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The intracellular stability of DLC1 is regulated by the 26S proteasome in human hepatocellular carcinoma cell line Hep3B

Hong-wei Luo [a,1], Qiu-ping Luo [a,1], Ying Yuan [b], Xiao-ying Zhu [a], Shi-feng Huang [a], Zhi Peng [a], Chun-li Li [a], Zong-gan Huang [c], Wen-li Feng [a,*]

[a] Department of Clinical Hematology, Key Laboratory of Diagnostic Medicine Designated by the Ministry of Education, Chongqing Medical University, Chongqing 400016, PR China
[b] Department of General Surgery, The First Affiliated Hospital, Chongqing Medical University, Chongqing 400016, PR China
[c] Department of Hematology, The First Affiliated Hospital, Chongqing Medical University, Chongqing 400016, PR China

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ABSTRACT

Deleted in liver cancer 1 (*DLC1*), a tumor suppressor gene identified in a primary human hepatocellular carcinoma, encodes a Rho GTPase-activating protein (RhoGAP). Although *DLC1* expression has been studied at the transcriptional level, little is known about its regulation at the protein level. Here we show that DLC1 is an unstable protein that is degraded by the 26S proteasome in human hepatocellular carcinoma Hep3B cells. In addition, five putative PEST motifs were identified in the N-terminus of DLC1. Unexpectedly, the N-terminus of DLC1 appeared to be stable. Furthermore, deletion of any one of the five PEST motifs except PEST2 decreased the stability of the N-terminus of DLC1, which suggests that the PEST motifs may play an unrevealed role in maintaining the stability of DLC1. These data indicated that the intracellular stability of DLC1 is regulated by the 26S proteasome via its PEST motifs.

1. Introduction

Deleted in liver cancer-1 (*DLC1*), a tumor suppressor gene identified in a primary human hepatocellular carcinoma, encodes a Rho GTPase-activating protein (RhoGAP) with selective activity against RhoA [1,2]. The RhoGAPs act as negative regulators of the Rho family of small GTPases by stimulating the intrinsic GTPase activity of Rho proteins and converting the active GTP-bound form of Rho to the inactive GDP-bound form [3]. It has been demonstrated that over-expression of DLC1 in DLC1 null cancer cell lines is able to suppress cell proliferation, decrease cell migration and invasion and prevent tumor formation in nude mice [4]. In contrast, knockdown of DLC1 with siRNA in the breast cancer cell line MCF7 demonstrated that a decrease of DLC1 expression results in cells acquiring a more migratory phenotype [5]. More recently, a mouse liver tumor model using short-hairpin RNA-mediated DLC1 knockdown confirmed that DLC1 functions as a tumor suppressor [6]. These studies support a tumor-suppressor role of DLC1.

As a tumor suppressor, the regulation of DLC1 at the mRNA and protein levels should be important for its function. Extensive studies have shown that the expression of DLC1 mRNA is downregulated or absent in various types of cancer due to genome deletion or aberrant DNA methylation of the DLC1 promoter [7]. Treatment of cancer cells with the demethylation agent 5-aza-2′-deoxycytidine or the histone deacetylase inhibitor trichostatin A (TSA) can induce endogenous DLC1 mRNA expression [8,9]. These studies suggest that epigenetic changes of DLC1 may be a mechanism of DLC1 inactivation at the transcriptional level in cancer cells. However, the molecular mechanisms that regulate DLC1 expression at the protein level have not been documented.

Protein degradation is an important means by which cells control normal protein levels. The 26S proteasome is an important degradation pathway through which the turnover of various proteins, including tumor suppressors, is regulated [10]. Protein turnover is often caused by signals that induce protein degradation. PEST motifs, enriched in proline (P), glutamate (E), serine (S), and threonine (T), have been found in many short-lived regulatory proteins and serve to target them to the 26S proteasome for degradation [11].

Here, we report that DLC1 is an unstable protein that is rapidly degraded by the 26S proteasome in human hepatocellular carcinoma Hep3B cells. Moreover, analysis of the primary sequence of DLC1 protein using the PESTfind algorithm revealed the presence of five putative PEST motifs located in its N-terminus. Surprisingly, the N-terminus of DLC1 appeared to be stable, and deletion of any one of the five PEST motifs, except for the second, led to decreased

Abbreviations: PEST, proline-glutamic acid-serine-threonine; CHX, cycloheximide; EGFP, enhanced green fluorescent protein; HEK, human epithelial kidney; RT-PCR, reverse transcription-polymerase chain reaction.

* Corresponding author. Fax: +86 023 68485005.
E-mail addresses: fengwlcqmu@sina.com, shreli@cc.kmu.edu.tw (W.-l. Feng).

[1] These authors contributed equally to this work.

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stability of the N-terminus of DLC1, suggesting that four of the five PEST motifs may play an unexpected role in maintaining the stability of DLC1.

2. Materials and methods

2.1. Plasmids

pcDNA3-DLC1 plasmid was kindly provided by Prof. Ng Irene Oi-Lin. DLC1, DLC1N (aa 1–600), and DLC1C (aa 601–1091) were amplified with primers P1/P4, P51/P52, and P61/P4 using pcDNA3-DLC1 as the template. The amplified products were inserted into pEGFP-N1 at the *Kpn* I/*Bam*H I, *Eco*R I/*Bam*H I, and *Kpn* I/*Bam*H I sites to obtain pDLC1-EGFP, pDLC1N-EGFP, and pDLC1C-EGFP, respectively. DLC1N with a PEST1 (aa 85–100) deletion was amplified by overlap extension PCR using the primers P51/P72 and P71/P52 with pDLC1N-EGFP as the template, and the PCR product was inserted into pEGFP-N1 at the *Eco*R I and *Bam*H I sites to generate plasmid pDLC1NP1-EGFP. Using the same strategy, the other plasmids expressing DLC1N lacking PEST motifs were generated. All constructs were confirmed by sequencing. The primers used in this study are shown in Table 1.

2.2. Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Human hepatocellular carcinoma cell line Hep3B was cultured in minimum essential medium (Gibco) supplemented with 10% FBS and 1.0 mM sodium pyruvate. All cells were maintained at 37 °C with 5% CO_2. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.3. Identification of PEST motifs

The program PESTfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind) was used to identify putative PEST motifs in DLC1 of mouse, rat, and human (GenBank accession No. NM_015802, NM_001127446, NM_006094, respectively). The results of a PESTfind analysis are a score ranging from −50 to +50. A score greater than +5 indicates a putative PEST motif.

2.4. RT-PCR

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen). RT-PCR reactions were performed as described previously [12]. The primer sequences were as follows: DLC1 forward, 5′-AGG CTT TGG AGG TCA GTC AT-3′; DLC1 reverse, 5′-TTT CCC ACA GGG TTC AAT CAA AT-3′; β-actin forward, 5′-GTG GAC ATC CGC AAA GAC-3′; β-actin reverse, 5′-AAA GGG TGT AAC GCA ACT AA-3′.

Table 1
Primers for amplification.

Primer[a]	Sequence
P1	5′-GGGGTACCGCCACCATGTGCAGAAAGAAGCCGGACAC-3′
P4	5′-CGGGATCCCGCCTAGATTTGGTGTCTTTGG-3′
P51	5′-CGGAATTCGCCACCATGTGCAGAAAGAAGCCGGACAC-3′
P52	5′-CGGGATCCCGTAGGAGTGAGTATTTCTGCAG-3′
P61	5′-GGGGTACCGCCACCATGCTAAAGCTAACGGCCCTGCTGG-3′
P71	5′-AAGTCCATTTTCGTTTCCGATGAGGACTAAT-3′
P72	5′-TCGGAAACGAAAATGGACTTTCCAAAGGG-3′
P81	5′-AGTTAGTCCGGTGGCTGGGGAGGCTGCCAG-3′
P82	5′-CCCCAGCCACCGGACTAACTCCGTCATCAG-3′
P91	5′-TCCGGGTCCTCCTCTTTCGTACCATGGGG-3′
P92	5′-ACGAAAGAGGAGGACCCGGAGCCTCAGTGC-3′
P101	5′-GTATCTGTTTCTTCTCCGACCACTGATTGAC-3′
P102	5′-GTCGGAGAAGAAACAGATACACCTGGATG-3′
P111	5′-AATCCCTTCTTCGGTCGTTGTCCACATCC-3′
P112	5′-CAACGACCGAAGAAGGGATTCTGGGGTTGG-3′

[a] Primers used are described in Section 2.

2.5. Western blot analysis

Rabbit anti-DLC1 (H-260), anti-GFP (B-2), and anti-β-Actin (C4) antibodies were purchased from Santa Cruz. Western blotting was performed as previously described [12].

2.6. Protein half-life determination

At 24 h after transfection, HEK293 cells cultured in a 100 mm dish were trypsinized and re-cultured in four 35 mm dishes. HEK293 or Hep3B cells were incubated with medium containing 100 μg/ml cycloheximide (Cat# 239764, Calbiochem, San Diego, CA) for the indicated times. MG132 (Cat# 474790, Calbiochem, San Diego, CA) was added to cell cultures at a final concentration of 10 μM/L 3 h before the addition of cycloheximide. The cells were harvested and 50 μg of cell lysate was analyzed by Western blotting with anti-DLC1 and anti-β-actin antibodies. The protein levels were calculated using densitometry-analyzing software, and the values of DLC1 were plotted against the values of β-actin. The ratio of DLC1/β-actin at 0 h was taken as 100%. The assay was repeated independently at least three times.

3. Results

3.1. Proteasome inhibition increases the levels of both endogenous and exogenous DLC1 protein

The 26S proteasome plays a central role in the regulation of the stability of various proteins [13]. To test the effect of proteasome inhibition on the levels of DLC1 protein, HEK293 and Hep3B cells were treated with the proteasome inhibitor MG132 for 12 h. The mRNA and protein levels of DLC1 were analyzed by semi-quantitative RT-PCR and Western blotting, respectively. DLC1 protein levels increased markedly in cells after MG132 treatment (Fig. 1A), whereas DLC1 mRNA levels were not affected (Fig. 1B). Furthermore, treatment of Hep3B cells with various concentrations of MG132 led to increased expression of DLC1 protein (Fig. 1C). To gain information on the regulation of exogenous DLC1 protein

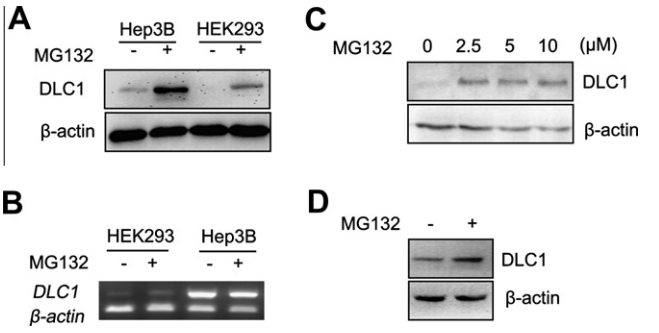

Fig. 1. Proteasome inhibition increases the levels of both endogenous and exogenous DLC1 protein. (A and B) HEK293 and Hep3B cells were treated with the proteasome inhibitor MG132 (10 μM) or DMSO for 12 h, and DLC1 protein and mRNA levels were analyzed by Western blotting and RT-PCR. (C) Hep3B cells were treated with MG132 at the indicated concentrations for 6 h. Cell lysates were subjected to Western blot analysis using an anti-DLC1 antibody. (D) HEK293 cells were transfected with plasmid pcDNA3-DLC1; 36 h after transfection, the cells were treated with MG132 (10 μM) or DMSO for 12 h. Cell lysates were subjected to Western blot analysis using an anti-DLC1 antibody.

levels by the 26S proteasome, the DLC1 expression vector pcDNA3-DLC1 was transiently transfected into HEK293 cells. Treatment of transfected cells with MG132 for 12 h led to significantly increased levels of exogenous DLC1 protein compared with vehicle-treated cells (Fig. 1D). Therefore, these data indicate that the levels of both endogenously and exogenously expressed DLC1 protein are regulated by the 26S proteasome.

3.2. DLC1 is an unstable protein that is degraded by the 26S proteasome

Given that proteasome inhibition by MG132 up-regulated DLC1 protein levels, we asked whether the stability of DLC1 protein was affected by the 26S proteasome. The half-life of endogenous DLC1 was subsequently examined in Hep3B cells by inhibiting new protein synthesis with cycloheximide (CHX). Hep3B cells were treated with 100 μg/ml CHX and the amount of DLC1 protein at 0–5 h was assessed by Western blot analysis. Without MG132 pretreatment, DLC1 in Hep3B cells degraded rapidly and exhibited a relatively short half-life of less than 4 h. However, treatment with the proteasome inhibitor MG132 prolonged its half-life in Hep3B cells (Fig. 2A). Moreover, the half-life of exogenous DLC1 protein was assessed in HEK293 cells transfected with pcDNA3-DLC1. There was a marked reduction in the stability of DLC1 at 3 h after CHX addition. The DLC1 level decreased by more than 50% compared with that of untreated cells (Fig. 2B). These data suggest that DLC1 is an unstable protein that is degraded by the 26S proteasome.

3.3. Five putative PEST motifs are located in the N-terminus of DLC1

DLC1 is a multidomain protein that includes an NH_2-terminal sterile alpha motif domain (SAM), a middle Rho GTPase-activating protein domain (RhoGAP), and a COOH-terminal steroidogenic acute regulatory-related lipid transfer domain (START) [14,15]. Analysis of the amino acid sequence of DLC1 using PESTfind revealed the presence of five putative PEST motifs with PESTfind scores greater than +5 in the region linking the SAM and RhoGAP domains (Fig. 3A). The sequences of mouse and rat DLC1 were also analyzed by PESTfind. Intriguingly, four of the five high-scoring putative PEST motifs are highly conserved among species (Table 2). PEST motifs are associated with proteasome-mediated degradation and have been found in several short-lived proteins [16,17]. To determine the involvement of the putative PEST motifs in DLC1 degradation, three EGFP-tagged DLC1 fusion proteins were constructed: pcDNA3-DLC1N (aa 1–600) containing the five PEST motifs, pcDNA3-DLC1C (aa 601–1091) containing the RhoGAP and START domains, and pcDNA3-DLC1 (Fig. 3A).

The three constructs were transfected into HEK293 cells and the impact of proteasome inhibition on the degradation of the EGFP-tagged fusion proteins was analyzed. The expression levels of DLC1C and DLC1 increased significantly after treatment with MG132. In contrast, MG132 had no effect on DLC1N levels (Fig. 3B). We then tested the half-lives of these EGFP-tagged fusion proteins. Cells expressing DLC1N, DLC1C and DLC1 were treated with CHX and their protein levels were analyzed by Western blotting with anti-GFP antibody at 0, 4, 8, and 12 h. DLC1C degraded rapidly with a half-life of less than 4 h, and the half-life of DLC1

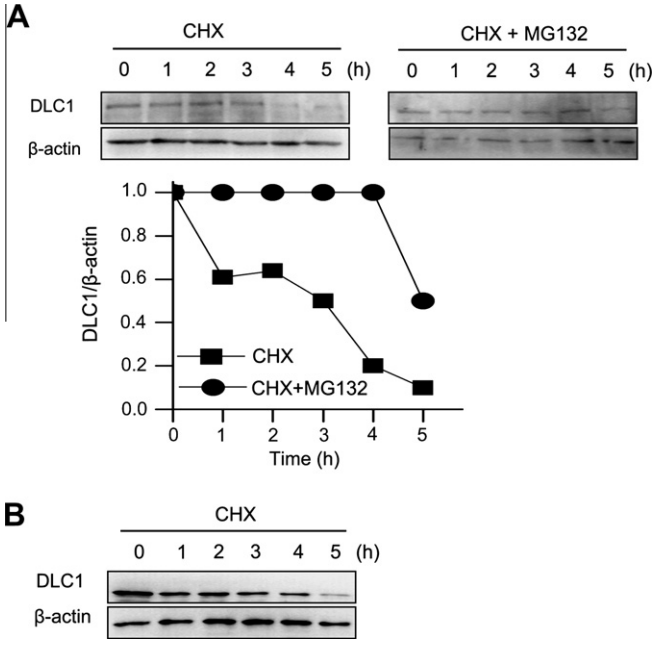

Fig. 2. DLC1 is an unstable protein that is degraded by the 26S proteasome. (A) Hep3B cells were treated with MG132 (10 μM) or DMSO for 3 h, and the protein synthesis inhibitor CHX (100 μg/ml) was then added. Cells were harvested at the indicated time points after CHX addition and examined for the expression of DLC1 and β-actin by Western blot analysis. A densitometric analysis of the DLC1 and β-actin bands using a Molecular Imager System (Quantity One) is shown in the graphs. The value of DLC1/actin at 0 h was taken as 100%. (B) HEK293 cells were transfected with plasmid pcDNA3-DLC1; 36 h after transfection, the cells were incubated with CHX (100 μg/ml) for the indicated times, after which cell lysates were subjected to Western blot analysis to detect DLC1.

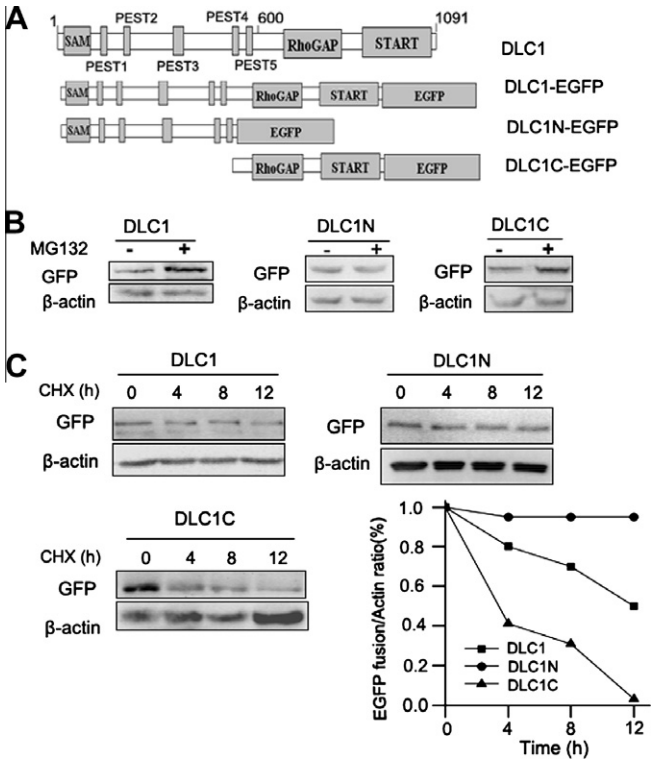

Fig. 3. Five putative PEST motifs are located in the N-terminus of DLC1. (A) Schematic representation of the five identified PEST motifs located in the linker region between the SAM and RhoGAP domains. Three plasmid constructs expressing full-length DLC1, the N-terminus of DLC1 (aa 1–600), and the C-terminus of DLC1 (aa 601–1091) all tagged with EGFP were constructed. (B) HEK293 cells transfected with the three plasmids encoding EGFP fusion proteins were treated with MG132 (5 μM) or DMSO for 12 h and the transfected cell lysates were analyzed by Western blotting with an anti-GFP antibody to evaluate the stability of each EGFP fusion protein. (C) The transfected HEK293 cells were incubated with CHX (100 μg/ml) for the indicated times and the half-lives of the EGFP fusion proteins were analyzed by Western blotting using an anti-GFP antibody. The graph represents the EGFP fusion/actin ratio as measured by densitometry. The value of EGFP fusion/actin at 0 h was taken as 100.

Table 2
PEST sequences are higly conserved in DLCI.

Species	Fragment	Sequences	PESTfind score
Mouse	PEST1	85-RSEDSDEDEPCAISGK-100	14.01
Rat	PEST1	85-RSEDSDEEEPCAISGK-100	14.36
Human	PEST1	85-RSDDSDEDEPCAISGK-100	13.66
Mouse	PEST2	-	-
Rat	PEST2	-	-
Human	PEST2	169-HAPPSEDAATPR-180	6.56
Mouse	PEST3	297-RSVSNSTQTSSSSSQSETSSAVSTPSPVTR-326	14.58
Rat	PEST3	296-RSVSNSTQTSSSSSQSETSSAVSTPSPVTR-325	14.58
Human	PEST3	295-RSVSNSTOTSSSSSOSETSSAVSTPSPVTR-324	14.58
Mouse	PEST4	490-KFSDEGDSDSALDSVSPCPSSPK-512	11.80
Rat	PEST4	489-KFSDFGDSDSALDSVSPCPSSPK-511	11.80
Human	PEST4	489-KFSDEGDSDSALDSVSPCPSSPK-511	11.80
Mouse	PESTS	523-RTPSDLDSTGNSLNEPEEPTDIPER-547	18.33
Rat	PEST5	522-RTPSDLDSTGNSLNEPEEPTDIPER-546	18.33
Human	PEST5	521-RTTPSDLDSTGNSLNEPEEPSEIPER-546	19.15

Occurrence of PEST sites within the amino acid sequence of DLCI from the mouse, rat, and human. Amino acid identity is underlined.

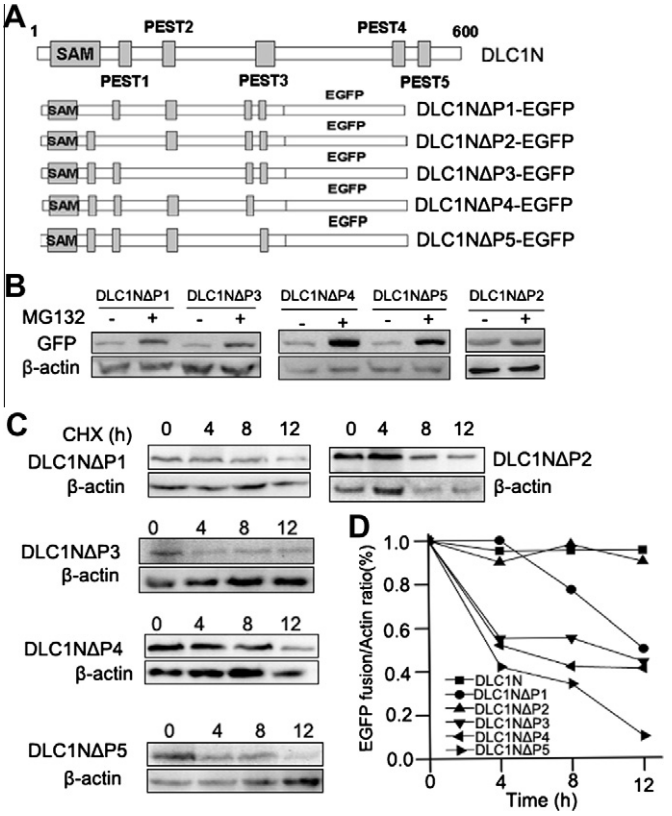

Fig. 4. Deletion of the PEST motifs decreases the stability of the N-terminus of DLC1. (A) Schematic of the five EGFP-tagged DLC1 mutants with individual PEST motifs deleted from the N-terminus. (B) HEK293 cells transfected with plasmids encoding the five PEST deletion mutants were treated with MG132 (10 μM) or DMSO for 12 h, and total cell lysates were analyzed by Western blotting to evaluate the stability of each EGFP fusion protein. (C) The transfected HEK293 cells were incubated with CHX (100 μg/ml) for the indicated times and the half-lives of the five PEST deletion mutants were analyzed by Western blotting using an anti-GFP antibody. (D) The densitometric analysis of DLC1N and its five PEST deletion mutants is shown in the graphs. The value of EGFP fusion/actin at 0 h was taken as 100%.

was about 12 h. However, DLC1N protein levels remained unchanged after treatment with CHX for 12 h (Fig. 3C). These data suggested that the N-terminus of DLC1 is more stable than either the full-length DLC1 or the C-terminus of DLC1. These putative PEST motifs located at the N-terminus of DLC1 seemed not to promote DLC1 instability.

3.4. Deletion of the PEST motifs decreases the stability of the N-terminus of DLC1

To clarify whether the five PEST motifs affects DLC1 stability, deletion mutagenesis was employed to delete the PEST motifs individually from DLC1N. The deletion mutants of DLC1 were named DLC1NΔP1 (lacking PEST1: aa 85–100), DLC1NΔP2 (lacking PEST2: aa 169–180), DLC1NΔP3 (lacking PEST3: aa 295–324), DLC1NΔP4 (lacking PEST4: aa 489–511), and DLC1NP5 (lacking PEST5: aa 521–546) (Fig. 4A). These plasmids were transiently transfected into HEK293 cells treated with MG132 or untreated. The levels of all the DLC1N mutants except DLC1NP2 were significantly increased by MG132 (Fig. 4B). Next, the half-lives of these mutants were measured after treatment with CHX. Compared with DLC1N, four of the five mutants exhibited shorter half-lives. In particular, DLC1NΔP5 was rapidly degraded with a half-life of less than 4 h, whereas DLC1NP2 showed a turnover rate similar to that of DLC1N (Fig. 4C and D). These data indicate that four of the five putative PEST motifs may play an unexpected role in enhancing the stability of DLC1.

4. Discussion

DLC1 functions as a regulator of actin cytoskeletal remodeling and is involved in various biological processes such as cell migration and proliferation. Loss of DLC1 has been shown in a variety of human cancers. Restoration of DLC1 in DLC1 null cell lines was previously shown to suppress cell growth and reduce tumorigenicity in nude mice [18,19]. These studies support a tumor-suppressor role of DLC1. However, the mechanisms involved in the regulation of DLC1 protein levels have remained unknown.

In this study, we demonstrated that DLC1 is an unstable protein that is rapidly degraded by the 26S proteasome in human hepatocellular carcinoma Hep3B cells. The protein levels of endogenous DLC1 were significantly higher in HEK293 and Hep3B cells after treatment with the proteasome inhibitor MG132. The protein levels of exogenous DLC1 were also increased by inhibition of the 26S proteasome, suggesting that both endogenous and exogenous DLC1 proteins are degraded by the 26S proteasome. Furthermore, treatment of Hep3B cells or DLC1-transfected HEK293 cells with the protein synthesis inhibitor CHX resulted in rapid down-regulation of DLC1 protein levels and revealed the relatively short half-life of DLC1. However, pretreatment of Hep3B cells with MG132 led to dramatic stabilization of DLC1. These data suggest that the instability of DLC1 protein is attributable to degradation by the 26S proteasome. Because DLC1 functions as a negative regulator of Rho GTPase protein, it is possible that inactivation of DLC1 by proteasome-mediated degradation would result in rapid activation of Rho protein. Like DLC1, some GAP proteins have previously been reported to be regulated by the 26S proteasome. The RasGAP neurofibromin has been observed to be degraded by the 26S proteasome upon treatment of cells with various growth factors [20]. Moreover, the RhoGAP p190-A has also been shown to undergo targeted destruction via the 26S proteasome [21]. Therefore, our

study reveals a new mechanism of DLC1 regulation at the protein level by the 26S proteasome.

PEST motifs are often found in proteins with short half-lives, and they are proposed to play a role in protein turnover. In the present study, we identified five putative PEST motifs located in the N-terminus of DLC1. Four of the five PEST motifs are conserved among species with high scores. Surprisingly, we found that the N-terminus of DLC1 appeared to be stable, and individual deletion of four of the five PEST motifs led to decreased stability and shortened half-life of the N-terminus of DLC1. In particular, PEST5 (aa 521–546) deletion resulted in rapid turnover of the N-terminus of DLC1, leading to a half-life shorter than 4 h. These data indicate that four of the five PEST motifs described in this study may enhance the stability of DLC1 protein by reducing protein degradation via the 26S proteasome. Consistent with our observations, the expression of the tumor suppressor PTEN was previously reported to decrease after deletion of its PEST motif [22]. One possible explanation for this is that the four PEST motifs or their adjacent sequences may serve as critical protein–protein interaction sites that stabilize DLC1. Consistent with this, it has been reported that a PEST domain of Fbx2, a brain-enriched F-box protein ubiquitin ligase subunit, can serve as a domain for protein–protein interaction to stabilize Fbx2 [23]. Therefore, further identification of the potential binding partner(s) of the PEST motifs of DLC1 is required to reveal the definite function of these PEST motifs. It is notable that the region where the five putative PEST motifs are located has been found to contain important binding sites for interactions of DLC1 with its binding partners [24,25].

In summary, we demonstrate that DLC1 is an unstable protein that is degraded by the 26S proteasome, and five putative PEST motifs located in the N-terminus of DLC1 seem to play an unexpected role in promoting DLC1's stability. Further investigation is required to unravel the molecular mechanisms governing DLC1 stability and to explore the potential contribution of DLC1's stability to its tumor-suppressive activity.

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