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The c-Cbl-mediated non-canonical TGFBR2 neddylation drives BRAF V600E melanoma metastasis

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Abstract

Background

Metastatic dissemination is the main cause of death in patients with cutaneous melanoma, particularly those with the $BRAF^{V600E}$ subtype, in whom therapeutic resistance often limits durable disease control. Aberrant neddylation has been implicated in cancer progression. However, the contribution of non-canonical neddylation to melanoma metastasis remains poorly defined. The current study examined whether c-Cbl-mediated non-canonical neddylation stabilizes transforming growth factor-β receptor 2 (TGFBR2) and drives metastatic progression in $BRAF^{V600E}$ melanoma.

Methods

Data from The Cancer Genome Atlas were analyzed to assess the prognostic relevance of NEDD8 in melanoma. Gain-of-function and loss-of-function approaches were used to define the roles of NEDD8 and TGFBR2 in melanoma cell migration. Neddylation was pharmacologically inhibited using MLN4924 and TAS4464. Transcriptomic analyses, Smad-responsive luciferase assays, cycloheximide chase assays, and His-NEDD8 pulldown assays were conducted to characterize the link between neddylation and transforming growth factor-β (TGF-β) signaling. Site-directed mutagenesis and rescue experiments were used to identify functionally relevant neddylation sites on TGFBR2. In vivo significance was evaluated using xenograft tumor growth and orthotopic metastasis models.

Results

Elevated NEDD8 expression was associated with a poor prognosis, particularly in $BRAF^{V600E}$ melanoma, and correlated with enhanced migratory capacity in melanoma cells. Genetic depletion or pharmacologic inhibition of neddylation markedly suppressed melanoma cell migration. Mechanistically, neddylation blockade attenuated TGF-β/Smad signaling, reduced Smad-dependent transcription, accelerated TGFBR2 degradation, and diminished NEDD8 conjugation of TGFBR2. Functional studies revealed that TGFBR2 is required for melanoma cell migration, and rescue experiments indicated that the prometastatic activity of TGFBR2 depends on non-canonical neddylation at K556 and K567. In vivo, TGFBR2 depletion suppressed xenograft tumor growth, reduced tumor burden and Ki-67 expression, and abolished lymph node and brain metastasis in orthotopic models. Notably, c-Cbl was identified as the upstream E3 ligase required for this pathway, with c-Cbl silencing reducing TGFBR2 neddylation, impairing Smad2/3 activation, and suppressing melanoma cell motility.

Conclusions

In this study, we identified a novel c-Cbl–TGFBR2 non-canonical neddylation axis that stabilizes TGFBR2, sustains prometastatic TGF-β signaling, and drives $BRAF^{V600E}$ melanoma progression and metastasis. Targeting this posttranslational regulatory pathway may represent a promising therapeutic strategy for metastatic BRAF-mutant melanoma.

Introduction

Cutaneous melanoma is the most lethal form of skin cancer, with mortality primarily driven by metastatic dissemination [1]. Among the genomic mutations associated with cutaneous melanoma, $BRAF^{V600E}$ is the most prevalent oncogenic driver, occurring in approximately 50% of cases and leading to the constitutive activation of the MAPK pathway, which promotes tumor progression and therapeutic resistance [2]. Although the management of advanced melanoma has been transformed by MAPK pathway inhibitors and immune checkpoint inhibitors, durable disease control remains limited in many patients [3, 4]. In patients with cutaneous melanoma, clinical benefits are often compromised by the rapid emergence of acquired resistance to targeted therapy and primary resistance to immunotherapy [5], which frequently occurs in immunosuppressive tumor microenvironments [6]. These problems highlight a need to extend investigations beyond genomic alterations and to define the posttranslational regulatory networks that sustain metastatic progression in $BRAF^{V600E}$ melanoma.

Neddylation is a ubiquitin-like posttranslational modification in which NEDD8 is conjugated to target proteins through a sequential E1 (NEDD8-activating enzyme [NAE]), E2, and E3 enzymatic cascade. Although NEDD8 is canonically recognized for its role in activating cullin-RING ligases and promoting ubiquitin-mediated proteolysis, emerging evidence has established a non-canonical paradigm in which NEDD8 modifies noncullin substrates to regulate their stability, localization, and signaling output [7]. Aberrant activation of the neddylation pathway has been implicated in tumor progression across multiple malignancies [8, 9]. However, the specific contribution of non-canonical neddylation and the identity of its relevant downstream substrates to the metastatic cascade of $BRAF^{V600E}$ melanoma remain poorly defined.

Transforming growth factor-β (TGF-β) plays a context-dependent role in cancer, functioning as a tumor suppressor during the early stages of cancer but transforming into a potent prometastatic driver in advanced stages [10]. TGF-β signaling has been linked to invasive plasticity, mesenchymal-like transcriptional programs, and metastatic dissemination in melanoma [11, 12]. Because transforming growth factor-β receptor 2 (TGFBR2) is required for the initiation of TGF-β signaling, the mechanisms that regulate receptor stability and abundance are likely to influence the magnitude and persistence of downstream oncogenic responses. According to the literature, ubiquitin-dependent receptor turnover, including regulation by Smurf2, attenuates TGF-β signaling through receptor complex degradation [13, 14]. However, whether a distinct posttranslational modification such as neddylation can stabilize TGFBR2 and amplify its prometastatic signaling output in the aggressive biological setting of $BRAF^{V600E}$ melanoma remains unknown.

In this study, we examined whether c-Cbl-mediated non-canonical neddylation stabilizes TGFBR2 and contributes to metastatic progression in $BRAF^{V600E}$ melanoma. Specifically, we explored whether this posttranslational regulatory mechanism sustains TGF-β/Smad signaling, promotes melanoma cell motility, and drives metastatic colonization in vivo. This study was designed to define a previously unrecognized posttranslational regulatory axis with potential mechanistic and translational relevance in metastatic BRAF-mutant melanoma.

Materials and methods

Collection of melanoma samples and transcriptional profiles

Clinical and transcriptional profiling data of melanoma derived from The Cancer Genome Atlas (TCGA) were obtained from the UCSC Xena (University of California, Santa Cruz) website (http://xena.ucsc.edu/welcome-to-ucsc-xena/).

Cell culture

All melanoma cell lines were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). A375 and A2058 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin–glutamine (PSG) solution, and 1 mM sodium pyruvate. C32, MeWo, and RPMI-7951 cells were cultivated in minimum essential medium supplemented with 10% FBS, 1% PSG, 1 mM sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 1% nonessential amino acids. All culture reagents were purchased from Gibco (Grand Island, NY, USA). All melanoma cell lines used in this study were routinely tested for mycoplasma contamination and tested negative.

Plasmid construction and site-directed mutagenesis

Human NEDD8 (BC104201, #HG15620-NH) and TGFBR2 (NM_003242.5, #HG10358-UT) cDNA clone expression plasmids were purchased from Sino Biological (Beijing, China) and subjected to sticky-end polymerase chain reaction (PCR) [15] with two paired primers:

- For TGFBR2 subcloning into a pIRES2-EGFP vector
    - Paired primer #1 forward: TCGAGATGGGTCGGGGGCTGCTCAG; reverse: CCTATTTGGTAGT GTTTAGGGAGCCGTC
    - Paired primer #2 forward: GATGGGTCGGGGGCTGCTCAG; reverse: AATTCCTATTTGGTAGTGTTTAGGGAGCCGTC
- For NEDD8 subcloning into a pLAS/3w-puro vector
    - Paired primer #1 forward: CTAGCATGCTAATTAAAGTGAAGACGCTGACC; reverse: CTCACTGCCTAAGACCACCTCCTCC

    - Paired primer #2 forward: CATGCTAATTAA AGTGAAGACGCTGACC; reverse: AATTCTCACTGCCTAAGACCACCTCCTCC
- For TGFBR2 subcloning into a pLAS/3w-puro vector
    - Paired primer #1 forward: CTAGCATGGGTCGGGGGCTGCTCAG; reverse: CCTATTTGGTAGTGTTTAGGGAGCCGTC
    - Paired primer #2 forward: CATGGGTCGGGGGCTGCTCAG; reverse: AATTCCTATTTGGTAGTGTTTAGGGAGCCGTC

PCR products with cohesive XhoI/EcoRI and NheI/EcoRI restriction sites were subcloned into pIRES2-EGFP and pLAS/3w-puro vectors, respectively. Both constructs were used as templates for site-directed mutagenesis using PCR with a PfuUltra II Fusion High-Fidelity DNA Polymerase kit (Agilent Technologies, Santa Clara, CA, USA) and the following paired primers:

- For K556R
    - Forward: ccgtcttcaggaatcctctcctccgagcagc
    - Reverse: gctgctcggaggagaggattcctgaagacgg
- For K567R (pIRES2-EGFP)
    - Forward: cgactgcagaattcctatctggtag tgtttagggagc
    - Reverse: gctccctaaacactaccagataggaattctgcagtcg
- For K567R (pLAS/3w-puro)
    - Forward: caagatctagaattcctatctggtagtgtttagggagcc
    - Reverse: ggctccctaaacactaccagataggaattctagatcttg

DpnI endonuclease (New England Biolabs, Hitchin, Hertfordshire, UK) was used to digest the methylated parental DNA template in the PCR products. Mutation sites are underlined in the DNA sequences of paired primers. All constructs were validated by DNA sequencing to ensure sequence fidelity and correct orientation.

Lentivirus-driven gene overexpression and knockdown

Briefly, the lentiviral shuttle vector pLAS/3w-puro, with or without the NEDD8 or TGFBR2 coding gene, was packaged into lentiviral particles through collaboration with the National RNAi Core Facility Platform (Taipei, Taiwan). Lentiviral particles containing control nonsilencing short hairpin RNA (shRNA, oligo sequence: CCGGACACTCGAGCACTTTTTG), NEDD8 shRNA (target sequences: sh1_GCTCATAATGAGGCATCATAT; sh2_GAAAGGAGATTGAGATTGACA), TGFBR2 shRNA (target sequences: sh1_GACCTCAAGAGCTCCAATATC; sh2_GCAGTCATTCTCTGGGTATAT; sh3_CT CTAGGCTTTATCGTGTTTA), or c-Cbl shRNA (target sequences: sh1_TGATCTGACCTGCAATGATTA; sh2_GCCGATGTGAAATTAAAGGTA; sh3_CCAGTGAGTTGGGAGTTATTA) were obtained from the National RNAi Core Facility Platform. Cells (~50% confluence) cultivated on six-well plates with fresh medium

supplemented with 5 μg/mL polybrene (Santa Cruz Biotechnology, Dallas, TX, USA) were infected overnight with lentiviral particles at a multiplicity of infection of 2–10. The infected cells were further subjected to puromycin (10 μg/mL) selection for 24 h to establish stable clones with or without target gene overexpression or knockdown. Finally, the efficiency of target gene overexpression or knockdown was validated using Western blot analyses with specific antibodies against the target genes.

Western blot analysis

Different aliquots of cell lysates (20–100 μg) were separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. These membranes were immersed in blocking buffer (5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20) for 2 h at room temperature to minimize nonspecific binding. The membranes were then incubated with primary antibodies against NEDD8 (1:1000, #A1163; ABclonal, Woburn, MA, USA), phospho-Smad2/3 (1:1000, #AP1343; ABclonal), Smad2/3 (1:1000, #A18674; ABclonal), TGFBR2 (1:1000, #41896S; Cell Signaling Technology, Danvers, MA, USA), His-tag (1:1000, #2366S; Cell Signaling Technology), EGFP (1:1000, #A11351; ABclonal), Ki-67 (1:1000, #A20018; ABclonal), c-Cbl (1:1000, #A7881; ABclonal), or GAPDH (1:5000, #A19056; ABclonal) overnight at 4 °C. After extensive washing, the membranes were incubated with species-specific horseradish-peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, protein–antibody complexes were detected using an enhanced chemiluminescence system (Amersham Biosciences, GE Healthcare, Billerica, MA, USA).

Cell migration assay

Before transwell cultivation in a Boyden chamber (NeuroProbe, Gaithersburg, MD, USA), a polycarbonate membrane (8-μm pore size, 25 mm × 80 mm; NeuroProbe) was precoated with 10 μg human fibronectin (Sigma-Aldrich, St. Louis, MO, USA) on the side facing the lower chamber, which contained a conditioned medium supplemented with 10% FBS. Next, the tested melanoma cells (1.5×10^4) were seeded in the upper chamber containing 50 μL of serum-starved medium (0.1% FBS). After 8 h of incubation, the remaining cells on the upper side of the membrane were removed, and the cells on the bottom surface were fixed with 100% methanol, stained with 10% Giemsa solution (Merck, Darmstadt, Germany) for 1 h, and counted under a microscope. All experiments were performed in triplicate and were repeated three times.

Cycloheximide chase assay

A2058 and RPMI7951 cells were pretreated with 1 mM pevonedistat (MLN4924) for 2 h, followed by treatment with or without 10 mM cycloheximide for specific time intervals to inhibit newly synthesized proteins. After incubation, whole-cell lysates were collected and subjected to Western blot analysis with a TGFBR2-specific antibody.

Pulldown assay

Before any pulldown assays were conducted, all Sepharose beads were equilibrated in a commercial cell lysis buffer (Abcam, Cambridge, UK). Total proteins (1 mg) collected from melanoma cells with His-tag NEDD8 overexpression in the absence or presence of 1 mM MLN4924 for 2 h were precleared with uncoated Sepharose beads to remove nonspecific binding proteins. These precleared cell lysates were further incubated with Sepharose beads coated with nickel–nitrilotriacetic acid (Ni^{2+}-NTA) overnight at 4 °C with gentle rotation to capture His-tag NEDD8-incorporated proteins. After incubation, these proteins were pulled down through centrifugation at 1,000 rpm for 10 min and subjected to a Western blot analysis with TGFBR2 and His-tag-specific antibodies.

Luciferase reporter assay

Luciferase reporter vectors containing a Smad binding element were obtained from Promega (Madison, WI, USA). Cells cultured on six-well plates were cotransfected with *Renilla* luciferase control vectors and a Smad-binding-element-containing firefly luciferase reporter vector for 24 h. Whole-cell lysates were then prepared and subjected to luciferase activity measurements by using a Dual-Glo Luciferase Assay System (Promega) in accordance with the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Animal experiments

B6.129S4-Il2rg^{tm1Wjl}/J mice with advanced severe immunodeficiency were obtained from the National Laboratory Animal Center (Taiwan). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC2024-0408). Luciferase-expressing melanoma cell variants, generated as previously described [16], were resuspended in phosphate-buffered saline at 5×10^7 cells/mL and mixed with Matrigel at a ratio of 1:1. Subsequently, 100 μL of cell suspension was orthotopically injected into the intradermal layer of the dorsal skin of each mouse under anesthesia. Tumor growth and lymph node and brain metastasis were monitored weekly using an in vivo imaging system.

Gene set enrichment analysis

A differentially expressed gene (DEG) analysis was conducted against microarray data from the GSE30531 dataset by using the GEO2R software on Gene Expression Omnibus's website. The resulting fold-change values were used to generate a ranked gene list for gene set enrichment analysis (GSEA) to identify the biological pathways associated with MLN4924 treatment. On the basis of normalized enrichment scores, ranked gene sets were analyzed using the GSEA algorithm against HALLMARK gene sets from the Molecular Signatures Database (https://www.gsea-msigdb.org/gsea/msigdb; accessed on January 1, 2022) to identify statistically significant enrichment patterns.

Statistical analysis

All statistical analyses were conducted using SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). A Kaplan–Meier survival analysis with a log-rank test was used to evaluate survival probabilities. The Mann–Whitney *U* test (two groups) and Kruskal–Wallis test (three or more groups) were used for nonparametric data analyses. In all tests, a *p* value of <0.05 was considered significant.

Results

NEDD8 upregulation is associated with a poor prognosis and enhanced migratory capacity in $BRAF^{V600E}$ melanoma

We analyzed the prognostic relevance of NEDD8 expression by conducting Kaplan–Meier survival analyses of overall, progression-free, and disease-specific survival in patients with melanoma whose data were obtained from TCGA. In this analysis, we used an optimal cutoff that maximized survival discrimination. Our results indicated that higher NEDD8 expression was associated with a poorer prognosis in melanomas with the $BRAF^{V600E}$ mutation, whereas, interestingly, it was associated with a more favorable prognosis in melanomas with wild-type (WT) BRAF (**Fig. 1A**). We observed that the $BRAF^{V600E}$ melanoma cell lines A2058 and RPMI7951, which exhibited higher total NEDD8 protein levels, exhibited a greater migratory capacity, whereas the A375 and C32 cell lines, which expressed lower NEDD8 levels, exhibited reduced migration (**Fig. 1B–C**). Consistent with these findings, we identified a strong correlation between endogenous NEDD8 protein level and migratory capacity across the tested melanoma cell lines (**Fig. 1D**). In addition, Kaplan–Meier survival analysis of the metastatic melanoma cohort revealed that higher NEDD8 expression was significantly associated with shorter progression-free survival ($p = 0.034$; **Fig. 1E**), suggesting a potential role of NEDD8-mediated neddylation in the metastatic progression of $BRAF^{V600E}$ melanoma.

NEDD8-mediated protein neddylation increases the migratory capacity of $BRAF^{V600E}$ melanoma cells

To determine the causal role of NEDD8 in melanoma cell migration, we examined the impact of ectopic NEDD8 overexpression in A375 cells, which have low endogenous NEDD8 levels and a low migratory capacity. Our results indicated that NEDD8 overexpression was associated with a significantly higher migratory capacity in A375 cells compared with parental and vector control cells (**Fig. 2A–B**). By contrast, NEDD8 silencing with two independent shRNA clones dramatically suppressed cellular migration in A2058 and RPMI7951 cells, which express higher endogenous NEDD8 protein levels and have a higher migratory capacity compared with parental and nonsilencing control cells (**Fig. 2C–F**). Significantly, the re-expression of NEDD8 in the NEDD8-silenced RPMI7951 cells restored the cellular migration ability, indicating the critical role of NEDD8-mediated protein neddylation in promoting the metastatic progression of $BRAF^{V600E}$ melanoma cells (**Fig. S1A–B**). Moreover, pharmacologic inhibition of protein neddylation with MLN4924 and TAS4464 [17], which are both NAE inhibitors, dose-dependently suppressed cellular migration in A2058 and RPMI7951 cells (**Fig. 2G**).

MLN4924 suppresses TGF-β signaling and TGFBR2 neddylation in highly migratory BRAF^{V600E} melanoma cells

To explore the mechanism through which neddylation inhibition suppresses the migratory phenotype of highly migratory BRAF^{V600E} melanoma cells, we reanalyzed microarray data from the GSE30531 dataset. Using GEO2R, we identified certain DEGs after 8 h of MLN4924 treatment in melanoma cells (**Fig. 3A**). Subsequent GSEA revealed that the TGF-β signaling pathway, as well as epithelial–mesenchymal transition, was among the most prominently suppressed pathways after MLN4924 treatment (**Fig. 3B**). We further confirmed that MLN4924 reduced the phosphorylated and active forms of Smad2/3, namely, the downstream effectors of TGF-β signaling, in a dose- and time-dependent manner, as determined through Western blot analysis in RPMI7951 and A2058 cells (**Fig. 3C–D, Fig. S2A–B**). Consistent with these findings, we observed that MLN4924 treatment dose-dependently reduced Smad-dependent transcriptional activity, as measured through a Smad-responsive luciferase reporter assay in RPMI7951 and A2058 cells (**Fig. 3E, Fig. S2C**).

Studies have indicated that c-Cbl-mediated neddylation of TGFBR2 antagonizes its ubiquitination-dependent degradation and stabilizes the receptor [18, 19]. In the present study, our cycloheximide chase assay revealed that MLN4924 markedly accelerated TGFBR2 degradation in RPMI7951 and A2058 cells (**Fig. 3F**, **Fig. S2D**). Moreover, our His-NEDD8 pulldown assay demonstrated that MLN4924 reduced the neddylated form of TGFBR2 in RPMI7951 cells (**Fig. 3G**). Together, these findings indicate that TGFBR2 neddylation is required to sustain TGF-β signaling and the prometastatic phenotype of BRAF^{V600E} melanoma cells.

TGFBR2 is required for the metastatic progression of BRAF^{V600E} melanoma cells

Consistent with NEDD8 expression, the endogenous expression levels of TGFBR2 were highly associated with cellular migration ability in the tested melanoma cell lines (**Fig. S3**). Therefore, we performed TGFBR2 knockdown in RPMI7951 cells by using three independent shRNA clones (**Fig. 4A**) and overexpressed the wild-type or neddylation-defective TGFBR2 gene in A375 cells (**Fig. 4B**) to determine whether TGFBR2 is required for melanoma cell migration. We observed that TGFBR2 knockdown significantly ($p < 0.001$) suppressed the migratory capacity of RPMI7951 cells (**Fig. 4C**), whereas ectopic expression of wild-type, but not neddylation-defective, TGFBR2 in A375 cells markedly enhanced cell migration (**Fig. 4D**). Moreover, compared with wild-type TGFBR2, the enforced expression of neddylation-defective TGFBR2 accelerated its protein degradation in A375 cells **(Fig. 4E**). Notably, re-expression of exogenous WT TGFBR2, but not the neddylation-deficient constructs, substantially restored the migratory ability of TGFBR2-silenced RPMI7951 cells (**Fig. 4F–G**).

We next established an orthotopic melanoma mouse model through an intradermal injection approach to determine the effect of TGFBR2 on metastatic progression in vivo. We observed that compared with the nonsilencing control group, TGFBR2 knockdown in RPMI7951 cells was associated with markedly lower tumor growth, as evidenced by reduced tumor volume over time (**Fig. 5A**) and reduced tumor

weight at the endpoint (**Fig. 5B**). At 6 weeks after transplantation, TGFBR2 knockdown markedly reduced lymph node metastasis in the tissue surrounding the ear and abolished brain metastasis, as confirmed by the bioluminescence imaging of luciferase-expressing cells (**Fig. 5C–D**) and hematoxylin and eosin staining (**Fig. 5E**). Moreover, Western blot analysis revealed reduced expression of TGFBR2 and the proliferation marker Ki-67 in xenografts derived from TGFBR2-silenced RPMI7951 cells (**Fig. 5F**). Together with rescue experiments involving neddylation-deficient TGFBR2 mutants, these findings support a functional requirement for TGFBR2 neddylation in sustaining the metastatic phenotype of $BRAF^{V600E}$ melanoma cells.

c-Cbl-mediated TGFBR2 neddylation drives the migratory phenotype of metastatic $BRAF^{V600E}$ melanoma cells

Studies have indicated that c-Cbl mediates the non-canonical neddylation of TGFBR2, which antagonizes its ubiquitination-dependent degradation and stabilizes the receptor [18, 19]. These findings are consistent with the broader concept that non-canonical neddylation regulates the fate of noncullin proteins and cellular receptors [20]. To further validate this mechanism, we performed c-Cbl knockdown in A2058 and RPMI7951 cells by using three independent shRNA clones (**Fig. 6A**). Our results indicated that c-Cbl knockdown markedly suppressed migratory capacity (**Fig. 6B**), reduced TGFBR2 neddylation (**Fig. 6C**), attenuated Smad2/3 phosphorylation (**Fig. 6D**), diminished Smad-dependent transcriptional activity (**Fig. 6E**), and accelerated TGFBR2 protein degradation (**Fig. 6F, Fig. S4**). Together, these findings support that c-Cbl-mediated TGFBR2 neddylation plays a key role in sustaining the prometastatic phenotype of $BRAF^{V600E}$ melanoma cells.

Discussion

Brain metastasis is a common and clinically devastating complication of advanced $BRAF^{V600E}$ melanoma [21]. However, the molecular mechanisms that enable brain colonization remain incompletely understood [22]. In the present study, we identified a c-Cbl-mediated non-canonical neddylation pathway that stabilizes TGFBR2 through NEDD8 conjugation at K556 and K567, thereby antagonizing ubiquitination-dependent receptor turnover and sustaining TGF-β/Smad-driven metastatic progression in $BRAF^{V600E}$ melanoma cells (Fig. 6G). These findings highlight TGFBR2 neddylation as a potential therapeutic vulnerability in metastatic BRAF-mutant melanoma. We established an orthotopic intradermal model involving RPMI7951 cells and observed brain metastasis in the nonsilencing control group but not in the TGFBR2-silenced group. Recent comprehensive reviews highlight the tumor microenvironment (TME) as a critical and emerging therapeutic target in melanoma brain metastasis [23], underscoring the urgent need for clinically relevant in vivo platforms. In this context, our orthotopic intradermal model provides a highly valuable and timely tool to investigate these complex TME interactions. Ultimately, we believe this platform will be instrumental in elucidating the biology of brain colonization and evaluating candidate antimetastatic therapies against $BRAF^{V600E}$ melanoma.

MLN4924 (pevonedistat) is a small-molecule NAE inhibitor that has demonstrated antitumor activity in both preclinical and clinical studies [24–27]. By directly targeting NAE and preventing NEDD8 activation, MLN4924 globally suppresses cellular neddylation [28]. However, because this strategy inhibits the neddylation required for essential functions in both malignant and normal cells, on-target systemic toxicity may constrain its therapeutic window [25, 29–31]. These findings suggest that selectively targeting non-canonical neddylation represents a more precise therapeutic strategy compared with global NAE inhibition.

In our study, we observed that c-Cbl depletion markedly reduced TGFBR2 neddylation, accelerated receptor loss, and suppressed melanoma cell migration in $BRAF^{V600E}$ RPMI7951 cells. Although additional studies are required to establish the feasibility of directly targeting this interface, our findings suggest that disrupting NEDD8 conjugation at K556 and K567 of TGFBR2 can be used as a more selective approach for attenuating prometastatic signaling in $BRAF^{V600E}$ melanoma.

Notably, c-Cbl is currently the only directly validated NEDD8 E3 ligase for TGFBR2. This ligase mediates receptor neddylation, which promotes clathrin-dependent endocytosis, stabilizes TGFBR2, and prolongs its signaling activity. Deneddylation by NEDP1 redirects the receptor toward ubiquitination-dependent lysosomal degradation [19]. In the present study, our functional, biochemical, and signaling evidence collectively supported that the c-Cbl–TGFBR2 neddylation axis plays a key role in metastatic $BRAF^{V600E}$ melanoma. We observed that c-Cbl knockdown suppressed melanoma cell migration, reduced NEDD8-conjugated TGFBR2, and attenuated TGF-β/Smad transcriptional activity. Together with prior evidence demonstrating that c-Cbl supports melanoma cell migration and invasion, these findings suggest that c-Cbl may functionally contribute to sustaining TGFBR2 neddylation and downstream prometastatic signaling rather than being merely associated with metastatic behavior [32]. Although previous structural research has explored whether c-Cbl directly activates the canonical NEDD8 E2 enzyme ubiquitin-conjugating enzyme [33], our pulldown data revealed a clear reduction in TGFBR2 neddylation upon c-Cbl depletion. These data support the crucial role of c-Cbl in this pathway but do not address the possibility of indirect or non-canonical mechanisms, which warrant further biochemical reconstitution. In the TGF-β context, the ATM–c-Cbl–TGFBR2 axis, which normally reinforces cytostatic responses after DNA damage, may be repurposed during tumor progression to sustain malignancy [18, 34]. Although Smurf2 promotes TGFBR2 ubiquitination and degradation [35], c-Cbl appears to counterbalance this route by stabilizing TGFBR2 through neddylation. These findings suggest that in $BRAF^{V600E}$ melanoma, this regulatory balance shifts toward c-Cbl-dependent receptor preservation, which helps maintain prometastatic TGF-β signaling and repurpose a physiological stress-response pathway to support cancer progression.

This study has several limitations that should be acknowledged. Although pharmacologic inhibition of NAE by MLN4924 and TAS4464 provides crucial mechanistic insights, these agents globally suppress neddylation and exert pleiotropic effects, which precludes attribution of the observed phenotypes exclusively to TGFBR2. In addition, although the His-NEDD8 pulldown assay supports NAE-dependent neddylation of TGFBR2, interpretation of results is influenced by purification conditions; if denaturation is

incomplete, tight protein associations may be present. Additional validation in endogenous settings, optimally through unbiased site-mapping approaches such as mass spectrometry, is required to confirm direct covalent modification and to define the full spectrum of relevant lysine residues. Although our data support a key role for c-Cbl in this axis, they do not definitively establish c-Cbl as a direct NEDD8 E3 ligase, and indirect or non-canonical mechanisms may be present. Previous in vivo studies on TGFBR2 depletion have validated the functional value of this receptor but have not yet provided pathway-selective evidence for the therapeutic targeting of neddylation or c-Cbl-mediated conjugation [36]. Moreover, although brain metastasis data suggest that this axis plays a role in metastatic fitness, organ-specific mechanisms such as blood–brain barrier interaction and immune microenvironment remodeling have not been directly examined. Finally, because of the context-dependent nature of TGF-β signaling and the use of selected $BRAF^{V600E}$ melanoma models in this study, the generalizability of our findings remains to be established. Additional studies incorporating site-specific editing, biochemical reconstitution, immune-competent or patient-derived models, and pathway-selective interventions are required to more rigorously define the translational relevance of this pathway.

Conclusions

This study identifies a previously unrecognized c-Cbl–TGFBR2 non-canonical neddylation axis as a key driver of metastasis in $BRAF^{V600E}$ melanoma. NEDD8 is associated with melanoma aggressiveness and functionally contributes to melanoma cell motility. Pharmacologic inhibition of neddylation suppresses cellular migration in parallel with the destabilization of TGFBR2 and attenuation of TGF-β/Smad signaling. Mechanistically, TGFBR2 neddylation at K556 and K567 is required for its prometastatic activity, and c-Cbl serves as a key upstream regulator sustaining this pathway. Collectively, these findings highlight a mechanistic link between non-canonical neddylation and metastatic signaling and identify the c-Cbl–TGFBR2 axis as a potential therapeutic vulnerability in aggressive BRAF-mutant melanoma.

Abbreviations

$BRAF^{V600E}$: Valine-to-glutamate substitution at codon 600 of BRAF

c-Cbl: Casitas B-lineage lymphoma proto-oncogene

DEG: Differentially expressed gene

EGFP: Enhanced green fluorescent protein

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GSEA: Gene set enrichment analysis

MLN4924: Pevonedistat

NAE: NEDD8-activating enzyme

NEDD8: Neural precursor cell expressed, developmentally downregulated 8

Ni^{2+}-NTA: Nickel–nitrilotriacetic acid

PCR: Polymerase chain reaction

PSG: Penicillin–streptomycin–glutamine

SEM: Standard error of the mean

shRNA: Short hairpin RNA

TCGA: The Cancer Genome Atlas

TGF-β: Transforming growth factor-β

TGFBR2: Transforming growth factor-β receptor 2

TME: Tumor microenvironment

WT: Wild-type

Declarations

Ethical approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Taipei Medical University (approval no. LAC2024-0408) and were performed in accordance with institutional guidelines for the care and use of laboratory animals. This study did not involve human participants, human tissue, or identifiable human data; the human transcriptomic and clinical data analysed were retrieved from the publicly available The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GSE30531) datasets. Consent to participate was therefore not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analysed during the current study are available in the following public repositories: TCGA melanoma clinical and transcriptomic data, accessed through UCSC Xena (https://xena.ucsc.edu/); and the microarray dataset GSE30531, available in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30531). All other data generated during this

study are included in this published article and its supplementary information files, or are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Leon Tsung-Ju Lee, Hui-Wen Chiu, and Yuan-Feng Lin conceived and designed the study. Leon Tsung-Ju Lee, Jing-Quan Zheng, Hsiao-Wei Lu, Che-Hsuan Lin, and Yu-Hsien Kent Lin prepared the research material and collected and analyzed the data. Leon Tsung-Ju Lee, Hui-Wen Chiu, and Yuan-Feng Lin drafted the manuscript. All authors have reviewed and approved the final version of the manuscript.

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Figures

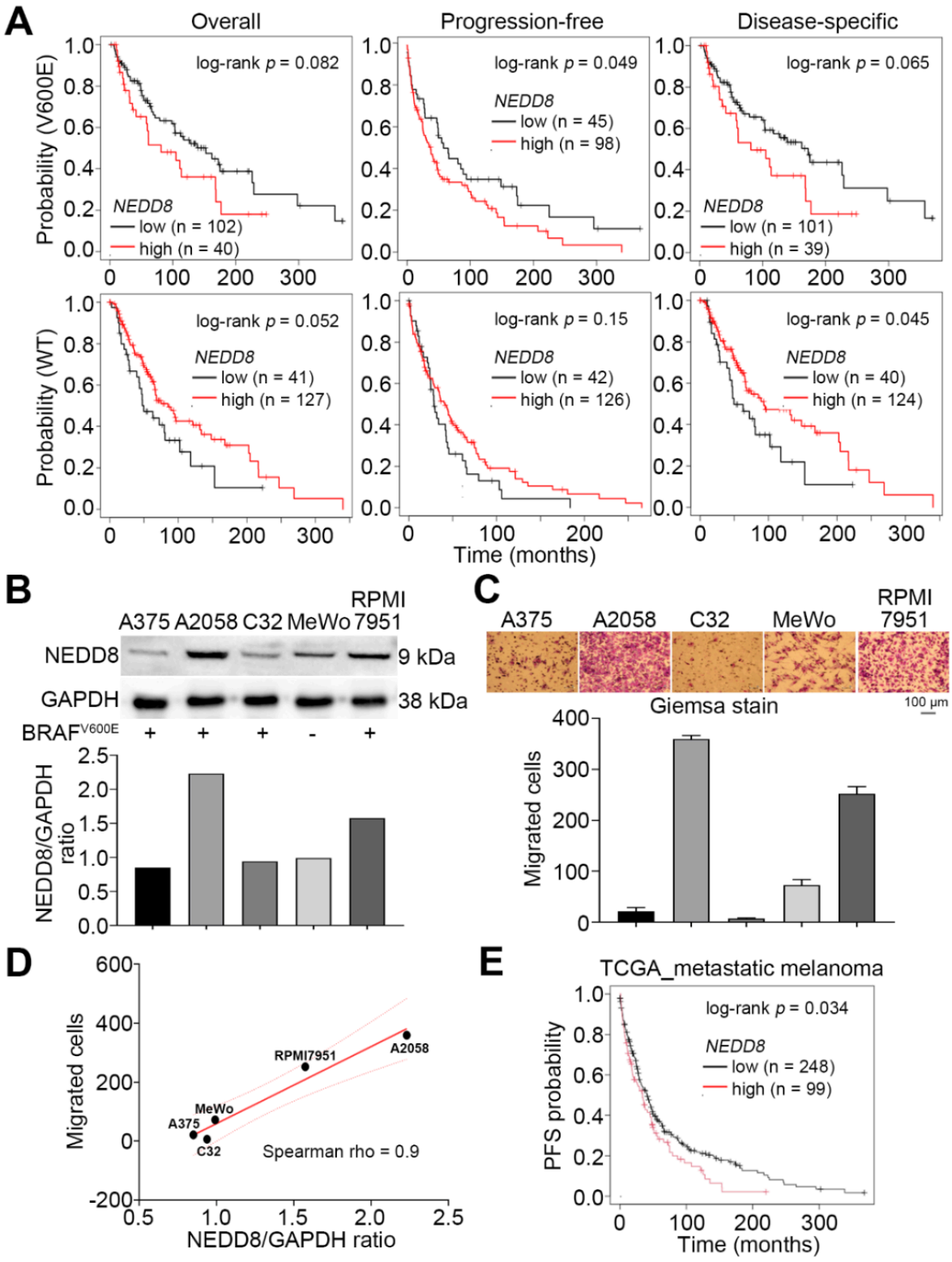


Figure 1

NEDD8 upregulation is associated with a poor prognosis in patients with BRAF^{V600E} melanoma and with an enhanced migratory ability of BRAF^{V600E} melanoma cells. (A) Kaplan–Meier survival analyses of NEDD8 mRNA levels at the maximum risk with overall, progression-free, and disease-specific survival probabilities against a TCGA cohort with melanoma harboring a BRAF^{V600E} mutation or WT BRAF gene. (B) Western blot analysis (upper) of endogenous NEDD8 and GAPDH protein levels and a histogram

(lower) of normalized NEDD8 protein levels in the tested melanoma cell lines. GAPDH was used as an internal control of protein loading. (C) Giemsa staining (upper) and a bar chart (lower) of migrating cells from the transwell cultivation of the tested melanoma cell lines. The error bars represent means ± SEMs from quadruplicate data. (D) Scatter plot of the correlation between NEDD8 protein level and cellular migratory ability in the tested melanoma cell lines. (E) Kaplan–Meier survival analysis of NEDD8 mRNA levels at the maximum risk with progression-free survival probability against a TCGA cohort with metastatic melanoma.

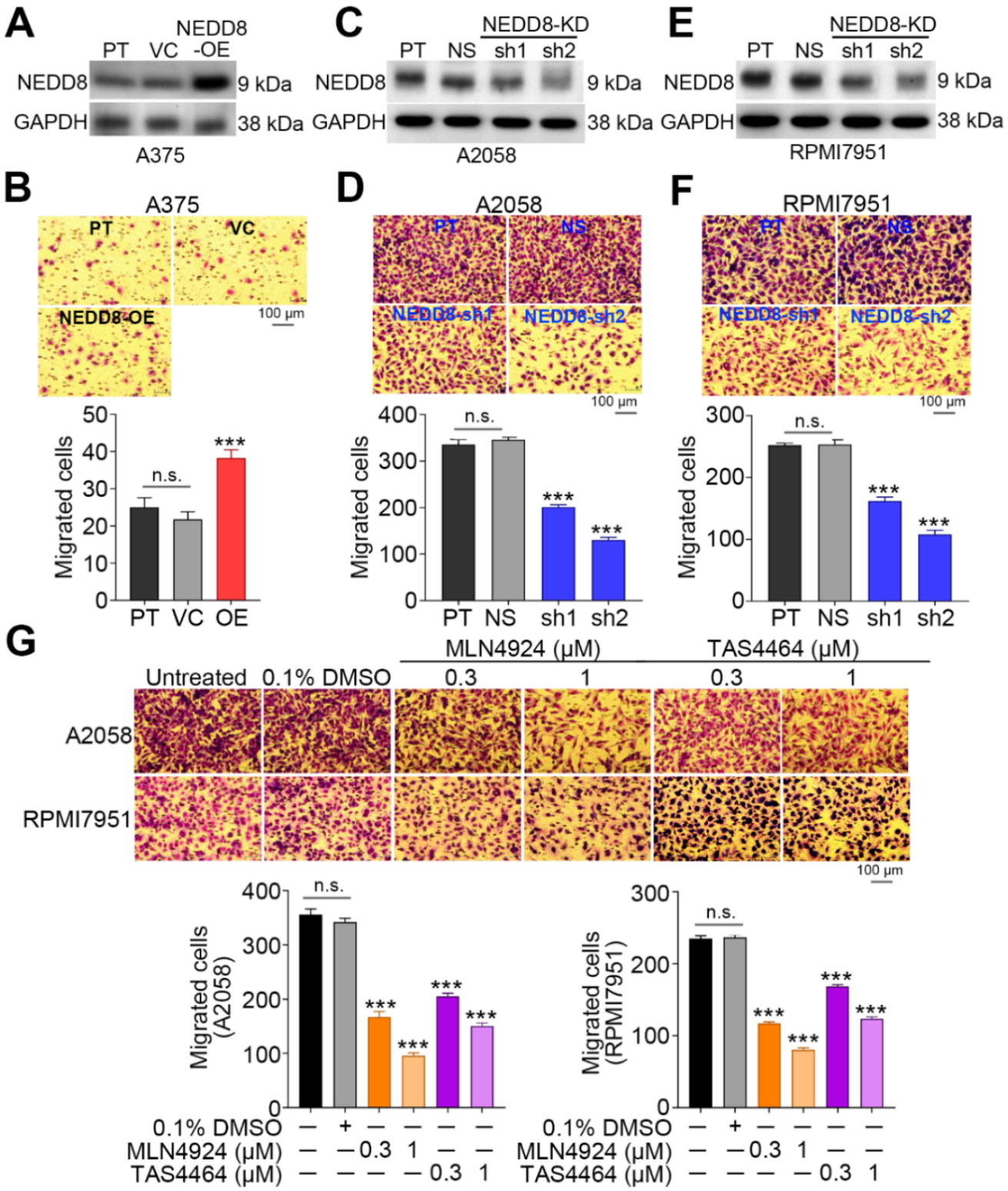


Figure 2

Targeting NEDD8-mediated protein neddylation suppresses the migratory ability of BRAF^{V600E} melanoma cells. Western blot analyses of NEDD8 protein levels (A, C, and E) and Giemsa staining/bar charts of migrating cells from the transwell cultivation (B, D, and F) of A375 cells stably transfected without (parental, PT) or with a vector control (VC) and exogenous NEDD8 gene (A and B) or A2058/RPMI7951 cells stably transfected without (PT) or with a nonsilencing (NS) control and two independent NEDD8 shRNA clones (C–F). OE and KD denote overexpression and knockdown, respectively. (G) Giemsa staining (upper) and bar charts (lower) of migrating cells from the transwell cultivation of A2058/RPMI7951 cells in the absence or presence of the NAE inhibitors MLN4924 and TAS4464 at the indicated concentrations. 0.1% dimethyl sulfoxide (DMSO) was used as a vehicle control. In panels (B), (D), (F), and (G), the error bars represent means ± SEMs from quadruplicate data. The symbols "n.s" and "***" denote not significant and $p < 0.001$, respectively, in the statistical analyses.

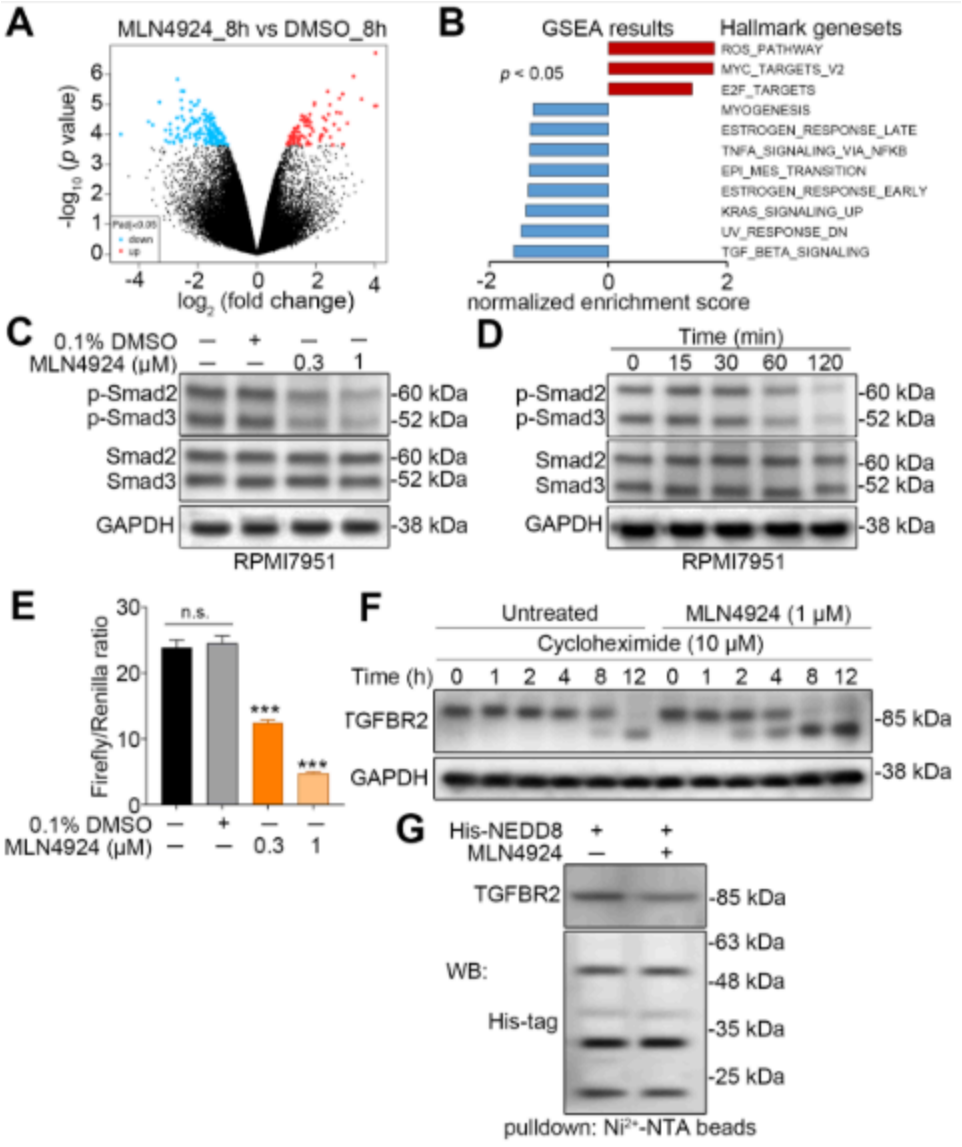


Figure 3

MLN4924 mitigates TGFb signaling by suppressing TGFBR2 neddylation in BRAF^{V600E} melanoma cells. (A) Volcano plot of DEGs after MLN4924 treatment for 8 h in the tested melanoma cells. (B) Histogram of normalized enrichment scores derived from GSEA simulation against the gene list ranked by fold change of gene expression (A) and hallmark gene sets. (C and D) Western blot analyses of the protein levels of phosphorylated Smad2/3 (p-Smad2/3), total Smad2/3, and GAPDH in RPMI7951 cells treated without and with MLN4924 at the indicated concentrations for 2 h (C) or at 1 mM for the designated time intervals (D). (E) Bar chart of firefly luciferase activity normalized to *Renilla* luciferase activity in RPMI7951 cells cotransfected with the Smad2/3-responsive firefly luciferase reporter and *Renilla* luciferase-expressing plasmids in the absence or presence of MLN4924 at the indicated concentrations. The error bars represent means ± SEMs from three independent experiments. The symbols "n.s." and "***" denote not significant and $p < 0.001$, respectively, in the statistical analyses. (F) Western blot analyses of TGFBR2 and GAPDH protein levels in the cycloheximide chase assay at the designated time intervals in RPMI7951 cells pretreated without or with MLN4924 at 1 mM for 2 h. In panels (C), (D), and (F), GAPDH was used as an internal control of protein loading. (G) Western blot analyses of TGFBR2 and His-tag protein levels in the pulldown assay with Ni^{2+}-NTA beads against cell lysates from RPMI7951 cells transfected with a His-tagged NEDD8 construct in the absence or presence of MLN4924 at 1 mM for 2 h.

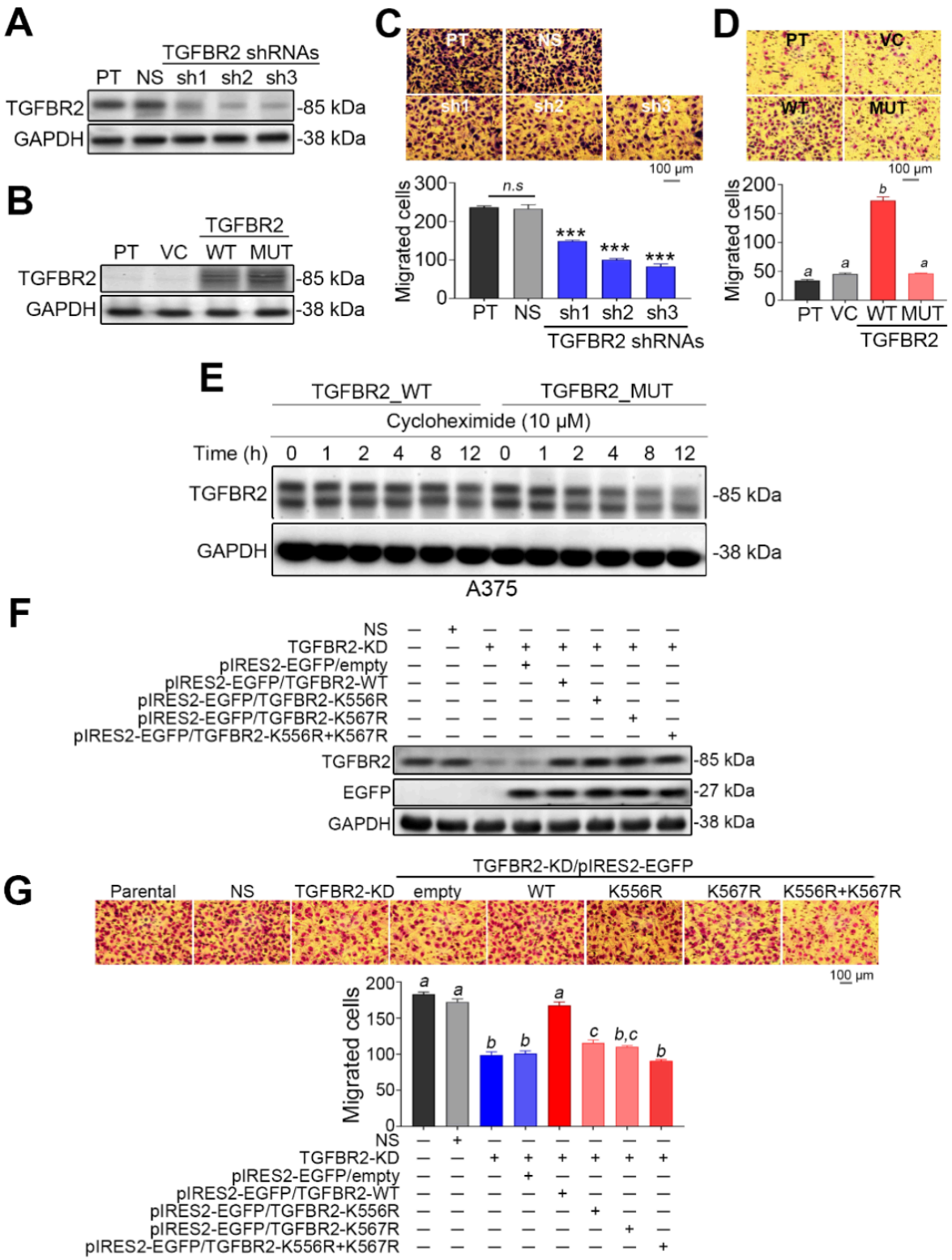


Figure 4

TGFBR2 neddylation is necessary for the migratory ability of BRAF^{V600E} melanoma cells. Western blot analyses of TGFBR2 protein levels (A and B) and Giemsa staining/bar charts of migrating cells from the transwell cultivation (C and D) of RPMI7951 cells stably transfected without (PT) or with a nonsilencing (NS) control and three independent TGFBR2 shRNA clones (A and C) or A375 cells stably transfected without (parental, PT) or with a vector control (VC) and exogenous WT/neddylation-site-mutated (MUT)

TGFBR2 genes (B and D). (E) Western blot analyses of TGFBR2 and GAPDH protein levels in the cycloheximide chase assay at the designated time intervals in A375 cells stably expressing wild-type (WT) or neddylation-defective mutant TGFBR2. (F and G) Western blot analyses of TGFBR2, EGFP, and GAPDH protein levels (F) and Giemsa staining/bar chart of migrating cells from the transwell cultivation (G) of parental/NS control RPMI7951 cells and TGFBR2-KD RPMI7951 cells without or with the transient transfection of an empty pIRES2-EGFP plasmid or a plasmid containing the WT or mutant TGFBR2 gene. EGFP and GAPDH were used as internal controls of transfection efficiency and protein loading, respectively. In panel (C), the symbols “n.s.” and “***” denote not significant and $p < 0.001$, respectively, in the designated experiments. In panels (D) and (G), different letters indicate significance at $p < 0.05$.

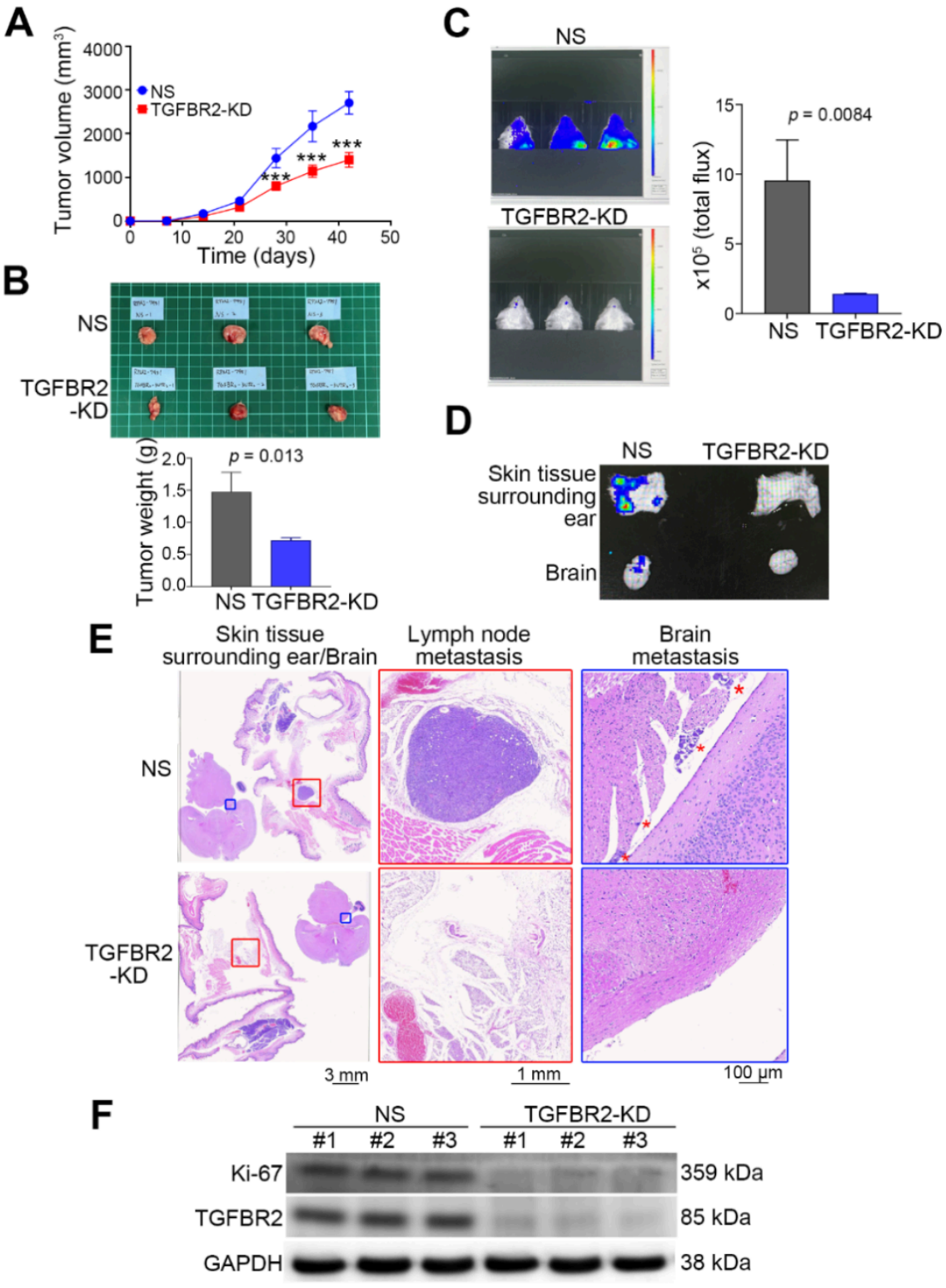


Figure 5

TGFBR2 knockdown abolishes the tumor growth and brain metastasis of RPMI7951 melanoma cells. Tumor volume (A), appearance (B, upper), and weight (B, lower) of nonsilencing (NS) control and TGFBR2-KD RPMI7951 cells after intradermal transplantation in an orthotopic mouse model at the indicated time intervals or at the end of the experiment. (C) Images captured using an in vivo imaging system against the brain area of tumor-bearing mice (left) and bar chart of luminescent intensity

measured from the brain area of tumor-bearing mice (right). The error bars represent means ± SEMs from the data of three mice. (D) Appearance of luciferase-expressing cells visualized using in vivo imaging system for skin tissues surrounding the ear and brain derived from the indicated tumor-bearing mice. (E) Hematoxylin and eosin staining for metastatic melanoma cells in the lymph nodes of skin tissues surrounding the ear and brain. (F) Western blot analysis of the protein levels of Ki-67, TGFBR2, and GAPDH in whole-cell lysates derived from the xenografts of nonsilencing (NS) control and TGFBR2-KD RPMI7951 cells. GAPDH was used as an internal control of protein loading.

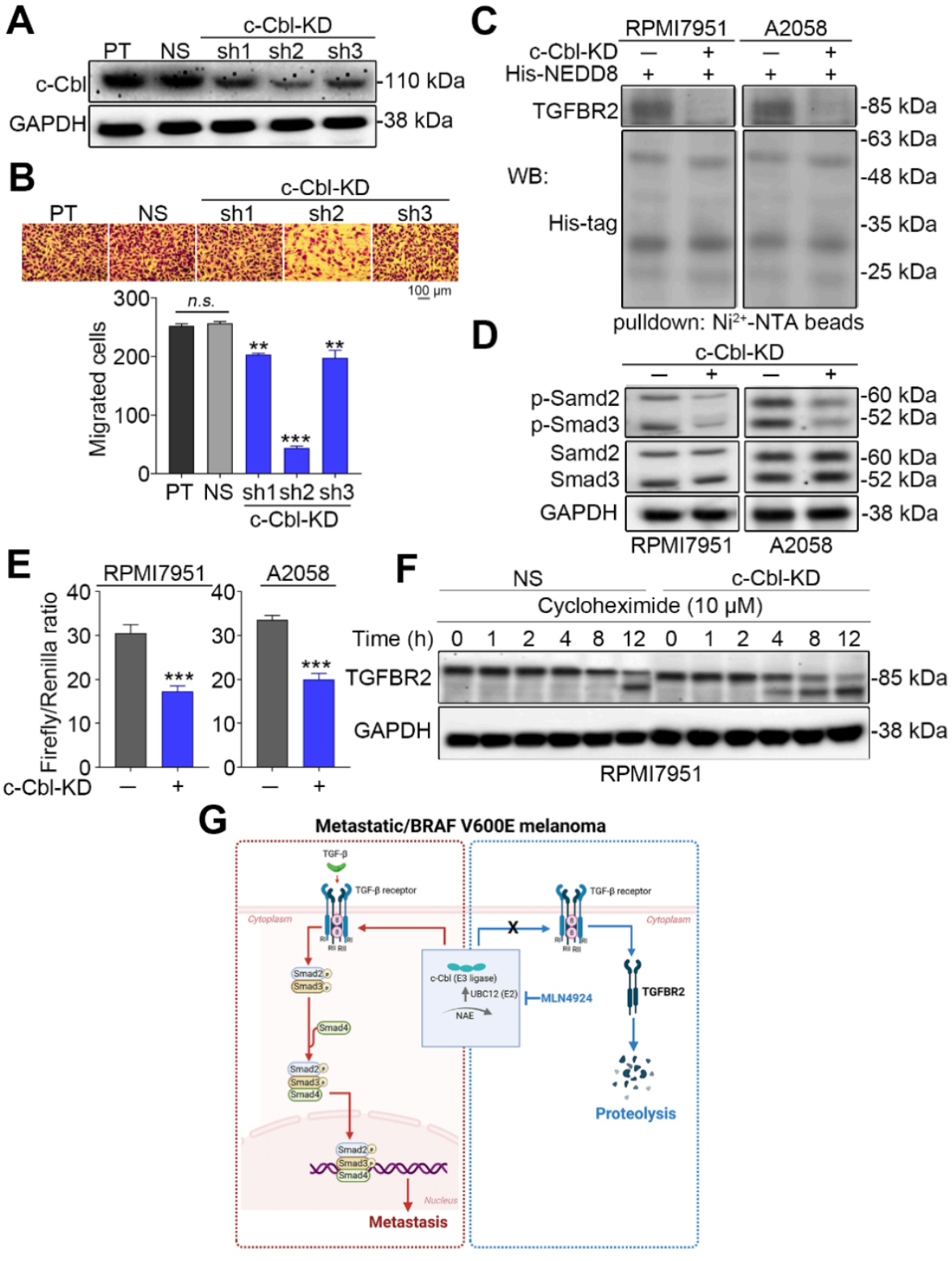


Figure 6

E3 ligase c-Cbl regulates TGFBR2 neddylation in metastatic BRAF^{V600E} melanoma cells. Western blot analysis of the protein levels of c-Cbl and GAPDH (A) and Giemsa staining (upper)/bar chart (lower) of migrating cells (B) from the transwell cultivation of RPMI7951 cells transfected without (PT) or with nonsilencing (NS) control and three independent c-Cbl shRNA clones. The error bars represent means ± SEMs from quadruplicate data. (C) Pulldown assay using Ni^{2+}-NTA beads against His-tagged NEDD8-conjugated TGFBR2 in A2058/RPMI7951 cells without or with c-Cbl knockdown (KD). Western blot analysis of total His-tagged NEDD8-conjugated protein used as an internal control. (D and E) Western blot analyses of the protein levels of phosphorylated (p)/total Smad2/3 (D) and transcriptional regulatory activity of Smad2/3 (E) in A2058/RPMI7951 cells without or with c-Cbl-KD. Data from three independent experiments are presented as means ± SEMs. (F) Western blot analyses of TGFBR2 protein levels in the cycloheximide chase assay at the designated time intervals in A2058 cells without (NS) or with c-Cbl-KD. In panels (B) and (E), the symbols “n.s.” and “***” denote not significant and $p < 0.001$, respectively, in the designated experiments. In panels (A), (D) and (F), GAPDH was used as an internal control of protein loading. (G) Proposed mechanism in which c-Cbl-mediated TGFBR2 neddylation drives the metastatic progression of melanoma.

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