The CUL4B/AKT/β-catenin axis restricts the accumulation of myeloid-derived suppressor cells to prohibit the establishment of a tumor permissive microenvironment

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Abstract

Cancer progression requires a permissive microenvironment that shields cancer from the host immunosurveillance. The presence of myeloid-derived suppressor cells (MDSCs) is a key feature of a tumor-permissive microenvironment. Cullin 4B (CUL4B), a scaffold protein in the Cullin 4B-RING E3 ligase complex (CRL4B), represses tumor suppressors through diverse epigenetic mechanisms and is overexpressed in many malignancies. We report here that CUL4B unexpectedly functions as a negative regulator of MDSC functions in multiple tumor settings. Conditional ablation of CUL4B in the hematopoietic system, driven by Tek-Cre, resulted in significantly enhanced accumulation and activity of MDSCs. Mechanistically, we demonstrate that the aberrant abundance of MDSCs in the absence of CUL4B was mediated by the downregulation of the AKT/β-catenin pathway. Moreover, CUL4B repressed the phosphatases PP2A and PHLPP1/2 that dephosphorylate and inactivate AKT to sustain pathway activation. Importantly, the CUL4B/AKT/β-catenin axis was downregulated in MDSCs of healthy individuals and was further suppressed in tumor-bearing mice and cancer patients. Thus, our findings point to a pro- and antitumorigenic role for CUL4B in malignancy, in which its ability to impede the formation of a tumor-supportive microenvironment may be context-specific.

Précis:
Surprising findings describe a previously uncharacterized antitumorigenic role for CUL4B in the hematopoietic system where it restricts the accumulation and activity of myeloid-derived suppressor cells to prevent the establishment of a tumor permissive microenvironment, underscoring mechanisms by which immunosurveillance may be compromised by certain therapeutic strategies.
Introduction

Cancer initiation and progression require a favorable microenvironment that includes immune cells, endothelial cells, pericytes, mesenchymal cells and many soluble factors(1, 2). Myeloid-derived suppressor cells (MDSCs) have emerged as one of the major players that enable cancer to escape immunosurveillance(1-4). In mice, MDSCs are characterized by the co-expression of CD11b and Gr-1 markers(5, 6). Their immunosuppressive activity is attributable to high activity of arginase 1 (ARG1) and/or inducible nitric oxide synthase (iNOS), production of NO and reactive oxygen species (ROS), as well as release of IL-10 and TGF-β(3, 4, 7-9). Although tumor- and host-secreted factors, including vascular endothelial growth factor (VEGF), stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, IL-6 and prostaglandin E2, have been reported to promote the expansion and activation of MDSCs during tumor development(3, 4, 8), little is known about the intracellular signaling pathways that regulate the expansion of MDSCs.

β-catenin is critical for Wnt signaling, a pathway that governs many developmental processes including specification of cell fate and the maintenance of stem cell pluripotency(10, 11). Many studies indicated that a tight regulation of Wnt signaling is required for normal hematopoiesis and lymphopoiesis(12-15). β-catenin was recently shown to negatively regulate the
functions of immunosuppressive cells including regulatory T (Treg) cells and MDSCs(16-18). However, how Wnt signaling is regulated in those immunosuppressive cells remains to be elucidated.

Cullin 4B (CUL4B), as a scaffold protein in the Cullin 4B-RING E3 ligase complex (CRL4B), participates in the regulation of diverse physiologically and developmentally controlled processes (19-22). Mutations in human CUL4B cause X-linked mental retardation (XLMR)(23, 24). Recently, CUL4B has been shown to be substantially up-regulated in various types of solid tumors and possess potent oncogenic properties. H2AK119 monoubiquitination catalyzed by CRL4B is critical for epigenetic inactivation of tumor suppressors by PRC2, SUV39H1/HP1/DNMT and SIN3A/HDAC complexes(25-27). In this study, we demonstrated that CUL4B, AKT and β-catenin function in an axis that negatively regulates MDSCs. CUL4B sustains the activation of AKT/β-catenin pathway by repressing the antagonists of AKT. The CUL4B/AKT/β-catenin axis was down-regulated in MDSCs and was further suppressed in tumor-bearing hosts. Our results establish a functional dichotomy of CUL4B in cancer cells and in tumor microenvironment.
Materials and Methods

Mice

*Cul4b*<sup>flox/flox</sup> mice, as previously described(28), were crossed to *Tek-Cre* transgenic mice to produce *Cul4b*<sup>flox/Y Tek-Cre<sup>+/−</sup></sup> (CKO) mice and *Cul4b*<sup>flox/Y Tek-Cre<sup>−/−</sup></sup> (WT) littermates controls. Mice were housed in a specific pathogen-free animal facility at Shandong University. All procedures involving mice were approved by the Animal Care and Use Committee of Shandong University School of Medicine.

Cell culture

B16/F0 (C57BL/6 murine melanoma cells), 4T1 (BALB/c murine mammary tumor cells) and RAW264.7 (BALB/c murine macrophage leukemia) were cultured at 37°C in DMEM complete media containing 10% FBS. EL4 (C57BL/6 murine lymphoma cells) were cultured at 37°C in RPMI-1640 containing 10% FBS. All cell lines were purchased from the authenticated ATCC (American Type Culture Collection) repository.

Histology

Tissues from *Cul4b*<sup>flox/Y Tek-Cre<sup>+/−</sup> R26R* mice were fixed in paraformaldehyde. 7-μm-thick sections were stained with X-gal and counterstained with eosin. Photomicrographs were taken using an Olympus IX71 with DP71 camera.
Tumor growth and metastasis

B16 (2 × 10^5), EL4 (2 × 10^5) or 4T1 (5 × 10^6) tumor cells were suspended in PBS and inoculated subcutaneously in the flank of mice. Tumor measurements were performed every 2 or 3 d with a vernier caliper, and volumes were calculated using the following formula: \( V = \frac{1}{2} \times \text{length [mm]} \times \text{width}^2 \). For the myeloid/tumor admix experiments, 2 × 10^5 MDSCs were mixed with 2 × 10^5 EL4 cells and implanted. For adoptive transfer assay, 3 × 10^6 MDSCs were injected intravenously into CD45.1 WT tumor-bearing mice on days 3 and 5 after tumor challenge. For lithium chloride (LiCl) treatment experiment, 5 days after EL4 transplantation, the WT or Cul4b CKO recipients were randomly divided into two groups, and treated daily with saline (0.9% NaCl/100 μl) or with LiCl (0.2 mmol/100 μl, GIBCO-BRL) intraperitoneally. In the tumor metastasis model, mice administered with B16/F0 cells (2 × 10^5) in 200 μl PBS via tail vein were sacrificed at day 21 and evaluated for tumor metastasis into lungs.

Isolation and differentiation assay of mouse MDSCs and Lin^−^ cells

MDSCs were purified by immunomagnetic separation according to the manufacturer’s protocol (Miltenyi Biotec). Cell purity (>95%) was determined by flow cytometric analysis using anti-CD11b and Gr-1 antibodies. MDSCs isolated from tumor-bearing mice were induced to differentiate in the presence or absence of 10 ng/ml GM-CSF (Sigma-Aldrich) for 3 and 5 days.
Lin^- cells were purified using a biotinylated lineage cell depletion cocktail (Miltenyi Biotec). The differentiation of Lin^- cells was as described (18).

**Isolation of human MDSCs cells**

Human peripheral blood was collected before treatment from cancer patients aged between 45 and 65. Control samples were collected from age- and gender-matched healthy controls. MDSC were isolated from freshly PBMC by using HLA-DR-negative selection and CD33-positive selection magnetic beads (Miltenyi Biotec). Purity was confirmed by flow cytometry using anti-HLA-DR and CD33 antibodies (BioLegend) (>90%). Use of human specimens was approved by the Institutional Review Board at Shandong University School of Medicine. Informed consent was obtained from all blood donors.

**T cell suppression assay**

Freshly isolated splenocytes (5 × 10^6 cells/ml) from C57BL/6 WT mice or OT-I mice were depleted of red cells and labeled with CFSE (1 μM; Invitrogen, Carlsbad, California) for 10 min at 37°C and washed with fresh culture media. Splenocytes from WT mice were stimulated with coated anti-CD3 antibody (10 μg/ml; BioLegend) and soluble anti-CD28 antibody (6 μg/ml; BioLegend). Splenocytes from OT-I mice were stimulated with SIINFEKL (10 pM; Sigma). MDSCs (1 × 10^5) purified from spleen were co-cultured with 2 × 10^5 CFSE-labeled splenocytes for 72 h in RPMI 1640 (10% FBS) in U-bottom 96-well plates. Cells were stained for anti-CD4 or anti-CD8 antibody and
analyzed for CFSE dilution using FACS CantoII (BD Bioscience).

**Flow cytometric analysis**

Single-cell suspensions were freshly prepared from mouse tissues. Cells were stained as described (17) with the antibodies listed in Table S1. Data were analyzed with FlowJo 7.6.5 (Treestar, San Jose, California) software.

**NO and ROS Measurements.**

The measurements of NO and ROS were as previously described(17). DCFDA (Invitrogen) was used to measure ROS production by purified G-MDSCs. Nitrite was quantified by a standard Greiss reaction (Invitrogen).

**Lentiviral plasmid, viral production and infection**

Lentivirus shCul4b, PLOC-CUL4B, PLOC-CA-β-catenin, shPhlpp2 and shPpp2cb as well as their controls were prepared in HEK293T cells, as described previously(29). For the differentiation assay of mouse MDSCs and Lin⁻ cells, 24 h after infected with viral particles, the cells were cultured in fresh medium containing GM-CSF or GM-CSF and IL-4 for 5 days.

**Gene expression and Chromatin immunoprecipitation (ChIP) analysis**

Quantitative real-time PCR, western blotting and ChIP assays were performed as previously described(20, 25). Primers and antibodies are listed in Table S1.
and S2.

**Statistical analysis**

Statistical analysis was performed using unpaired Student's t test to calculate a two-tailed $P$ value between two groups. Differences were considered significant at $P < 0.05$. The data are recorded as the Mean ± SEM of the indicated number of mice or biological replicates and as the Mean ± SD of the indicated number of technical replicates.
Results

Increased accumulation of CD11b^Gr-1^ cells in Cul4b CKO mice

Cul4b heterozygous mice exhibit impaired vascularization in placentas (28). An elevation in monocytes was also detected in patients with CUL4B mutation (24). To test the function of CUL4B in angiogenesis and hematopoiesis, we generated Cul4b^lox/yl^Tek-Cre^+/− mice, referred to as Cul4b CKO hereinafter. CUL4B was effectively ablated in endothelial cells and hematopoietic tissues such as blood, spleen and bone marrow in Cul4b CKO mice (Fig. 1A and 1B). Cul4b CKO mice did not exhibit overt histological defects (data not shown), and the percentages of lymphocytes, dendritic cells, macrophages and NK cells in spleen and the various hematopoietic progenitors in bone marrow were similar between two groups (Supplementary Table S3 and S4). But the CD11b^Ly6G^ neutrophils and CD11b^Ly6C^ monocytes, the two subsets of CD11b^Gr-1^ immature myeloid cells (30), were increased in the spleen of Cul4b CKO mice. Consistently, flow cytometry analyses revealed a significant increase in the population of CD11b^Gr-1^ cells in peripheral blood, spleen, and bone marrow of Cul4b CKO mice (Fig. 1C).

To determine whether CD11b^Gr-1^ cells in Cul4b CKO mice possess immunosuppressive activity, we examined their inhibitory effects on T cell proliferation in vitro. As shown in Figure 1D, Gr-1^ cells from Cul4b CKO mice, but not those from Cul4b WT mice, significantly inhibited CD8^ T cell
proliferation under both antigen- and mitogen-driven stimulatory conditions. These results suggest that the CD11b⁺Gr-1⁺ cells that are highly accumulated in Cul4b CKO mice probably represent MDSCs.

**Cul4b CKO mice confer a tumor-supportive microenvironment that is dependent on CD11b⁺Gr-1⁺ cells**

Because MDSCs are generally expanded in the presence of tumor and favor tumor establishment and progression, we next determined whether the increased abundance of CD11b⁺Gr-1⁺ cells in Cul4b CKO mice would promote tumor growth. The tumor masses formed by injected tumor cells, EL4 lymphoma cells or B16/F0 melanoma cells, were significantly larger in Cul4b CKO mice than in WT mice (Fig. 2A and Supplementary Fig. S1A). In accordance with accelerated tumor growth in Cul4b CKO mice, MDSCs were expanded more drastically (Fig. 2B and Supplementary Fig. S1B). We next determined whether lack of CUL4B has distinct effects on the two MDSC subsets and found that both granulocytic (G-MDSCs) and monocytic (M-MDSCs) MDSCs were significantly elevated in Cul4b CKO mice compared with WT mice (Fig. 2C), suggesting that CUL4B has a negative effect on both subpopulations.

To determine whether the increased abundance of MDSCs is responsible for enhanced tumor growth in Cul4b CKO mice, we tested the effect of MDSCs admixed with EL4 cells. MDSCs from Cul4b CKO mice were significantly more
potent in promoting tumor growth than those from WT mice (Supplementary Fig.S1C). Consistently, when adoptively transferred into tumor-bearing CD45.1^+ WT recipients, CD45.2^+ Cul4b-deficient MDSCs were more potent than their WT counterparts in promoting tumor growth (Fig. 2D). The fact that MDSCs from CKO mice possessed a stronger tumor-promoting property while their retention in recipients was indistinct from that of their WT counterparts (Fig. 2E) suggests that Cul4b-deficient MDSCs individually have stronger tumor-promoting property. Thus, the MDSCs in CKO mice could have contributed to a tumor-permissive environment by their increased abundance and by the enhanced activity of the individual cells. The function of CD8^+ T cells and NK cells, on the other hand, appeared to be unimpaired by lack of CUL4B (Supplementary Fig.S2). Strikingly, even BALB/c 4T1 mammary cancer cells were able to form tumor grafts and grow in C57BL/6 Cul4b CKO (Fig. 2F), suggesting that Cul4b-deficient mice are greatly immunocompromised. We also determine the effect of CUL4B depletion on metastasis of intravenously injected B16/F0 melanoma cells to lung. As shown in Figure 2G, both the number and the volume of micrometastatic nodules were markedly increased in the Cul4b CKO mice.

**The immunosuppressive activities of CD11b^+Gr-1^+ cells are negatively regulated by CUL4B**

We next determined whether tumor-associated MDSCs are functionally more potent in Cul4b CKO mice than in WT. Remarkably, G- and M-MDSCs from
Cul4b CKO tumor-bearing mice were more suppressive on CD8+ T cell proliferation than those from WT mice in both antigen- and mitogen-driven T cell stimulatory conditions (Fig. 3A). The frequencies of CD4+ and CD8+ T cells in tumors from Cul4b CKO mice were significantly lower than those in WT mice (Fig. 3B). Consistently, the frequencies of CD4+ and CD8+ T cells in spleen were significantly decreased in tumor-bearing Cul4b CKO mice (Fig. 3B). We also examined the frequencies of Treg cells in spleen, thymus and tumor. While the numbers of Treg cells remained largely unchanged in thymus and spleen of Cul4b CKO mice, an increased abundance of Treg cells was detected in tumor grafts of Cul4b CKO mice (Fig. 3C). To determine whether increased Treg cells in tumor were secondary to the increased accumulation and activity of MDSCs, we examined the effect of MDSCs on Treg induction. As shown in Figure 3D, MDSCs isolated from Cul4b CKO mice were more efficient in inducing Treg cells than those from WT mice.

MDSCs have been shown to suppress antitumor immunity through the production of NO and ROS by M-MDSCs and G-MDSCs, respectively(8). Compared with those isolated from WT mice, MDSCs from Cul4b CKO mice produced ROS and NO at significantly higher levels (Fig. 3E - 3G). Furthermore, Cul4b CKO MDSCs exhibited higher levels of Arg1, Nox2, Nos2, Cox2 and IL-10 transcripts (Fig. 3H). Taken together, these results indicate that lack of CUL4B does not only permit a greater accumulation of MDSCs but also enhances the immunosuppressive activity per cell, thus conferring a more
permissive microenvironment for tumor progression.

**The differentiation of MDSCs is impaired in *Cul4b* CKO mice**

We evaluated the proliferation and apoptosis of MDSCs in bone marrow in vivo in EL4 tumor-bearing *Cul4b* CKO and WT mice, but found no difference (Fig. 4A and 4B). Similarly, no significant differences in the proliferation or apoptosis of CD11b+Gr-1+ cells were observed in naive WT and CKO mice (Supplementary Fig. S3A and S3B). These results indicate that the enhanced accumulation of MDSCs in *Cul4b* CKO mice may not be caused by abnormal proliferation or apoptosis.

We next examined whether MDSCs are more favorably generated from hematopoietic progenitors in the absence of CUL4B. Lineage negative (Lin−) hematopoietic progenitor cells isolated from bone marrow were cultured for 5 days in the presence of IL-4 and GM-CSF to induce myeloid differentiation. There was a significantly increased generation of MDSCs, marked by the expression CD11b and Gr-1, from *Cul4b*-deficient progenitors compared with WT progenitors (Fig. 4C).

Because MDSCs could differentiate into dendritic cells (DCs) (CD11b+CD11c+) and macrophages (CD11b+F4/80+) upon appropriate stimulations(31), we tested whether the increased accumulation of MDSCs in *Cul4b* CKO mice could also be caused by impaired differentiation of MDSCs into dendritic cells.
or macrophages. Indeed, when treated with GM-CSF for 3 or 5 days, 

*Cul4b*-deficient MDSCs gave rise to lower percentages of CD11b<sup>+</sup>CD11c<sup>+</sup> and CD11b<sup>+</sup>F4/80<sup>+</sup> cells than WT MDSCs (Fig. 4D and 4E, Supplementary Fig. S3C). S100A8 and S100A9, which are known to inhibit MDSC maturation(32), were significantly up-regulated in MDSCs isolated from *Cul4b* CKO mice (Fig. 4F).

In accordance with the impaired differentiation in *Cul4b* CKO mice, immunoblotting showed that the level of CUL4B was dramatically reduced when Lin<sup>−</sup> hematopoietic progenitor cells were induced to undergo myeloid differentiation. Conversely, CUL4B expression was increased when MDSCs were induced to undergo maturation (Fig. 4G). Taken together, these results indicate that the increased production of MDSCs from hematopoietic progenitors and their impaired differentiation into mature myeloid cells could be both responsible for the enhanced accumulation of MDSCs in *Cul4b* CKO mice.

**Aberrant accumulation of MDSCs was mediated by down-regulation of β-catenin**

It was reported that loss of β-catenin in myeloid cells resulted in accelerated tumor growth and a greater accumulation of MDSCs, whereas expression of a constitutively active β-catenin mutant in myeloid cells inhibited tumor growth and reduced accumulation of MDSCs(17). Our recent study showed that
CUL4B could activate Wnt/β-catenin signaling in human hepatocellular carcinoma(29). Thus, we next examined the effect of Cul4b ablation on the level of β-catenin in MDSCs. Immunoblotting showed that lack of CUL4B resulted in a significantly decreased level of β-catenin in naive Gr-1+ cells as well as in two subsets of MDSCs from tumor-bearing mice (Fig. 5A). Because phosphorylation of β-catenin by GSK3β is crucial for its degradation and the phosphorylation of GSK3β itself reflects its inactivation, we next examined the phosphorylation status of GSK3β in MDSCs from Cul4b CKO and WT mice. As shown in Fig. 5A, the levels of GSK3β phosphorylated at Ser9, an inactive form of GSK3β, were significantly reduced in Cul4b-deficient MDSCs, indicating that increased kinase activity of GSK3β, which phosphorylates β-catenin for its degradation, could be responsible for the increased accumulation of MDSCs caused by lack of CUL4B. Indeed, when proteasome inhibitor MG132 was applied to Cul4b-deficient MDSCs, the reduction of β-catenin level was greatly attenuated (Supplementary Fig. S4A). Furthermore, two inhibitors of GSK3β, SB216763 and lithium chloride (LiCl), could efficiently block the reduction of β-catenin level caused by Cul4b ablation (Fig. 5B). Consistently, increased generation of MDSCs from Lin− progenitors and the impaired differentiation of MDSCs into CD11b+CD11c+ and CD11b+F4/80+ cells caused by lack of CUL4B were rescued by either of the two inhibitors as well as by ectopic expression of constitutively active β-catenin (Supplementary Fig. S4B and Fig. 5C). The ability of LiCl to decrease the accumulation of
MDSCs was also demonstrated in CKO mice in vivo (Fig. 5D). Strikingly, LiCl greatly impeded the tumor growth in *Cul4b* CKO mice (Fig. 5E) as well as tumor-induced expansion of MDSCs (Fig. 5F). Taken together, these results indicate that increased GSK3β activity, and consequently β-catenin down-regulation, may mediate the enhanced accumulation of MDSCs in *Cul4b* CKO mice.

**Up-regulation of AKT phosphatases PP2A and PHLPP1/2 in the absence of CUL4B**

Since Ser9 of GSK3β is phosphorylated by AKT(33), we next determined the level of AKT phosphorylated at Thr308 and Ser473, which marks maximal AKT activity(34, 35). We found that the levels of phosphorylated AKT were markedly decreased in naive Gr-1+ cells and in both subsets of MDSCs in CKO mice, whereas the amount of total AKT was not altered (Fig. 6A), indicating that the decreased GSK3β phosphorylation at Ser9 is due to a decreased AKT kinase activity. Decreased level of AKT phosphorylation could be attributable to decreased kinase activities upstream of AKT or increased activities of phosphatases that dephosphorylate AKT. However, no significant difference in the levels of phosphorylated PDK1 (the kinase of AKT at the site of Thr308) and PTEN (a negative regulator of AKT) was found between *Cul4b*-deficient and control MDSCs (Supplementary Fig. S5). Interestingly, when AKT phosphatases were examined, the expression levels of PP2A (AKT
phosphatase for Thr308), PHLPP1 and PHLPP2 (AKT phosphatases for Ser473)(35-37), were found to be significantly elevated in Cul4b-deficient MDSCs (Fig. 6B). Consistently, the phosphorylation level of PKCβII, another substrate of PHLPP1/2(38), was significantly decreased in Cul4b-deficient MDSCs (Supplementary Fig. S6).

Because CUL4B is a transcriptional co-repressor (25), we next examined the mRNA levels of the genes encoding these phosphatases. Quantitative real-time PCR (qRT-PCR) revealed that the mRNA levels of genes encoding different isoforms of PP2A, Ppp2r1a, Ppp2r1b, Ppp2ca and Ppp2cb, as well as those of Phlpp1 and Phlpp2 were much higher in Cul4b-deficient MDSCs than in WT MDSCs (Fig. 6C). Moreover, chromatin immunoprecipitation (ChIP) assay showed that CUL4B could directly bind to the promoters of the phosphatase genes in MDSCs (Supplementary Fig. S7A). To determine whether the enhanced accumulation of MDSCs in Cul4b CKO mice is due to the up-regulation of AKT phosphatases, we examined the generation of MDSCs from Phlpp2- or Ppp2cb- depleted Lin- cells. Importantly, depletion of either Phlpp2 or Ppp2cb significantly attenuated the generation of MDSCs from Cul4b-deficient Lin- cells (Supplementary Figure S7B and Fig. 6D), suggesting that downregulation of AKT mediates the enhanced production of MDSCs.

The negative regulation of PP2A and PHLPP1/2 by CUL4B was also confirmed
in Cul4b knockdown RAW264.7 cells as well as in cells overexpressing human
CUL4B (Fig. 6E). Quantitative ChIP assay showed that depletion of Cul4b in
RAW264.7 cells resulted in a significantly reduced recruitment of CUL4B,
HDAC and EZH2 to the promoters (Fig. 6F and Supplementary Fig. S7C).
Consistently, the levels of H3K27me3 and H2AK119ub1 were also markedly
decreased, whereas those of acetylated H3 and H4 were increased, providing
further evidence that CUL4B is critical for the transcriptional repression of
these phosphatases. Furthermore, while overexpression of CUL4B could lead
to a remarkable decrease in the expression of Phlpp1, Phlpp2, Ppp2ca, and
Ppp2cb, treatment with HDACs inhibitor TSA, EZH2 inhibitor DNZep or both
could efficiently attenuate the reduction in the expression of these genes (Fig.
6G). Taken together, these results indicate that CRL4B/PRC2/HDAC
complexes can repress AKT phosphatases PP2A and PHLPP1/2 by promoting
H2AK119 monoubiquitination, H3K27 trimethylation, as well as H3 and H4
deacetylation, en route to activate AKT/GSK3β/β-catenin signaling pathway.

**CUL4B/AKT/β-catenin axis is down-regulated in MDSCs**

The data shown above clearly demonstrated a critical role of CUL4B in
activating the AKT/β-catenin cascade that limits the accumulation of MDSCs.
To determine whether this finding bears general implications, we measured the
levels of CUL4B in MDSCs and other hematopoietic cells in wild-type mice. As
shown in Fig. 7A, Cul4b was remarkably down-regulated in bone marrow and
spleen MDSCs when compared to other hematopoietic cells. Similarly, CUL4B in MDSCs isolated from human peripheral blood, based on the absence of HLA-DR and expression of CD33, was also significantly down-regulated when compared to other leukocytes (Fig. 7B). We speculated that the CUL4B/AKT/β-catenin pathway may generally restrain the population of MDSCs and that a down-regulation of CUL4B is probably required for the generation of MDSCs from their progenitors and for the maintenance of their immunosuppressive ability. Indeed, there was a significant reduction in the levels of CUL4B, β-catenin, p-GSK3β, and p-AKT in MDSCs isolated from tumor-bearing mice when compared with those from tumor-free mice (Fig. 7C). Consistently, the levels of AKT phosphatases PP2A and PHLPP1/2 were significantly increased (Fig. 7C).

We next determined whether CUL4B/AKT/β-catenin signaling pathway is altered in MDSCs of patients with cancer. Consistent with previous reports(2-4), the percentages of MDSCs were significantly higher in 22 patients with cancer than in age-matched healthy donors (Fig. 7D). Furthermore, MDSCs from cancer patients were more suppressive on CD4⁺ and CD8⁺ T cell proliferation than those from healthy controls (Fig. 7E). Importantly, the expression levels of CUL4B, measured by qRT-PCR, were negatively correlated with the percentages of MDSCs (Fig. 7F). Western blot analysis showed a significant reduction in the levels of CUL4B, β-catenin,
p-GSK3β (Ser9) in MDSCs of cancer patients compared with those of healthy donors (Fig. 7G). Correspondingly, PP2A was significantly up-regulated (Fig. 7G). Taken together, these results indicate that CUL4B/AKT/β-catenin signaling pathway is generally down-regulated in MDSCs during tumor progression.

Discussion

CUL4B has recently been shown to be highly expressed in a variety of human malignancy and is positively correlated with tumor progression (25, 26, 29, 39, 40). In the current study, we demonstrated that CUL4B functions to limit the expansion and activity of MDSCs that are an integral part of the immunosuppressive tumor microenvironment. The increased accumulation of MDSCs in mice that lack CUL4B in hematopoietic cells greatly facilitated tumor growth and metastasis, and even tolerated allogeneic tumor growth. Thus, while CUL4B may drive the malignancy of cancer cells, it negatively regulates a cancer-supporting microenvironment in hematopoietic system. Thus, the function of CUL4B in cancer development is dichotomous.

Our finding that the negative regulation of MDSCs by CUL4B is mediated by β-catenin is consistent with two recent reports of β-catenin being a negative regulator of MDSCs (17, 18). We showed that pharmacologic inhibition of GSK3β drastically reduced the growth of tumor grafts in Cul4b CKO mice.
Importantly, our findings provided an insight into the mechanism by which β-catenin is regulated. We demonstrated that AKT, which phosphorylates and inactivates GSK3β(33), is positively regulated by CUL4B. CUL4B functions to sustain AKT activity by transcriptionally repressing AKT phosphatases, such as PP2A and PHLPP1/2, via H2AK119 monoubiquitination. The fact that the repression of those phosphatases by CUL4B can be compromised by HDAC and EZH2 inhibitors suggests that the functions of PRC2 and HDACs, which are physically associated with CRL4B, are also essential for the epigenetic silencing of those phosphatases. Interestingly, unlike in solid tumors in which EZH2 acts as an oncogene, inactivating somatic mutations in EZH2 cause myelodysplastic/myeloproliferative disorders, implicating EZH2 as a tumor suppressor in myeloid malignancy(41, 42). Thus, EZH2 appears to behave similarly in this regard (41-43). Because EZH2 is essential for the function of PRC2, it is possible that an EZH2/AKT/β-catenin axis may operate in suppressing myeloid malignancy. The negative regulation of MDSCs by AKT described here bears some analogy to the reduction of AKT activity required for the development and function of Treg cells, another kind of immunosuppressive cells contributing to tumor immune evasion(44, 45), although dephosphorylation of AKT occurs only at Ser473, but not at Thr308, in activated Treg cells. These findings suggest that the AKT signaling is regulated differently in Treg and MDSCs. Indeed, the numbers of Treg cells remained largely unchanged in thymus and spleen of Cul4b CKO mice.
Although there was an increased abundance of Treg cells in tumor grafts in *Cul4b* CKO mice, it could be secondary to the increased accumulation and activity of MDSCs. PTEN, a primary negative regulator of AKT, was shown to be repressed by CUL4B in cancer cells (25). However, the level of PTEN was not found to be elevated in *Cul4b*-deficient hematopoietic cells including MDSCs. Thus, the CUL4B/AKT/β-catenin axis described here could be cell type specific.

Our studies showed that whether CUL4B functions as a promoter or as a suppressor of tumorigenesis is context dependent. Because AKT/β-catenin signaling cascades in carcinomas are often sought as therapeutic targets, systemic down-regulation of those pathways may sabotage the anti-tumor immune defense due to the expansion and activation of immunosuppressive cells such as MDSCs and Treg cells. On the other hand, the aberrant accumulation of MDSCs, and its promotion of tumor growth, in *Cul4b* CKO mice could be managed by the use of GSK3β inhibitors. Lithium, commonly used for treatment of bipolar disorders, may help restore cancer-targeting immunosurveillance.

**Author contributions**

YQ, CS and YG conceived the project, directed research, analyzed the results and wrote the manuscripts; YQ performed the majority of the experiments and data analysis; JY, HH, SZ and BJ assisted with isolated and characterized
mouse samples; QY and JL collected patient samples and performed clinical analysis.

Reference


Figure legends

Figure 1. Lack of CUL4B in endothelial and hematopoietic cells leads to enhanced accumulation of CD11b+Gr1+ cells.

A. X-gal staining of spleen and heart from Cul4b^loxP/Y Tek-Cre*+/− Rosa26 mice. Scale bar represents 100 μm (top) and 20 μm (bottom).

B. CUL4B expression levels in various tissues of WT and CKO mice.

C. CD11b+Gr-1+ cells in hematopoietic tissues of 6-week-old WT and CKO mice.

D. Flow cytometry analysis of CD4+ and CD8+ T cell proliferation (shown as CFSE dilution) in the presence of WT (blue line) and Cul4b CKO (red line) Gr-1+ cells. Splenocytes only (dashed line) was used as a control. The percentages of gated proliferative cells were summarized on the right. Results represent mean ± SEM (n = 8-13). Data are representative of more than three independent experiments. *, P< 0.05; **, P< 0.01.

Figure 2. Lack of CUL4B in hematopoietic system promotes tumor growth and metastasis.

A. EL4 lymphoma cells were injected subcutaneously into 6-8 week old WT or CKO mice, and tumor growth was monitored for 16 d. Then the tumors were resected and weighed.

B. Percentages of MDSCs in the blood, spleen, bone marrow and tumor site from mice bearing EL4 tumors.
C. Flow cytometry analysis of CD11b+Ly6G-Ly6C_{high} and CD11b+Ly6G+Ly6C_{low} cells from spleen and bone marrow from EL4-bearing WT or CKO mice. Numbers in the plots indicate the percent of gated cells.

D. Adoptive transfer of CD45.2^{+} Cul4b CKO or WT MDSCs into tumor-bearing CD45.1^{+} WT recipients were performed on days 3 and 5 after tumor transplantation. Tumor growth was evaluated for 2 weeks.

E. CD45.2^{+} (donor) and CD45.1 (host) MDSCs in blood of tumor-bearing mice on days 7 after tumor transplantation.

F. Growth of 4T1 mammary tumors in WT or Cul4b CKO mice.

G. B16/F0 metastasis to lung in WT and Cul4b CKO mice.

Results represent mean ± SEM (n = 6-10). Data are representative of more than three independent experiments. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

Figure 3. CUL4B inhibits the immunosuppressive activity of MDSCs.

A. Flow cytometry analysis of CD4^{+} and CD8^{+} T cell proliferation (shown as CFSE dilution) in the presence of WT (blue line) and Cul4b CKO (red line) M-MDSCs or G-MDSC. Numbers indicate the percentages of gated proliferative cells (right).

B. Flow cytometry analysis of CD4^{+} and CD8^{+} T cells from tumors and spleens of mice bearing EL4. The summary was shown on the right.

C. Percentages of CD4^{+}CD25^{+}Foxp3^{+} Treg cells in spleen, thymus and tumor of mice bearing EL4.
D. The percentage of CD4+CD25+Foxp3+ Treg cells induced in vitro. Splenocytes from WT mice were cultured with WT or Cul4b CKO MDSC and stimulated with anti-CD3 (10 μg/ml), anti-CD28 (2 μg/ml) and TGF-β (5 ng/ml).

E and F. Level of ROS (mean fluorescence intensity, MFI) in G-MDSC subsets isolated freshly or stimulated with PMA was measured using DCFDA staining.

G. NO release in supernatant of 1 × 10^5 M-MDSCs was assayed by a standard Greiss reaction with or without lipopolysaccharide (LPS) stimulation.

H. Transcript levels of the indicated genes in MDSCs from spleens of WT and CKO mice. Results are presented as the fold change over WT controls. Results represent mean ± SEM (n = 6-8). Data are representative of three independent experiments. **, P< 0.01; ***, P< 0.001.

Figure 4. Increased generation and impaired maturation of MDSCs in Cul4b CKO mice.

A. Flow cytometry analysis of BrdU incorporation in bone marrow CD11b+Gr1+ cells (left). Each dot in the plots indicates one individual mouse (right).

B. Splenocytes were prepared 16 days after EL4 cell inoculation and then stained for CD11b, Gr-1, and Annexin V. Each dot in the plots indicates one individual mouse (right).

C. Enriched Lin− cells isolated from bone marrow of WT and CKO mice were cultured with GM-CSF and IL-4 for 5 d in complete culture medium. The cells were then analyzed for CD11b and Gr-1 markers using flow cytometry.

E. Quantitative summary of MDSC differentiation shown in D.

F. Expression of S100A8 and S100A9 in MDSCs isolated from spleens of tumor-bearing mice. Results are presented as the fold change over WT controls.

G. Dynamic changes of CUL4B level in myeloid cells. Lane 1, Lin^- cells; Lane 2, Lin^- cells treated with IL-4 and GM-CSF for 5 days; Lane 3, Gr-1^ cells; Lane 4 and Lane 5, Gr-1^ cells treated with GM-CSF for 3 and 5 days, respectively.

Results represent mean ± SEM (n = 5-8). Data are representative of three independent experiments. **, P < 0.01; ***, P < 0.001, NS, not significant.

**Figure 5. Increased accumulation of MDSCs in Cul4b CKO mice was mediated by down-regulation of β-catenin.**

A. Expression levels of CUL4B, β-catenin, p-GSK3β (S9), GSK3β and GAPDH (loading control) in Gr-1^ cells isolated from naive (left) and M-MDSC, G-MDSC isolated from tumor-bearing (right) WT and CKO mice.

B. Bone marrow MDSCs from of tumor-bearing mice were cultured with GM-CSF, SB (SB216763) or LiCl for 24h, and cell lysates were analyzed for CUL4B, β-catenin and GAPDH (loading control).

C. Phenotypes of cells generated from Lin^- (left) and Gr-1^ cells (right) isolated from bone marrow of tumor-bearing WT and CKO mice cultured for 5
d with the indicated conditions. CA-β-catenin, constitutively activated β-catenin. Numbers indicate the percentages of differentiated cells. Results represent mean ± SEM (n = 6).

D. Negative regulation of CD11b+Gr-1+ by LiCl in vivo. Percentages of CD11b+Gr-1+ cells in blood from WT or CKO mice treated with saline or LiCl for 5 days are shown. Results represent mean ± SEM, n = 8 (WT), n = 9 (CKO).

E. Inhibition of tumor growth in CKO mice by LiCl. Five days after tumor injection, the mice were treated daily with saline or LiCl. Tumor growth was monitored for 15 d.

F. Inhibition of MDSC expansion in CKO mice by LiCl. Percentages of MDSCs (CD11b+Gr1+) are shown. Results represent mean ± SEM (n = 8).

For A-C, data are representative of three independent experiments. *, P< 0.05; **, P< 0.01. ***, P< 0.001. NS, not significant.

**Figure 6. Lack of CUL4B leads to up-regulation of AKT phosphatases PP2A and PHLPP1/2.**

A and B. Western blot analysis of phosphorylated AKT and AKT phosphatases in MDSCs.

C. mRNA levels of AKT phosphatases in MDSCs from Cul4b CKO mice. Data are shown as mean ± SEM (n = 6).

D. Generation of CD11b+Gr-1+ cells from Lin- cells transfected with shPhlpp2 or shPpp2cb or the shContol lentiviral.
E. Western blots of the indicated proteins in RAW264.7 cells in which CUL4B was knocked down or overexpressed.

F. Quantitative ChIP assay of the recruitment to or modification of the indicated proteins at the Phlpp2 and Ppp2cb promoters in RAW264.7 cells after transfection with control shRNA or shRNA targeting Cul4b. Results are represented as fold change over control. Data are shown as mean ± SD (n = 8).

G. RNA and protein were extracted from RAW264.7-CUL4B cells (or RAW264.7-NC cells) treated with TSA, DNZep or both. The mRNA levels of target genes were determined by qRT-PCR (left). Western blots were performed with the indicated antibodies (right). Data are shown as mean ± SD (n = 8).

Data are representative of three independent experiments. *, P< 0.05; **, P< 0.01. ***, P< 0.001. NS, not significant.

Figure 7. CUL4B/AKT/β-catenin axis is down-regulated in mouse and human MDSCs.

A. Cul4b expression in Gr1+ and Gr1- cells isolated from bone marrow (BM) and spleen (SP) of WT mice.

B. CUL4B expression in MDSCs and non-MDSCs from healthy donors.

C. Western blots of the indicated proteins in MDSCs isolated from spleen of naive (N), B16 and EL4 tumor-bearing WT mice.
D. The percentages of CD33*HLA-DR* cells in the peripheral blood of healthy donors and cancer patients determined by flow cytometry. \( n = 25 \) for healthy donors; \( n = 22 \) for cancer patients.

E. HLA-DR*CD33* cells isolated from PBMC of healthy donors and cancer patients were co-cultured for 72h with CSFE-labeled T cells from healthy donors and stimulated with anti-CD3/anti-CD28 in mitogen-driven experiments. Data are shown as mean ± SEM (\( n = 4 \)).

F. Negative correlation of CUL4B expression, measured by qRT-PCR, with the percentages of MDSCs in cancer patients (\( n = 22 \)).

G. Western blots of the indicated proteins in MDSCs isolated from healthy donors and cancer patients (left). Graph (right) shows protein levels based on band intensity after normalization to loading control, and healthy donors were assigned as 1. Data are shown as mean ± SEM (\( n = 8 \)). Data are representative of three independent experiments. *, \( P < 0.05 \); ***, \( P < 0.001 \).
Figure 3

(A) Mitogen-driven proliferation and antigen-driven proliferation

(B) Tumor and Spleen CD8+ T cells

(C) Treg cells in Spleen, Thymus, and Tumor

(D) Treg cells in Spleen, MDSCs

(E) Cell Count

(F) ROS (mean fluorescent intensity)

(G) Nitrite (micromolar)

(H) Gene expression (relative to Gapdh)

Authors: [Names]

Institution: [Institution]

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The CUL4B/AKT/β-catenin axis restricts the accumulation of myeloid-derived suppressor cells to prohibit the establishment of a tumor permissive microenvironment

Yanyan Qian, Jupeng Yuan, Huili Hu, et al.

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