CRL4A-FBXW5–mediated degradation of DLC1 Rho GTPase-activating protein tumor suppressor promotes non-small cell lung cancer cell growth

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Edited* by Douglas R. Lowy, National Cancer Institute, Bethesda, MD, and approved September 5, 2013 (received for review April 4, 2013)

DLC1 **encodes a RhoA GTPase-activating protein and tumor suppressor lost in cancer by genomic deletion or epigenetic silencing and loss of *DLC1* gene transcription. We unexpectedly identified non-small cell lung cancer (NSCLC) cell lines and tumor tissue that expressed *DLC1* mRNA yet lacked DLC1 protein expression. We determined that DLC1 was ubiquitinated and degraded by cullin 4A–RING ubiquitin ligase (CRL4A) complex interaction with DDB1 and the FBXW5 substrate receptor. siRNA-mediated suppression of cullin 4A, DDB1, or FBXW5 expression restored DLC1 protein expression in NSCLC cell lines. FBXW5 suppression-induced DLC1 reexpression was associated with a reduction in the levels of activated RhoA-GTP and in RhoA effector signaling. Finally, FBXW5 suppression caused a DLC1-dependent decrease in NSCLC anchorage-dependent and -independent proliferation. In summary, we identify a posttranslational mechanism for loss of DLC1 and a linkage between CRL4A-FBXW5–associated oncogenesis and regulation of RhoA signaling.**

Rho-selective GTPase-activating protein | Rho GTPase-activating protein 7 | STARD12

Rho family small GTPases function as extracellular signal-regulated on-off switches that cycle between an active GTP-bound state and an inactive GDP-bound state. Of the 20 human Rho family GTPases, the best studied are RhoA, Rac1, and Cdc42 (1). Rho-selective guanine nucleotide exchange factors (RhoGEFs) promote GDP-GTP exchange and formation of active Rho-GTP, whereas Rho-selective GTPase-activating proteins (RhoGAPs) stimulate hydrolysis of the bound GTP to return the GTPase to its inactive Rho-GDP form (2, 3). Rho-GTP binds preferentially to its downstream effectors, stimulating a diversity of cytoplasmic signaling cascades that control actin organization, cell morphology and polarity, cell cycle progression and cell proliferation, cell survival and migration, and gene expression (4).

In light of their key role in regulating fundamental processes in cell behavior, it is not surprising that the aberrant activation of Rho family small GTPases contributes to cancer and other human disorders (5–8). However, in contrast to the Ras small GTPase, where direct mutational activation leads to insensitivity to inactivation by Ras-selective GTPase-activating proteins (RasGAPs), Rho GTPases are more commonly activated through indirect mechanisms (2, 3). In human cancers, persistent RhoGEF activation or loss of RhoGAP stimulation are common mechanisms leading to aberrant Rho activation. For example, we determined that the P-Rex1 RhoGEF was up-regulated transcriptionally in melanoma through persistent activation of the ERK mitogen-activated protein kinase pathway and the related P-Rex2 isoform was found mutationally activated in melanoma (9, 10).

With regard to RhoGAPs, one of the most frequent and common mechanisms involves loss of expression of Deleted in Liver Cancer 1 (*DLC1*) in liver, breast, lung, ovarian, kidney, colon, stomach, prostate, and other cancers (3, 11, 12). *DLC1* encodes a GAP primarily for RhoA and related isoforms. Initially discovered as a gene lost in liver cancer by genomic deletion (13), subsequent studies found that the frequency of *DLC1* genomic deletion was comparable to the frequency seen with the*TP53* tumor suppressor gene in lung, colon, breast, and other cancers (14). Other studies also identified loss of *DLC1* mRNA expression through promoter methylation rather than genomic deletion in a wide variety of human cancers (15–21). For example, loss of the *DLC1* mRNA expression was found in primary non-small cell lung cancer (NSCLC) tumors and cell lines, due to aberrant DNA methylation rather than genomic deletion (20). Ectopic reexpression of DLC1 impaired growth, supporting a tumor suppressor role in lung cancer (20, 22).

In our evaluation of DLC1 function in NSCLC, we identified a subset of NSCLC patient tumors and cell lines that retained *DLC1* mRNA but surprisingly not protein expression, prompting our speculation that DLC1 loss in cancer may also occur posttranslationally. We determined that DLC1 protein loss was mediated by ubiquitination and proteasome degradation. We then searched for the E3 ligase involved and we identified and established a role for a cullin 4A–RING ubiquitin ligase (CRL4A) complex interaction with the FBXW5 substrate receptor in DLC1 protein loss. Suppression of FBXW5 expression restored DLC1 protein expression, resulting in suppression of RhoA activity and effector signaling, causing DLC1-dependent impairment in NSCLC growth. Our studies establish a posttranslational mechanism of DLC1 loss important for NSCLC biology and define a link between CRL4 and regulation of Rho GTPase signaling.

Significance

The *DLC1* tumor suppressor gene is commonly lost in cancer by genomic deletion or epigenetic silencing, leading to loss of gene transcription. *DLC1* encodes a GTPase-activating protein for the RhoA small GTPase, and DLC1 loss of expression results in aberrant RhoA activation and signaling. Unexpectedly, we found that a subset of non-small cell lung cancer patient tumors and cell lines retained *DLC1* mRNA but not protein expression. We determined that the CUL4A–DDB1–FBXW5 E3 ubiquitin ligase complex is responsible for loss of DLC1 protein expression. Suppression of FBXW5 function restored DLC1-dependent lung cancer cell growth suppression. Our observations identify a mechanism for posttranslational loss of DLC1 function in cancer and substrate for CRL4A-FBXW5–driven cancer growth.

Author contributions: T.Y.K., S.J., and Y.X. designed research; T.Y.K., T.G.W., J.R.L., G.J.W., and N.L.T. performed research; S.J. and Y.X. contributed new reagents/analytic tools; T.Y.K., T.G.W., J.R.L., G.J.W., and N.L.T. analyzed data; and T.Y.K., T.G.W., Y.-J.B., and C.D. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1306358110/-/DCSupplemental.

Results

DLC1 Protein Is Lost in DLC1 mRNA-Positive NSCLC Cell Lines and Tumor Tissue. A previous study found loss of *DLC1* transcription in lung tumors, but no analyses of DLC1 protein expression or association with lung subtype, oncogene mutation, or clinical parameters was determined (20). To address these issues, we first used Oncomine (www.oncomine.org) analyses of available datasets that revealed a reduction in *DLC1* mRNA expression in lung tumors compared with normal lung in the majority of datasets, for example, with a reduction seen in all lung cancer types from gene expression analysis of 186 human lung carcinomas (23) (Fig. S1). We next evaluated DLC1 expression by immunohistochemistry (IHC) staining of a lung tumor tissue microarray using a DLC1 antibody that we validated for IHC detection of DLC1 protein expression (Fig. S2). We found 65% of lung adenocarcinomas (n = 106) and 79% of squamous cell lung carcinomas (n = 91) with lost or reduced DLC1 expression (Fig. 1*A*). Finally, no significant correlation between DLC1 protein loss and patient survival, or mutant *KRAS* or *EGFR* mutation status, was observed (Table S1).

We previously determined that DLC1 protein was lost in six of nine NSCLC cell lines (22). To assess the basis for loss of DLC1 protein expression, we used RT-PCR analysis to determine whether loss of *DLC1* mRNA expression correlated with loss of protein expression. Unexpectedly, we discovered that three NSCLC cell lines (A549, H23, and SW900) that lacked detectable DLC1 protein nevertheless expressed *DLC1* transcripts (Fig. 1 *B* and *C*). To determine whether this situation was present in primary patient tumors, nine tumors that were negative by IHC were evaluated by real-time quantitative reverse transcription–PCR (qRT-PCR). Two patient tumors showed *DLC1* transcript levels comparable to or higher than a tumor that expressed high DLC1 protein (Fig. 1*D*). Thus, a similar frequency of protein-negative, transcript-positive NSCLC cell lines (3 of 10) and tumors (2 of 9) was seen.

We therefore speculated that the undetectable DLC1 expression in these cells may occur by proteasome-mediated protein degradation. Consistent with this possibility, we found that treatment with the MG132 proteasome inhibitor resulted in DLC1 accumulation in all three cell lines (Fig. 1*E*, *Upper*) and increased DLC1 level in a dose-dependent manner in SW900 cells (Fig. 1*E*, *Lower*). Because proteasomal protein degradation is mediated by covalently conjugating polyubiquitin chains to target proteins, we next investigated whether DLC1 is polyubiquitinated. Using an in vivo ubiquitination assay, where HA-tagged DLC1 and FLAG-tagged ubiquitin were ectopically coexpressed, we observed that DLC1 was polyubiquitinated upon MG132 treatment (Fig. 1*F*). Together, these results show that ubiquitin-mediated protein degradation contributes to the loss of DLC1 expression in multiple NSCLC cell lines.

CUL4A and DDB1 Complex with DLC1 and Regulate DLC1 Protein Stability. The cullin–RING ligases (CRLs) constitute the largest E3 ligase family and they target a wide array of substrates for

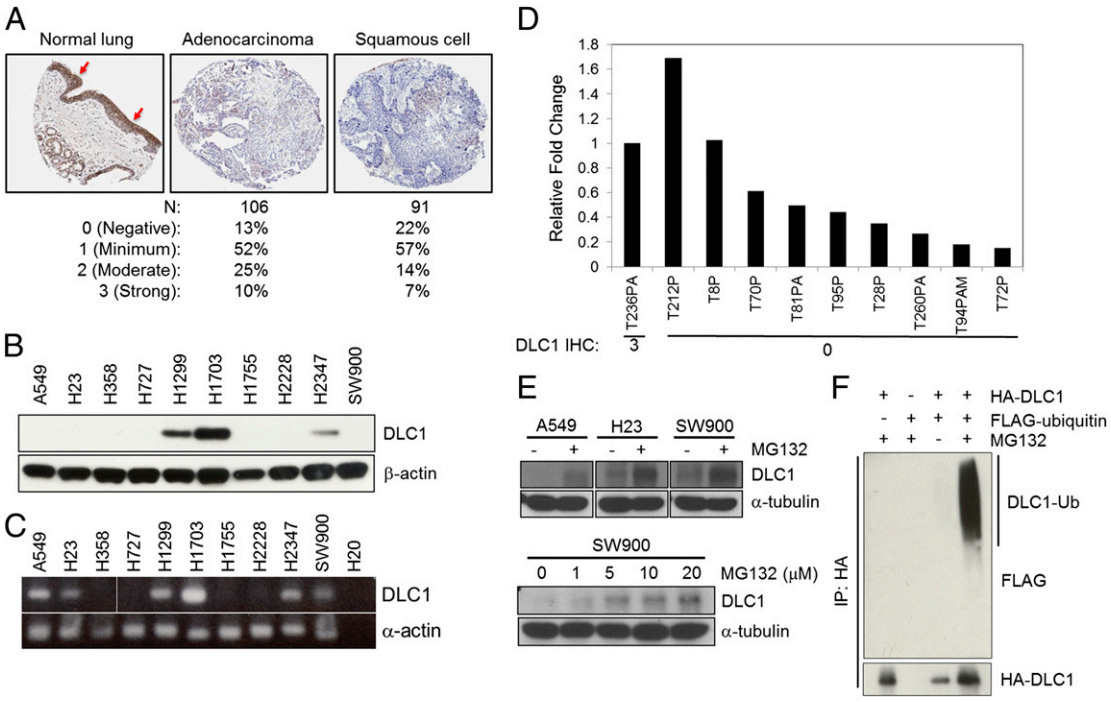

Fig. 1. DLC1 protein is lost in *DLC1* mRNA-positive NSCLC cell lines and tumor tissue, which is mediated by the ubiquitin–proteasome pathway. (*A*) Immunohistochemical staining analyses of a lung tumor tissue microarray. Shown are representative DLC1 staining of normal and lung tumor tissue. The summary of IHC scoring distribution of 106 lung adenocarcinomas and 91 squamous cell lung carcinomas is shown. The scoring as determined by a board-certified pathologist for DLC1 comprised staining intensity and extensiveness with the following: 0, negative; 1, weak; 2, moderate; 3, strong. (*B*) DLC1 expression was determined in 10 NSCLC cell lines by immunoblot analysis with anti-DLC1 antibody. Blot analysis for β-actin was done to verify equivalent total protein loading. (*C*) RT-PCR analysis of *DLC1* mRNA expression in the NSCLC cell lines. Analysis of β-actin mRNA expression was done to verify equivalent efficiency of cDNA synthesis. (*D*) qRT-PCR analyses of *DLC1* mRNA expression in NSCLC patient tumors. Nine NSCLC tumors that were scored as negative (0) by IHC analysis were evaluated for *DLC1* mRNA expression. One NSCLC tumor with strong staining (3) was used as a control for the mRNA level for tumors with high DLC1 protein expression. Analysis of β-actin mRNA expression was used as an endogenous control. (*E*) DLC1-negative/*DLC1* mRNA-positive NSCLC cells (A549, H23, and SW900) were treated with vehicle (DMSO) or 10 μM MG132 for 12 h, and SW900 cells were treated with indicated concentration of MG132 for 12 h. Total cell lysates were subjected to immunoblot analysis with anti-DLC1 and anti–α-tubulin antibodies. (*F*) HA-tagged DLC1 and FLAG-tagged ubiquitin were coexpressed in HEK293 cells, followed by treatment with either DMSO or 10 μM MG132 for 6 h. DLC1 was immunoprecipitated with anti-HA antibody and immunoblot analysis was done with anti-FLAG antibody to detect addition of FLAG-ubiquitin to DLC1. DLC1 expression from total cell lysates was detected with anti-HA antibody (*Lower*).

degradation (24–26). The seven cullin proteins (CUL1, 2, 3, 4A, 4B, 5, and 7) can associate with a different family of substrate receptors, potentially forming as many as 300–500 distinct CRLs. To determine whether cullin proteins may mediate DLC1 degradation, we carried out coimmunoprecipitation analysis with a panel of FLAG-tagged dominant-negative forms of each cullin protein that retain sequences for interaction with their substrates but not with Rbx-1 and E2 conjugating protein. We found that GFP- or HA-tagged DLC1 coimmunoprecipitated strongly with CUL4A, and to a lesser degree, with CUL1 (Fig. 2*A* and Fig. S3*A*). This result suggested that CUL4A-containing CRLs are the main E3 ubiquitin ligases for DLC1 degradation. Interestingly, DLC1 failed to bind to CUL4B, a highly related paralog of CUL4A (83% amino acid sequence identity). In most of cases, both CUL4A and CUL4B target the same substrates, but some target proteins are specific to CUL4A (p27kip1 and p53) (27) or CUL4B (WDR5) (28). A different subcellular localization may determine CUL4 paralog substrate specificity. Whereas CUL4B is predominantly nuclear localized, CUL4A is cytoplasmic, and may therefore target the cytoplasmic and focal adhesion-associated DLC1 (29).

CUL4 associates with DDB1, a linker protein that recruits DDB1-binding and WD40 repeat (DWD box; also known as DCAF or CDW) proteins (~90 human members) to form a functional E3 ubiquitin ligase complex (30). To examine whether DLC1 binds CUL4A–DDB1 complex, we first carried out coimmunoprecipitation analyses with DLC1 and full-length or N-terminal truncation mutants (ΔN52 or ΔN100) of CUL4A that lack the DDB1 binding domain (31). DLC1 coimmunoprecipitated with full length but not with N-terminal truncated CUL4A (Fig. 2*B*), suggesting a requirement for DDB1 in the DLC1 interaction with CUL4A. We further observed the interaction between DLC1 and DDB1 by demonstrating that ectopically expressed DDB1 coimmunoprecipitated with ectopically expressed DLC1 (Fig. 2*C* and Fig. S3*B*). We also demonstrated that endogenous DLC1 was associated with immunoprecipitated endogenous DDB1 in NSCLC cells (Fig. 2*D*). To address the possibility that the CUL4A–DDB1 complex regulates DLC1 degradation, we examined DLC1 levels after siRNA suppression of endogenous CUL4A, CUL4B, or DDB1. Depletion of either CUL4A or DDB1 but not CUL4B markedly increased DLC1 levels in NSCLC cells (Fig. 2*E*). These data indicate that the CUL4A–DDB1 E3 ligase can complex with and promote DLC1 degradation.

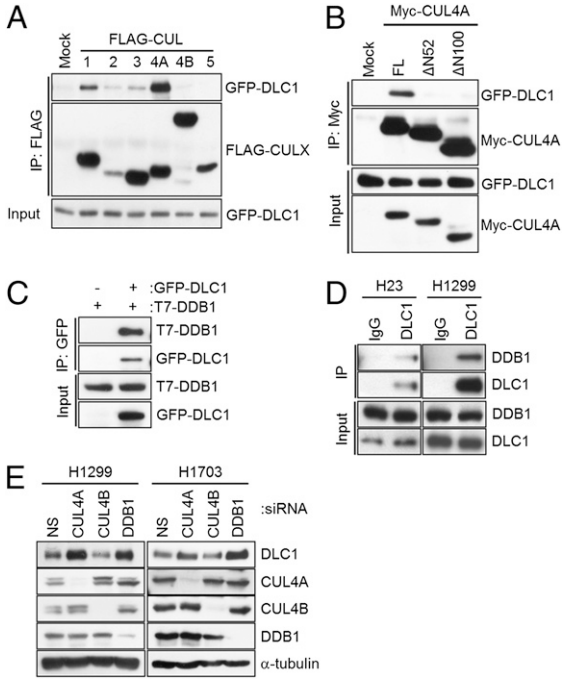

Fig. 2. CUL4A–DDB1 complex interacts with DLC1 and regulates DLC1 stability. (*A*) HEK293 cells were cotransfected with plasmids expressing GFP-tagged DLC1 and FLAG-tagged truncation mutants of the indicated cullin proteins for 24 h, followed by treatment with 10 μM MG132 for 4 h. Cell lysates and anti-FLAG immunoprecipitates were subject to immunoblot analysis with the indicated antibodies to determine FLAG-CUL proteins, which interact with DLC1. (*B* and *C*) HEK293 cells were cotransfected with plasmids expressing GFP-tagged DLC1 and Myc-tagged full-length or N-terminal truncated mutants of CUL4A (*B*) or with plasmids expressing GFP-tagged DLC1 and T7 epitope-tagged DDB1 (*C*) for 24 h, followed by treatment of 10 μM MG132 for an additional 4 h. Total cell lysates and anti-Myc (*B*) or -GFP (*C*) immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies to determine the residues in CUL4A for DLC1 binding (*B*) or DLC1 interacts with DDB1 (*C*). (*D*) H23 and H1299 cells were treated with 10 μM MG132 for 6 h. Immunoprecipitates with normal IgG or DLC1 antibodies were subjected to immunoblot analysis with the indicated antibodies to detect an endogenous DLC1 and DDB1 association. (*E*) H1299 and H1703 cells were transfected with siRNAs targeting the indicated genes for 2 d. Immunoblot analysis was done with anti-DLC1 antibody to determine DLC1 level, with anti-CUL4A, CUL4B, or DDB1 antibodies to examine the knockdown of each protein, or with anti–α-tubulin to verify equivalent total protein loading.

FBXW5 Promotes DLC1 Protein Degradation. To identify the DWD protein substrate receptor involved in DLC1 degradation, we evaluated five well-characterized DWD proteins: CDT2, CSA, DDB2, FBXW5, and VprBP. Transient overexpression of FBXW5 but not the other DWD proteins substantially decreased the levels of coexpressed DLC1 (Fig. 3*A*). Consistent with this result, DLC1 coprecipitated only with FBXW5 (Fig. 3*B*). We further demonstrated the interaction between ectopically expressed FBXW5 with endogenous DLC1 (Fig. 3*C*) and between endogenous FBXW5 and DLC1 (Fig. 3*D*), suggesting that FBXW5 is a substrate receptor for CUL4A-DDB1–mediated DLC1 degradation.

Human FBXW5 is composed of two recognized domains: an N-terminal F-box motif and seven WD40 repeats. To determine the FBXW5 domains required for interaction with DLC1, FBXW5 deletion mutants (ΔF and ΔWD with deletion of the F-box motif and WD40 domain, respectively) were used for a coimmunoprecipitation assay. Full length or ΔF but not ΔWD coimmunoprecipitated with DLC1, suggesting that FBXW5 binds to DLC1 via the WD40 repeats (Fig. 3*E*). To evaluate whether FBXW5 regulates DLC1 degradation, we evaluated four lentiviral shRNA constructs expressing short hairpin RNA (shRNA) targeting FBXW5 and identified one shRNA with effective knockdown of FBXW5 (Fig. S4). After FBXW5 depletion, we determined that DLC1 polyubiquitination was decreased significantly (Fig. 3*F*) and the level of endogenous DLC1 was greatly increased in H1299 cells (Fig. 3*G*, *Left*). Knockdown of FBXW5 by using two nonoverlapping siRNAs also led to an increase in DLC1 level (Fig. S5*A*), suggesting that FBXW5 facilitates CUL4A–DDB1 degradation of DLC1. Furthermore, we observed that siRNA-mediated knockdown of FBXW5 increases the half-life of DLC1 (Fig. S5*B*). Next, we explored the possibility whether FBXW5 is responsible for the diminished levels of DLC1 protein in *DLC1* mRNA-positive cells (Fig. 1 *B* and *C*), and we found that silencing *FBXW5* was associated with elevated DLC1 expression in all three NSCLC cell lines (Fig. 3*G*, *Right*). FBXW5 appears to be the predominant E3 ligase for DLC1 in H23 cells, because the level of DLC1 in FBXW5-depleted cells was not further increased by the additional treatment with MG132 (Fig. 3*H*). These data demonstrate that FBXW5 is the receptor protein associated with CUL4A–DDB1 to promote DLC1 degradation in DLC1 protein-deficient, *DLC1* mRNA-positive NSCLC cells. Finally, because protein level and stability of DLC1 was increased in DLC1 protein-positive cells by suppression of FBXW5 expression, in some NSCLC cells both transcriptional and post-translational mechanisms of regulation determine the steady-state levels of DLC1.

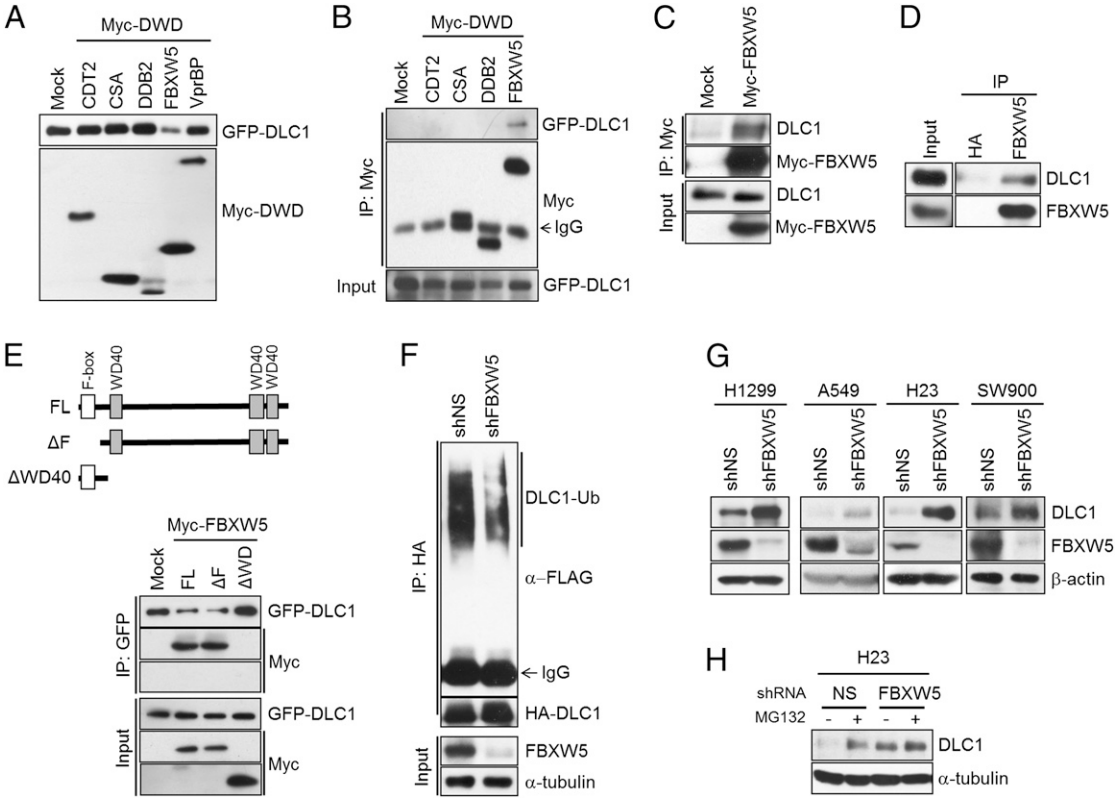

Fig. 3. FBXW5 binds to DLC1 for promoting its degradation. (*A* and *B*) HEK293 cells were cotransfected with plasmids expressing GFP-tagged DLC1 and indicated Myc-tagged DWD box-containing proteins for 24 h (*A*) or followed by treatment with 10 μM MG132 for 4 h (*B*). Cell lysates or anti-Myc immunoprecipitates were analyzed by immunoblot analysis with the indicated antibodies to determine a DWD protein that destabilizes DLC1 (*A*) or a DWD protein that binds to DLC1 (*B*). (*C*) H1299 cells transfected with plasmids expressing Myc-tagged FBXW5 for 24 h were treated with 10 μM MG132 for 4 h. Cell lysates and anti-Myc immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies to detect a Myc–FBXW5 interaction with endogenous DLC1. (*D*) Equal amount of cell lysates from MG132-treated H1299 cells (10 μM, 6 h) were immunoprecipitated with anti-HA or anti-FBXW5 antibodies, followed by immunoblot analysis with the indicated antibodies to detect an endogenous DLC1 and FBXW5 interaction. (*E*) HEK293 cells were cotransfected with plasmids expressing GFP-tagged DLC1 and Myc-tagged full length, F-box (ΔF), or WD40-domain (ΔWD) truncation mutants of FBXW5 for 24 h, followed by treatment with 10 μM MG132 for 4 h. Cell lysates and anti-GFP immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies to determine FBXW5 residues responsible for binding to DLC1. (*F*) HEK293 cells were transfected with HA-tagged DLC1 and FLAG-tagged ubiquitin for 24 h and infected with lentivirus expressing shRNA targeting FBXW5 or nonspecific shRNA (NS) for 2 d, followed by the treatment with 10 μM MG132 for 6 h. Anti-HA immunoprecipitates were subjected to immunoblot analysis with anti-FLAG antibody to detect DLC1 polyubiquitination. Cell lysates were analyzed with anti-FBXW5 and α-tubulin antibodies to determine the knockdown of FBXW5 protein and equivalent protein loading, respectively. (*G*) NSCLC cells were infected with lentivirus expressing shRNA targeting FBXW5 or NS shRNA, and selected with puromycin for 2 d, and then subjected to immunoblot analysis with anti-DLC1 antibody to determine DLC1 level, anti-FBXW5 antibody to examine knockdown of FBXW5, or anti–α-actin antibody to determine equivalent protein loading. (*H*) H23 cells which stably expressing NS or FBXW5 shRNA were treated with treated with DMSO or 10 μM MG132 for 6 h. Cell lysates were analyzed with anti-DLC1 or α-tubulin antibodies to determine DLC1 level and equivalent protein loading, respectively.

FBXW5 Depletion-Mediated Restoration of DLC1 Inhibits Rho GTPase Signaling and NSCLC Cell Proliferation. Because DLC1 functions as a RhoGAP activity, we reasoned that DLC1 accumulation in FBXW5-depleted cells might cause a reduction in RhoA activation and signaling. To this end, we examined the effects of DLC1 restoration on RhoA downstream effector signaling. The ROCK serine/threonine kinases are key effectors of RhoA-dependent growth transformation (32) and DLC1 has been shown to be a negative regulator of ROCK activation and signaling (33). RhoA activation of ROCK leads to phosphorylation of cofilin. Stable FBXW5 knockdown in H23 cells was associated with greatly reduced levels of phosphorylated cofilin (Fig. 4*A*). To investigate whether the reduced cofillin phosphorylation was mediated by DLC1 RhoGAP activity, we overexpressed wild-type or GAP-dead mutant (R718E) of DLC1 in H23 cells and measured RhoA-GTP levels by Rho-binding domain (RBD) pull-down assay and cofilin phosphorylation level. Overexpression of wild-type but not GAP-dead DLC1 reduced RhoA-GTP levels and cofilin phosphorylation level (Fig. 4*B*).

Because DLC1 can function as a tumor suppressor in NSCLC, we next determine whether the DLC1 restoration caused by FBXW5 depletion might have effects on NSCLC cell growth. We observed that stable knockdown of FBXW5 and the subsequent increase in DLC1 in H23 cells was associated with a significant impairment in cell proliferation (Fig. 4 *C* and *D*). FBXW5 has also been shown to act as a substrate receptor for the SCF (SKP–Cullin–F box) ubiquitin ligase complex, targeting HsSAS-6 or Eps8 for ubiquitination and degradation, to regulate centrosome duplication or mitotic progression, respectively (34, 35). Thus, FBXW5 may have functions independent of DLC1 in NSCLC. However, we found that the growth-inhibitory activity seen upon suppression of FBXW5 was significantly reversed by concurrent suppression of DLC1 (Fig. 4*C*). We also observed similar results for NSCLC cell line anchorage-independent growth in soft agar (Fig. 4*E*). Suppression of FBXW5 caused a ∼90% reduction in soft agar colony formation, whereas concurrent suppression of DLC1 restored colony formation to ∼90% the level seen with control nonspecific shRNA treatment. These results suggest that DLC1 is a significant substrate for FBXW5-dependent NSCLC tumor cell proliferation. When taken together, these observations suggest that FBXW5-dependent DLC1 degradation contributes to NSCLC growth.

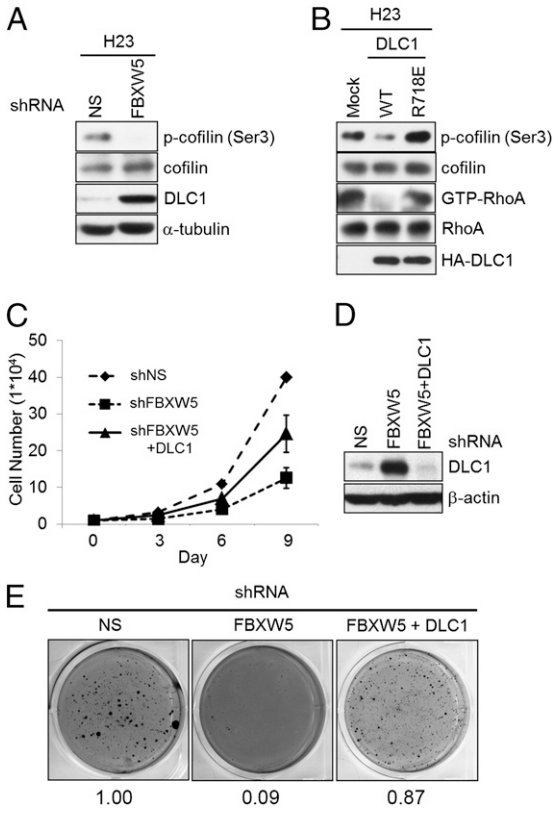

Fig. 4. FBXW5 depletion-associated DLC1 increase inhibits RhoA activation and signaling and NSCLC cell proliferation. (*A*) Cell lysates from H23 cells stably expressing NS or FBXW5 shRNA were subjected to immunoblot analysis with the indicated antibodies to determine whether DLC1 stabilization caused by FBXW5 suppression is associated with a decrease in the level of phosphorylated cofilin. (*B*) Cell lysates from H23 cells stably expressing HA-tagged wild-type DLC1 (WT) or mutant DLC1 (R718E; GAP-dead) were assessed by pull-down analysis with GST–Rhotekin–RBD. Precipitated and total cell lysates were immunoblotted with anti-RhoA antibody to detect RhoA-GTP and total RhoA, respectively. Cell lysates from the stable cell lines were analyzed with anti-total or –phospho-cofilin (serine 3) to determine whether cofilin phosphorylation level is associated with DLC1 RhoGAP activity, or with anti-HA antibody to determine DLC1 expression. (*C*) H23 cells stably expressing shRNA for GFP or DLC1 were further infected with NS or FBXW5 shRNA lentiviral particles and selected with puromycin. Cell proliferation was monitored by quantitation of cell number every 3 d. Data shown are the average of triplicate wells for each time point. (*D*) Cell lysates from the H23 stable cells in *C* were analyzed with anti-DLC1 antibody to monitor DLC1 protein levels and anti–α-tubulin antibody to verify equivalent protein. (*E*) Colony formation of the H23 stable cells in soft agar was monitored and quantitated. Colony formation was then normalized to NS shRNA (1.00), relative to colony formation seen with shRNA silencing of FBXW5 (0.09) or combination silencing of FBXW5 together with DLC1 (0.87).

Discussion

The loss of expression of DLC1 comprises one of the most widely observed mechanisms by which Rho GTPases become aberrantly activated in cancer (12). Loss of *DLC1* gene transcription has been attributed to genomic deletion at a frequency comparable with that seen with the *TP53* tumor suppressor gene in lung, colon, breast, and other cancers (14). Epigenetic gene silencing including promoter methylation has also been described to be a common mechanism of *DLC1* gene expression loss in cancer. Our study describes a ubiquitination–proteasome degradation mechanism through a CUL4A–DDB1–FBXW5 complex that accounts for the loss of DLC1 protein in a significant subset of NSCLCs. We also determined that this loss of DLC1 expression contributes to aberrant RhoA activation and signaling, promoting NSCLC growth. We previously determined that FBXW5 facilitated CUL4–DDB1 degradation of the Tsc2 tumor suppression. Thus, our studies identify a second target for this E3 ligase complex, both tumor suppressors, and additionally establish a link between CRL4 and regulation of RhoA and the actin cytoskeleton.

A previous study found a significant decrease or absence of *DLC1* mRNA expression was found in 95% of primary NSCLC (20 of 21) and 58% of NSCLC cell lines (11 of 19) (20). Because no protein expression analyses were done in patient tumors or evaluated in specific lung cancer subtypes, we performed IHC analyses of DLC1 protein expression in a lung tumor tissue microarray. We found that 65% of lung adenocarcinomas and 79% of squamous cell lung carcinomas exhibited lost or reduced DLC1 expression, indicating that DLC1 protein expression is reduced in a majority of NSCLC. Compared with the frequency of alterations in other genes in NSCLC (e.g., *TP53*, 26%; *EGFR*, 23%; *KRAS*, 16%; *CDKN2A*, 15% mutation) (COSMIC), DLC1 loss represents one of the most frequent genetic alterations found in this cancer.

A previous study showed that DLC1 protein is regulated by the 26S proteasome in a human liver cancer cell line (36), but no mechanism for DLC1 protein degradation nor biological consequences was elucidated. Here, we show that the CUL4A–DDB1–FBXW5 complex regulates DLC1 degradation in NSCLCs. We previously identified FBXW5 as the receptor protein for CUL4A/B-DDB1–dependent degradation of the Tsc2/tuberin tumor suppressor (37), a GAP for the Rheb small GTPase.

To date, ∼30 substrate proteins are known to be targeted by CRL4 complexes and most substrates are associated with chromatin formation, DNA replication, and DNA damage repair (24). Only two substrates have been reported to be related to cancer, as demonstrated by the degradation of Merlin and TSC2 tumor suppressor by VprBP (38) and FBXW5 (37), respectively. Therefore, we here add another tumor suppressor, DLC1, which is regulated by CRL4 complexes, implicating that CRL4 has cancer-related roles in addition to DNA-related functions.

In addition to CUL4–DDB1, FBXW5 has also been shown to act as a substrate receptor for the CUL1–SKP1 E3 ubiquitin ligase complex, targeting HsSAS-6 or Eps8 for ubiquitination and degradation, to regulate centrosome duplication or mitotic progression, respectively (34, 35). In contrast, FBXW5 through CUL4–DDB1 promotes sumoylation rather than ubiquitination of the Myb transcription factor, to alter its nuclear localization and transcriptional activity (39). Thus, FBXW5 may have functions independent of DLC1 in NSCLC. However, because we found that the growth-inhibitory activity seen upon suppression of FBXW5 were significantly reversed by concurrent suppression of DLC1, in NSCLC cells, a predominant function of FBXW5 is the targeted degradation of DLC1.

Interestingly, we did not observe increased TSC2 protein when FBXW5 was depleted in NSCLC cell lines, suggesting context-dependent roles for FBXW5. The stability of another RhoGAP protein, p190RhoGAP, was not changed by FBXW5 depletion, indicating the substrate specificity of FBXW5 (Fig. S5*C*). The molecular mechanisms that regulate DLC1 protein stability among NSCLC cell lines are not clear at present. It was reported that the Polo-like kinase 4 (PLK4) inactivates FBXW5 by phosphorylation on Ser151 residue (34) and PLK4 is down-regulated in cancer (40). Therefore, an abnormal activation of FBXW5 by PLK4 down-regulation could be a possible mechanism for a more rigorous degradation of DLC1 protein in DLC1 protein-negative NSCLCs. However, we did not observe any significant difference in PLK4 protein levels in DLC1 mRNA/protein-positive cells and DLC1 mRNA positive/protein-negative cells (Fig. S6). We also compared the protein level of each component of the E3 ubiquitin ligase complex to determine whether their expression correlated with loss of DLC1 protein. We did not detect any notable differences in CUL4A and DDB1 protein levels between two groups, and furthermore, the level of FBXW5 was less in DLC1 mRNA-positive/protein-negative cell lines (Fig. S6). These results suggest that the modification of DLC1 rather than differential expression of CRL4A

components is likely the basis for DLC1 ubiquitination–degradation in NSCLC. The DLC1 functional residues reported thus far were not related with DLC1 stability (Fig. S7). An important future direction will be the identification of DLC1 residue(s) critical for regulating DLC1 stability. Because binding to F-box proteins commonly requires phosphorylation of the substrate, DLC1 phosphorylation will be a key determinant for stimulating DLC1 degradation. Hence, pharmacologic approaches that modulate protein kinase function to prevent DLC1 degradation may be a viable therapeutic approach to restore DLC1 tumor suppressor function in lung cancer.

In summary, we identified a mechanism in which DLC1 tumor suppressor function is lost by protein degradation by the CRL4A–FBXW5 ubiquitin ligase complex. We also demonstrated that DLC1 restoration is partially responsible for the FBXW5 knockdown-mediated suppression of NSCLC cell growth and suggested that RhoA signaling through RhoA–ROCK–cofilin pathway via RhoGAP activity of DLC1 might be a cellular mechanism for DLC1 restoration effects on NSCLC cell growth. Our studies identify a target for CRL4A–FBXW5 that provides a link with Rho GTPase regulation. With substantial evidence for Rho GTPases in cancer (41), our identification of DLC1 as a substrate for CRL4A–FBXW5 further diversifies the cellular processes that when deregulated can facilitate Rho GTPase-driven cancer cell growth.

Materials and Methods

Immunohistochemical evaluation of DLC1 protein expression was performed on a lung tumor tissue microarray as described previously (42). Specimen blocks chosen for the tissue microarray met the criteria of nonnecrotic, nonirradiated, or chemo-treated lung cancer tissue. NSCLC subtypes included adenocarcinoma (n = 106) and squamous cell carcinoma (n = 91). Tumor tissue staining for DLC1 was performed on a BondMax autostainer (Leica Microsystems) using a purified mouse monoclonal antibody from BD Biosciences. Full methods for cell culture, immunohistochemistry, constructs, siRNA and transfection, immunoblotting and immunoprecipitation, qRT-PCR, cell proliferation and colony formation, in vivo ubiquitination assays, and RhoA activation assay are available in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Nishar P. Malik for anti-FBXW5 antibody and Michelle Mathews in the University of North Carolina at Chapel Hill Translational Pathology Laboratory (TPL) for expert technical assistance in validation of the anti-DLC1 antibody for immunohistochemistry analyses of formalin-fixed, paraffin-embedded tissues. We thank the patients and clinical staff, particularly Jeffrey Allen, MD. The TPL is supported, in part, by National Cancer Institute Grant 3P30CA016086, National Institute of Environmental Health Sciences Grant 3P30ES010126, Department of Defense Grant W81XWH-09-2-0042, and the University of North Carolina University Cancer Research Fund. Financial support was provided by National Cancer Institute Grants CA129610 (to C.D.) and CA130940 (to N.L.T.) and a Susan Komen postdoctoral fellowship (to T.Y.K.).

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