as an endpoint of a unique process of morphological change

To determine the consequences of the altered morphologies, we continuously monitored the same DLC1 positive cells. It was observed that 10% of the DLC1 positive cells that eventually progressed to apoptosis underwent the same process of morphological changes, which began with cytoplasmic extensions, followed by membrane blebbing and

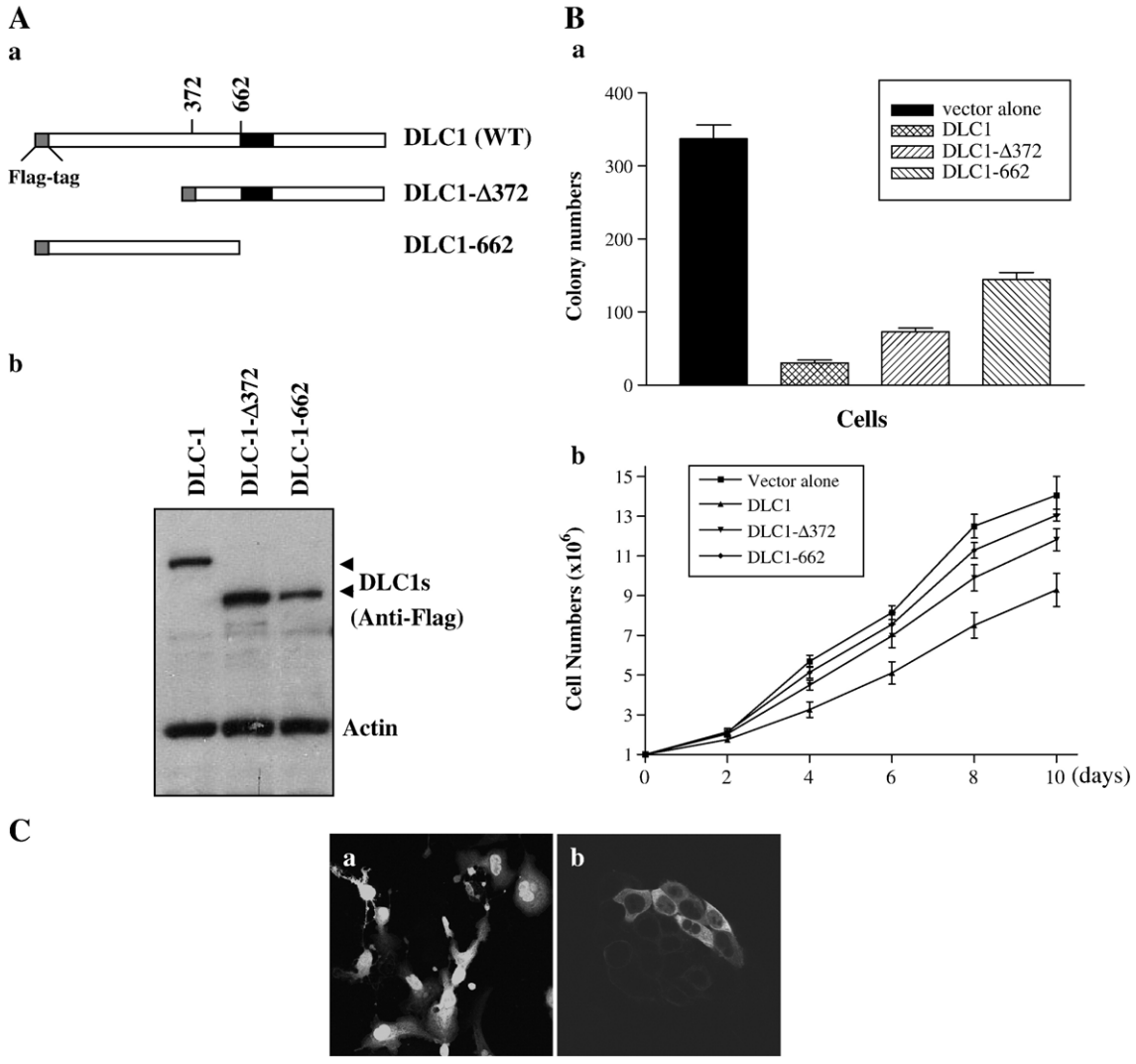


Fig. 4 – Functional analysis of DLC1 mutants in NCI-H358 cells. (A) (a) Schematic representation of DLC1, DLC1-Δ372 and DLC1-662 cDNAs. The *semi-solid box* denotes the Flag tag at the N-terminus of each cDNA; the *solid box* denotes the RhoGAP domain. The numbers refer to the residues from which the deletions were made. (b) Western blotting. Expression of mutant proteins was confirmed using anti-Flag. (B) Colony formation (a) and cell proliferation (b) assays following transfection with pcDNA3.1Zeo(+)-DLC1, -DLC1-Δ372, -DLC1-662, or vector alone. (C) Confocal microscopy. At 24 h after transfection with pEGFP-N1-DLC1-Δ372 or - DLC1-662, DLC1-Δ372 (a), but not DLC1-662 (b), positive cells exhibited cytoplasmic extensions, membrane blebbings and PNT. (D) Time lapse observation. Began at 24 h after transfection with pEGFP-N1-DLC1-662 (a), -DLC1-Δ372 (b) or -DLC1-R718E (c). (E) Caspase 3 activity assay. Performed at 24 h after transfection with pcDNA3.1Zeo(+)-DLC1, -DLC1-Δ372, -DLC1-662, -DLC1-R718E, or vector alone. (F) *In vitro* cell migration assay. Performed in the cells stably transfected with pcDNA3.1Zeo(+)-DLC1, -DLC1-Δ372, -DLC1-662, -DLC1-R718E, or vector alone.

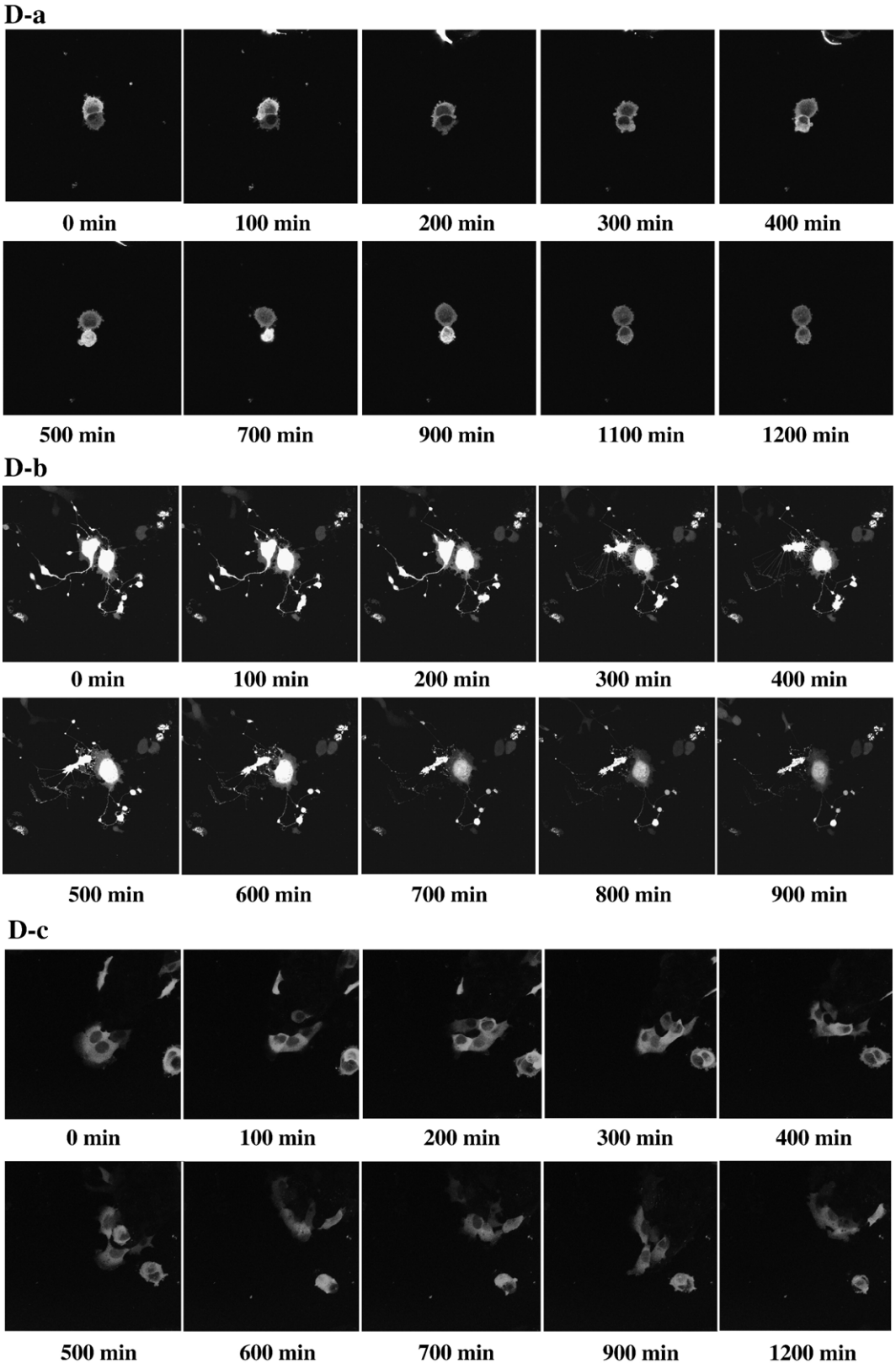


Fig. 4 *(continued)*.

progressive cell shrinkage, and ended by cell decomposition. In these cells, DLC1 protein was initially detected mainly in the cytoplasm, but in both the cytoplasm and nucleus shortly before the cells became fully apoptotic (Fig. 3A), suggesting DLC1 PNT occurred prior to apoptosis. The remaining DLC1 positive cells, however, did not become apoptotic and the

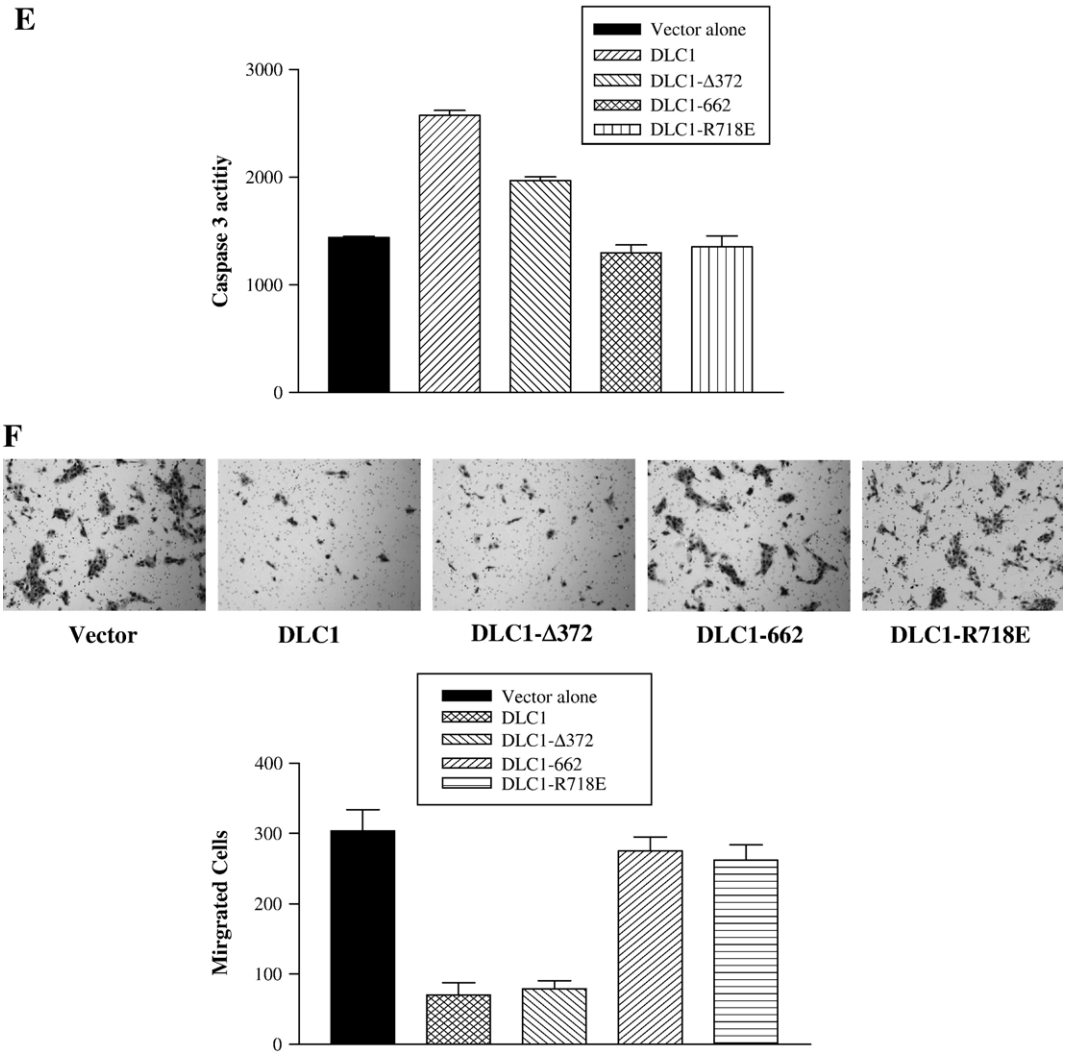


Fig. 4 (*continued*).

DLC1 protein remained mainly in the cytoplasm. DLC1 PNT was confirmed by Western blotting (Fig. 3B).

To support DLC1 induction of apoptosis, we examined the activity of caspase 3. As compared with vector alone, WT DLC1 approximately doubled caspase 3 activity (Fig. 3C). The caspase 3 activation by DLC1 was confirmed by Western blotting, in which PARP protein cleavage was increased by WT DLC1 (Fig. 3D).

The RhoGAP domain of DLC1 is required for inhibiting NSCLC cell growth and migration, and for inducing morphological changes, PNT and apoptosis

In our initial attempt to determine critical DLC1 functional domain(s), we generated two equal-sized DLC1 mutants, DLC1-Δ372 and DLC1-662, by deleting 5′ and 3′ regions from WT DLC1, respectively (Fig. 4A). Each mutant DLC1 was first examined for their effect on tumor cell growth. Colony formation and cell proliferation assays showed that the 3′ deletion in DLC1-662 significantly reduced WT DLC1's inhibition of tumor cell growth, whereas the 5′ deletion in DLC1-Δ372 exhibited a less significant effect (Fig. 4B). Subsequently, the GFP-tagged DLC1-Δ372 and DLC1-662 were examined for their effects on cell morphology, apoptosis and PNT. In DLC1-662 positive cells, morphological changes, PNT and cell death were not observed, whereas, DLC1-Δ372 positive cells displayed cell extensions, membrane blebbings and apoptotic morphologies (Figs. 4C-a+b and D-a+b). A reduction of stress fibers and filopodia was observed in most DLC1-Δ372, but not in DLC1-662, positive cells (data not shown). Also, the DLC1-662 mutant had no effect on caspase 3 activity, whereas the DLC1-Δ372 mutant exhibited a lower caspase 3 activation than WT DLC1 (Fig. 4E). However, dramatically different from WT DLC1, almost all DLC1-Δ372 positive cells exhibited an earlier PNT which occurred in first 12 h after gene transfection (Figs. 4C-a and 4D-b). These findings strongly suggested that the RhoGAP domain deleted in DLC1-662 is essential for WT DLC1 functions. To verify the importance of the RhoGAP domain, we tested a DLC1-R718E mutant which possesses an altered residue in the RhoGAP domain and abrogates WT DLC1's ability to inactivate RhoA and Cdc42 *in vitro* [29]. Cells transfected with DLC1-R718E did not exhibit PNT, morphological changes, caspase 3 activation (Figs. 4D-c and E), or changes in stress fibers and filopodia (data not shown), and as pre-

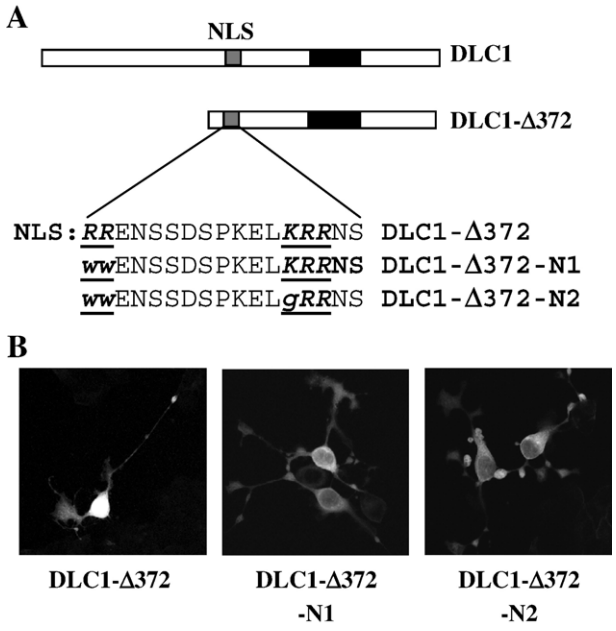


Fig. 5 – A bipartite NLS is required for DLC1 PNT. (A) Schematic representation of amino acid sequence of a bipartite NLS in DLC1. Point mutations introduced into the NLS of DLC1-Δ372 to generate DLC1-Δ372-N1 and DLC1-Δ372-N2 mutants are indicated. (B) As compared with DLC1-Δ372, the DLC1-Δ372-N1 and DLC1-Δ372-N2 proteins were sequestered in the cytoplasm at 24 h after transfection. The DLC1-related morphological changes were not affected by NLS mutations.

viously demonstrated, DLC1-R718E was not able to inhibit NCI-H358 cell growth [27]. These results underscore the pivotal role of the RhoGAP domain in newly revealed DLC1 properties and its implication in DLC1's anti-oncogenic activities.

DLC1's ability to inhibit cell migration and invasion was demonstrated in liver and breast cancer cells [12,14]. Thus, we examined the effect of WT DLC1 and its mutants on migration and invasion of NCI-H358 cells. *In vitro* Matrigel invasion assay demonstrated that parental NCI-H358 cells were not invasive. However, *in vitro* migration assay showed that WT DLC1 and DLC1-Δ372, but not DLC1-662 and DLC1-R718E, significantly inhibited cell migration (Fig. 4F).

Identification of DLC1's nuclear localizing sequence (NLS)

It is generally believed that PNT depends on a NLS(s) [30]. Online PROSITE protein sequence search revealed a bipartite NLS consensus sequence at residues 415–430 of WT DLC1, located at the very N-terminal portion of the DLC1-Δ372 mutant protein (Fig. 5A). The NLS is *R*R*E*NSSDSPK*E*L*KRR*, in which the first two *R*s (arginine) and the last three basic amino acids, one *K* (lysine) and two *R*s, were critical residues for NLS function. To establish the role of the NLS, we introduced point mutations at the critical residues of the NLS in DLC1-Δ372 to generate DLC1-Δ372-N1 and DLC1-Δ372-N2 mutants which were then tagged with GFP and transfected into NCI-H358 cells (Fig. 5B). In contrast to the DLC1-Δ372 protein which showed almost 100% PNT during early gene transfection (Figs. 4C-a and D-b), both DLC1-Δ372-N1 and DLC1-Δ372-N2 proteins appeared only in the cytoplasm at 12 h and remained in the cytoplasm thereafter. However, the NLS mutations did not affect DLC1-induced morphologies (Fig. 5B). These analyses established DLC1's PNT function and its dependence on a NLS consensus sequence.

Nuclear translocation represents a physiological activity of the DLC1 protein

Next we localized DLC1 protein in normal human lung epithelial cells by IHC staining. A monoclonal antibody against DLC1, which detected a distinct band between 120 kDa and 190 kDa by Western blotting and positively stained DLC1 protein mainly in the cytoplasm by IHC in WT DLC1-transfected NCI-H358 cells (Figs. 6A and B), was utilized to stain a normal human lung tissue specimen by IHC. Most alveolar epithelial cells were stained positive for DLC1 protein which concentrated more abundantly in the nucleus than in the cytoplasm (Fig. 6C). These findings suggest that DLC1 PNT may represent a physiological activity in normal human lung epithelial cells.

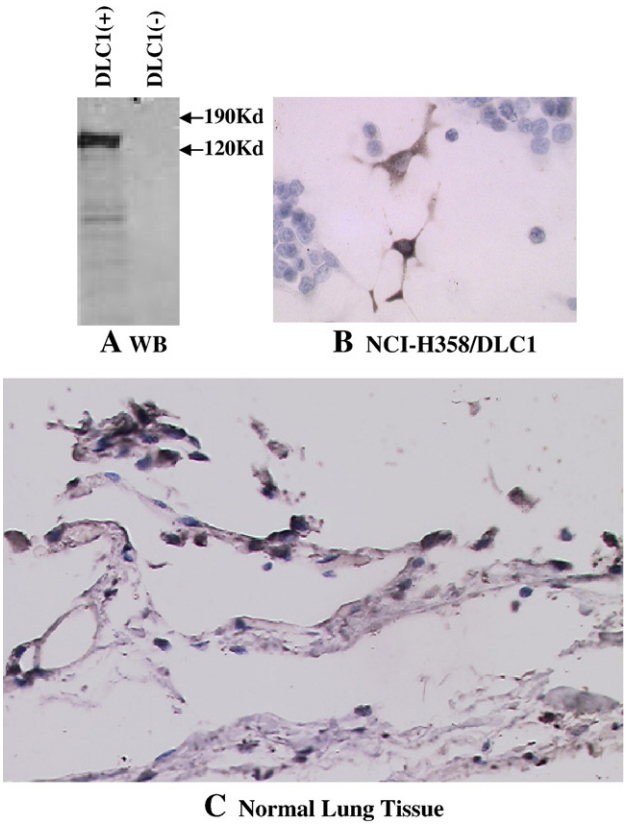
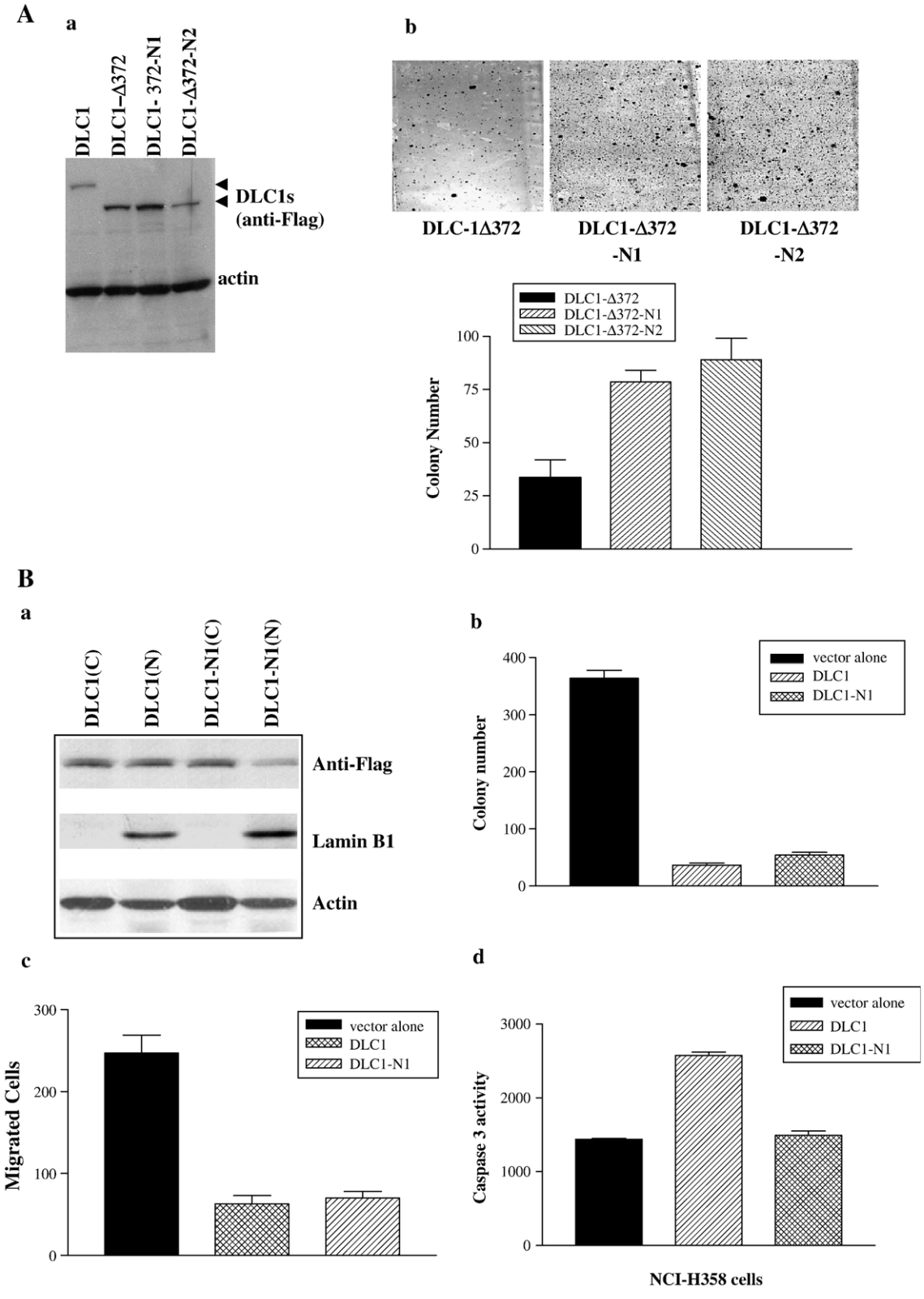


Fig. 6 – Localization of the DLC1 protein in normal human lung epithelial cells. An anti-DLC1 antibody which detected a distinctive band between 120 kDa and 190 kDa by Western blotting (A) and stained positive in the cells exhibiting cytoplasmic extensions by IHC after transfection with pcDNA3.1(+)-DLC1 (B) were used in IHC on a normal human lung tissue (C). Most lung alveolar epithelial cells on the upper part of the figure exhibited positive DLC1 staining with signals more concentrated in the nucleus (C).

DLC1 PNT is required for induction of tumor cell apoptosis

To determine the function of PNT of DLC1, we carried out colony formation and migration assays in NCI-H358 cells transfected with DLC1-Δ372, -Δ372-N1 or -Δ372-N2. It was found that both DLC1-Δ372-N1 and -Δ372-N2 formed significantly more colonies than DLC1-Δ372 (Fig. 7A-a+b), although they did not show a difference in cell migration (data not shown), suggesting that the PNT contributes to DLC1-Δ372's inhibition of tumor cell growth, but not to migration. To determine whether the NLS mutations can affect WT DLC1's functions, we generated a DLC1-N1 mutant, in which the first two arginines in the NLS of



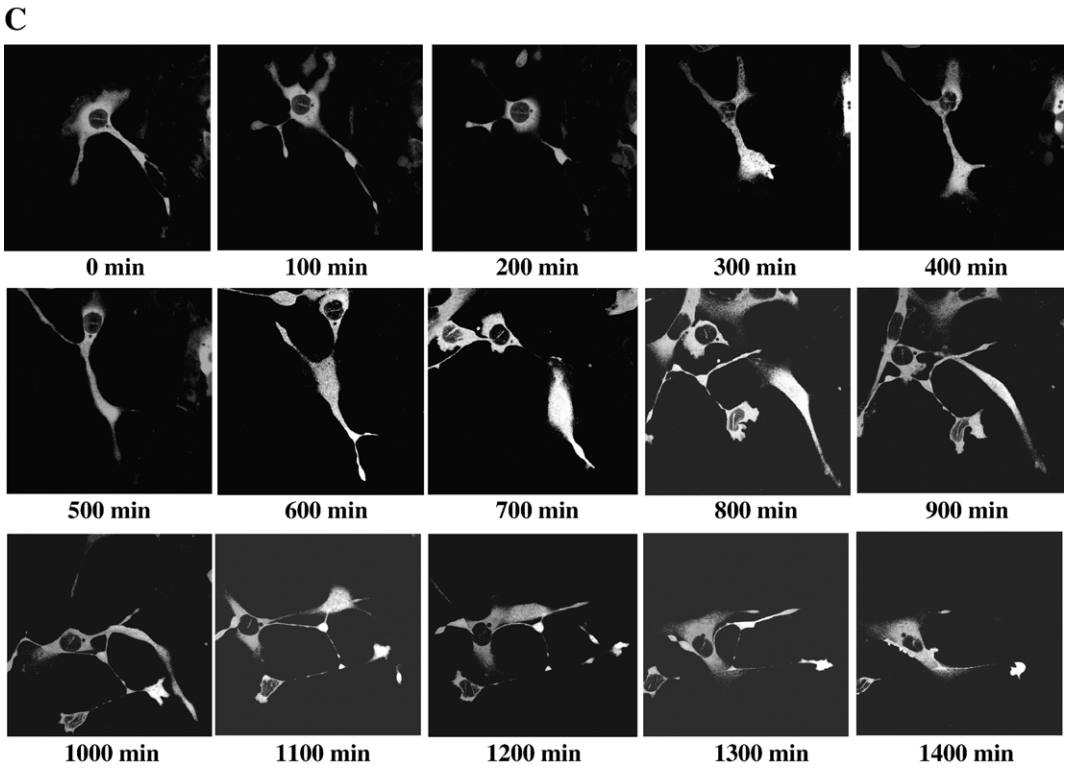


Fig. 7 – Distinct cellular locations determine different DLC1 tumor suppression functions. (A) Colony formation assay showed that the cells transfected with DLC1-Δ372-N1 or -Δ372-N2 (on pEGFP-N1) formed more colonies than the cells transfected with DLC1-Δ372 (b). Protein expression of DLC1-Δ372 mutants was confirmed by Western blotting (a). (B) (a) Western blotting showed a reduced protein level for NLS-deficient WT DLC1 (DLC1-N1) in the nucleus (denoted as N), but the same level in the cytoplasm (denoted as C) as compared with WT DLC1. The NLS-mutated DLC1-N1 still showed an inhibition in colony formation (b) and *in vitro* cell migration (c), but failed to activate caspase 3 (d). pcDNA3.1Zeo(+)-DLC1-N1 was used in gene transfection. Caspase 3 activity was measured at 24 h after gene transfection. (C) Time lapse observation, which began at 24 h after gene transfection, revealed cytoplasmic extensions, but neither PNT nor cell death in DLC1-N1 cells.

WT DLC1 were converted into two tryptophans, and tested its effects on colony formation, migration and apoptosis. A reduced level of the DLC1-N1 protein in the nucleus as compared with WT DLC1 was detected by Western blotting (Fig. 7B-a). WT DLC1 and DLC1-N1 had a similar effect on colony formation and cell migration (Figs. 7B-b and c). However, DLC1-N1 failed to activate caspase 3 (Fig. 7B-d). Live images of DLC1-N1 showed typical cell extensions, but neither PNT nor apoptotic morphologies (Fig. 7C). All these observations suggested that the PNT is primarily associated with DLC1 proapoptotic activity.

A serine-rich domain is involved in the regulation of DLC1 PNT

The finding that DLC1-Δ372 exhibited a much robust PNT than WT DLC1 suggested the existence of a regulatory region for PNT in the first 372 amino acids of WT DLC1. To localize this region, the DLC1-Δ291 and DLC1-Δ209 mutants were generated by deleting the first 291 and 209 amino acids of WT DLC1, respectively. Each new GFP-tagged mutant was then examined for cellular location in NCI-H358 cells. It was found that the DLC1-Δ291 protein still exhibited an early PNT, whereas the DLC-Δ209 protein appeared only in the cytoplasm (Fig. 8). These findings supported the existence of a regulatory region for DLC1 PNT located between residue 209 and 291. On-line protein domain search revealed that this region overlapped with the C-terminal half of a serine rich domain, suggesting that the serine-rich domain is involved in regulating DLC1 PNT.

Discussion

Here we show that DLC1's induction of programmed cell death in NSCLC is preceded by changes in cell morphology that begin with cytoplasmic extensions and membrane blebbings. The morphological changes are functions of the DLC1 RhoGAP domain which causes actin cytoskeleton reorganization resulting in the reduction of stress fibers and filopodia, most likely through inhibition of RhoA and Cdc42 activities [11,19]. While the mechanism involved in membrane blebbing remains to be elucidated, the cytoplasmic extensions seem to be the consequence of an unbalanced Rac1 activity with resultant enhancement of lamillipodia formation (or cytoplasmic extensions) due to inhibition of Rho activities [31].

The sequential occurrence of morphological changes and apoptosis may reflect an interaction between actin cytoskel-

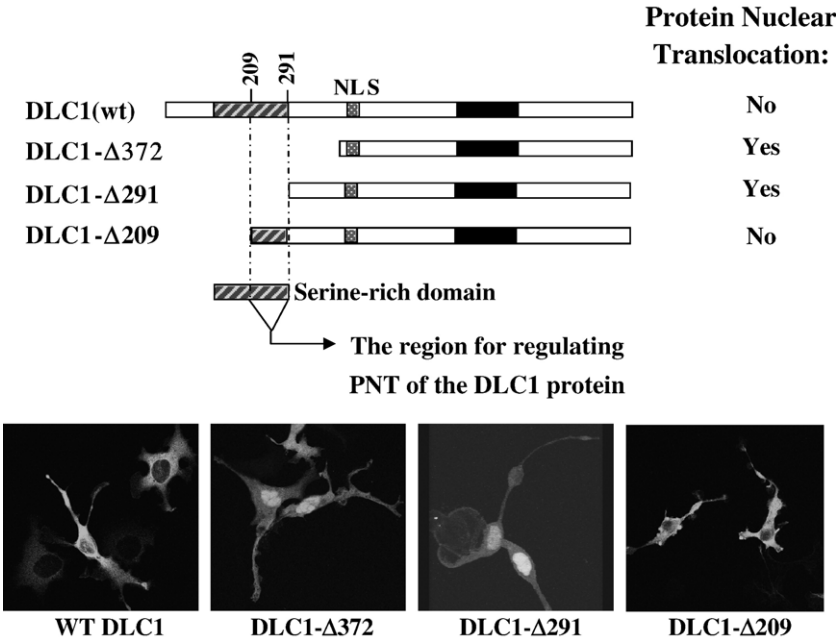


Fig. 8 – A serine-rich domain is responsible for regulating DLC1 PNT. Schematic WT DLC1, DLC1-Δ372, -Δ291 and -Δ209 are represented. The solid box denotes the RhoGAP domain. The NLS, serine-rich domain and amino acid residues for deletion mutations, are indicated. The PNT for each DLC1 mutant observed under a confocal microscope at 12 h after transfection is indicated and each GFP-fluorescent image (brighter in black/white) is presented.

eton reorganization and apoptosis machinery. Actin cytoskeletal disruption damages the mitochondrial membrane causing cytochrome *c* release and caspase activation with the cleavage of downstream proteins including actin cytoskeleton proteins [32]. In healthy cells, intact actin filaments bind and sequester DNase I, an endonuclease that cleaves genomic DNA. Actin cytoskeleton disruption releases DNase I causing nuclear fragmentation [33]. Therefore, collapsing of actin cytoskeleton and nuclear fragmentation observed during DLC1-induced apoptosis are likely the consequences of caspase 3 activation and DNase I release, respectively.

In addition to the initial morphological changes, DLC1-induced cell death is also dependent on DLC1 PNT as documented by lack of any typical manifestations of apoptosis by WT DLC1 in the absence of PNT and by the failure of DLC1-N1 to activate caspase 3. The occurrence of DLC1 PNT in normal lung epithelial cells suggests that DLC1 PNT represents a physiological activity in maintaining homeostasis of normal lung epithelial tissue probably by inducing apoptosis in response to different apoptotic stimuli, that include mutagenic–carcinogenic insults [34].

Most likely, DLC1 PNT is dependent on a complex intramolecular regulation. Point mutation analyses demonstrated that a bipartite NLS is the basis for DLC1 PNT. However, the NLS is not the only prerequisite for DLC1 protein nuclear movement. All RhoGAP domain-deficient DLC1 mutant proteins with intact NLS are sequestrated in the cytoplasm, indicating that the RhoGAP domain is required for DLC1 PNT as well. Given our finding on the role of the RhoGAP domain in regulating actin cytoskeleton organization and the association of actin protein with nuclear pore complexes (NPC) [35], it is likely that DLC1 affect NPC through a RhoGAP-dependent mechanism to facilitate its PNT. While both the NLS and the RhoGAP domain are positive regulators, a serine rich domain appears to be a negative regulator for DLC1 PNT. Recently, phosphorylation of Serine 322 of rat DLC1 was demonstrated in rat adipocytes treated with insulin [36]. Serine phosphorylation or dephosphorylation is a common mechanism for the regulation of a variety of protein functions, including inter-compartment trafficking [26]. This finding explains why the PNT for DLC1-Δ372 is enhanced after N-terminal deletion from WT DLC1, whereas the PNT for WT DLC1 is delayed. Whereas the serine rich domain could be the nuclear import regulatory domain, it may also be the domain that controls the stability or duration of nuclear residency of DLC1. The interactions among the NLS, RhoGAP domain and serine-rich domain warrant investigations for a better understanding of DLC1 PNT and its relevance to tumor suppressive function.

Our analyses provide evidence that distinct subcellular locations determine different aspects of DLC1 tumor suppression functions. Cytoplasmic sequestration after NLS point mutations reduced DLC1-Δ372's ability to inhibit tumor cell growth, but had minimal effects on WT DLC1's induction of morphological changes and inhibition of tumor cell growth and migration. Thus, it is likely that DLC1 exerts its pro-apoptotic activity in the nucleus and growth suppression function in the cytoplasm. DLC1 interaction with PLCδ1 that is also imported into the nucleus [19,37] suggests that DLC1's proapoptotic function in the nucleus could be related to the interaction with PLCδ1 while cell growth inhibitory activity in the cytoplasm would be mainly due to the actin cytoskeletal reorganization.

In summary, we present novel data showing that DLC1 functions as both a cytoplasmic and nuclear tumor suppressor

gene. In addition to transcriptional silencing by genomic deletion and/or aberrant DNA methylation, two major mechanisms for DLC1 inactivation, cytoplasmic sequestration may represent another important mechanism employed by tumor cells to inhibit DLC1 induction of apoptosis. Diverse mechanisms responsible for DLC1 inactivation in tumor cells further emphasize the importance of the DLC1 tumor suppressor gene in tumor development.

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