

RNF125 Modulates Tumor Immunity by Promoting Ubiquitination of PD-L1

Yumeng Peng

Guangxi Medical University

Huan Yang

Guangxi Medical University

Zihui Li

Guangxi Medical University

Huilong Li

Guangxi Medical University

Xiaolin Qiu

Hainan Medical University

Meng Wei

Guangxi Medical University

Zhiwei Chen

Guangxi Medical University

Zhihong Tang

Guangxi Medical University

Tao Wei

Guangxi Medical University

Xiaobo Wang

Guangxi Medical University

Tao Bai

Guangxi Medical University

Congwen Wei

Academy of Military Medical Sciences

Feixiang Wu (✉ doctorwufx@sina.com)

Affiliated Tumor Hospital of Guangxi Medical University <https://orcid.org/0000-0002-2886-3524>

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Abstract

In recent years, the incidence of tumors has been increasing, and the overall cure rate by traditional treatment methods does not exceed 20%. One of the most effective and promising strategies for comprehensive treatment of tumors is immunotherapy, such as treatment with the PD-1/PD-L1 antibody. Here, we showed that ring finger protein 125 (RNF125), an E3 ligase in the RING domain family, could interact with PD-L1 to reduce the stability of PD-L1 protein. In addition, RNF125 downregulated the expression of PD-L1 by promoting its ubiquitination at K48, whereas a mutation in the RING domain of RNF125 disrupted this function. A significant positive correlation between RNF125 and genes involved with tumor immunity was determined in cancer samples, as determined using data from The Cancer Genome Atlas (TCGA). Furthermore, we elucidated the effects of RNF125 on the occurrence and development of tumors in mice. Analyses of wild-type and RNF125 knockout mice transplanted with MC-38 cells revealed enhanced MC-38 tumor growth in KO mice. These data indicated that RNF125 could participate in tumor immunity by promoting the K48-linked ubiquitination of PD-L1 to affect the occurrence and development of tumors, providing a potential target for enhancing therapeutic efficacy for human cancer treatment.

Introduction

The current clinical treatment methods for tumors mainly include surgical resection, radiotherapy and chemotherapy, but they all have limitations[1]. Clinical use of tumor immunotherapies have significantly prolonged the survival time of patients with melanoma, prostate cancer and lung cancer, causing immunotherapy to gradually become the fourth type of tumor treatment after surgery, radiotherapy and chemotherapy. It is believed to be the only way to completely eliminate cancer cells[2].

Treatments that target immune checkpoints, such as those that target programmed cell death protein 1 (PD-1) and its ligand PD-L1, have been approved for treating human cancers and have had robust clinical benefit[3, 4]. However, many cancer patients fail to respond to anti-PD-1/PD-L1 treatment, and the underlying reason for this is not well understood[5, 6]. Recent studies have revealed that the response to PD-1/PD-L1 inhibition may correlate with PD-L1 expression levels in tumor cells[7, 8]. So it is important to mechanistically understand the pathways controlling PD-L1 protein expression and stability, which can offer a molecular basis for improving the clinical response rate and efficacy of PD-1/PD-L1 inhibition in cancer patients.

Studies have shown that ubiquitination is important for PD-L1 regulation and that it plays an important role in PD-L1 mediation of immune checkpoint-related signaling pathways. For example, TNF α secreted under inflammatory conditions can promote the expression of the ubiquitinating enzyme CSN5, thereby inhibiting the ubiquitination and degradation of PD-L1. Inhibition of CSN5 by curcumin reduces the expression of PD-L1 in tumor cells and makes them more sensitive to CTLA4-mediated immunotherapy[9]. In addition, studies have reported that CMTM6, which exists on the cell surface, can reduce the ubiquitination of the PD-L1 protein, increase the half-life of PD-L1, and further enhance the

ability of tumor cells to suppress T cells. Moreover, CMTM4, which is in the CMTM family, acts as a CMTM6A supplement and is also involved in PD-L1 ubiquitination[10, 11]. A recent study has shown that the regulation of PD-L1 protein levels is periodic. CyclinD, CDK4 and Cullin 3 can combine with the E3 ligase SPOP to control the expression of PD-L1 by regulating the proteasome. The further study have also shown that in mouse tumors and human prostate cancer specimens, SPOP with loss-of-function mutations can not ubiquitinate and degrade PD-L1, thereby increasing the level of PD-L1 protein[12]. In view of the complexity of PD-1/PD-L1-mediated immune checkpoint-related signaling pathways and the diversity of ubiquitination regulation mechanisms, looking for new molecules that regulate PD-L1 in different tumor types and different backgrounds, determining PD-L1 ubiquitination sites and identifying the mechanism of PD-L1 action will provide a molecular basis for studying the different clinical response rates of immune inhibitors.

In this study, for the first time, we provide evidences that RNF125 functions to promote ubiquitination of PD-L1. Specifically, PD-L1 is speculated to be a candidate substrate of the E3 ligase RNF125 since a mutation in RNF125 that disrupts its ubiquitin ligase activity results in RNF125 being unable to reduce the expression of PD-L1. We believe our investigation of RNF125 in the immune checkpoint pathway will not only help us to further uncover a novel molecular mechanism for regulating PD-L1 protein stability, but will also reveal the potential to enhance therapeutic efficacy of human cancer treatments.

Materials And Methods

Plasmids and siRNA Mammalian expression vectors encoding N-terminus Flag, HA, and Myc tagged-proteins were constructed by inserting the corresponding PCR-amplified fragments into pcDNA3 (Invitrogen). The target sequences of siRNA for human RNF125 was 5' - CCGUGUGCCUUGAGGUGUUTT - 3'.

Cell Culture and Transfection HEK293T, Hep-G2, MHCC97-H cells were cultured in DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin and 100 mg/mL streptomycin. Transient transfections were performed with jetPRIME reagent (Polyplus) following the manufacturer's instructions. All experiments were performed with mycoplasma-free cells

Western Blotting and Immunoprecipitation Whole-cell lysates were prepared by sonication in modified RIPA buffer. 60–80 µg of protein per lane were loaded onto SDS-PAGE gels, followed by western blot analysis. When necessary, figures were cropped using Adobe Photoshop software (Adobe). Whole-cell lysates for coimmunoprecipitations were prepared by sonication in NP40 buffer (1% NP40, 150 mM NaCl and 40 mM Tris pH 7.5). Clarified lysates were then incubated with primary antibodies for 3hrs, then with protein G-agarose (Roche) for one hour, followed by extensive wash with NP40 buffer. Supplementary Table S1 listed the primary antibodies used in this study.

Correlation analyses The mRNA expression and clinical data for liver, prostate and breast cancers were downloaded from the GDAC (Genome Data Analysis Center) Firehose at the BROAD Institute. The expression values were the normalized Log2 RSEM RNA-Sequencing data[13]. Pearson correlation was

calculated for expression of RNF125 with expression of PD-L1. The Benjamini & Hochberg adjusted p-value was calculated to measure the statistical significance of the observed correlations.

Quantitative real-time PCR (QRT-PCR) First-strand cDNA was synthesized as described previously[14]. QRT-PCR was performed in the iQ5 Real-time PCR System (Bio-Rad) using iTaq universal SYBR Green supermix (Bio-Rad). Each sample was analyzed in triplicate with GAPDH as the internal control. Supplementary Table S2 listed the primer sequences used for different genes in this study.

Animal model After culturing MC-38 cells in vitro and passage to a suitable state, a cell suspension was prepared. Adjusting the cell concentration to 2×10^6 /ml with PBS under aseptic conditions, the tumor cell suspension was aseptically inoculated on the back of C57BL/6 mice, and the tumor cell inoculation amount was 2×10^5 /head. The growth of tumor-bearing mice was observed daily, and the volume of the tumor was measured every other day from the 6th day of the tumor. A vernier caliper was used to calculate the longest diameter (D) and the shortest diameter (d) of the tumor. Tumor volume $V = D \times d^2 / 2$.

Statistical analysis Statistical analysis was performed using SPSS 17.0 (SPSS Inc, Chicago, IL) and R 2.13.0 ([http:// www.r-project.org](http://www.r-project.org)). All statistical tests were two-sided tests, and P values < 0.01 were considered to be statistically significant.

Results

RNF125 negatively regulates the stability of PD-L1

In view of the important role of PD-L1 in tumor immunity, we used PD-L1 as the target in this project to identify new PD-L1 regulatory molecules and determine their regulatory mechanism and physiological significance. Thus, we firstly verify the interaction between RNF125 and PD-L1. We transfected cells with Flag-RNF125 and Myc-PD-L1. Western blot analysis showed a significant association between Flag-RNF125 and Myc-PD-L1 (Fig. 1A).

To confirm that RNF125 could regulate the expression of PD-L1, we used transient transfection to introduce successively increasing concentrations of Myc-RNF125 and HA-PD-L1 into HEK293T cells, and then we harvested the cells 24 hours later. Whole cell lysates were used for western blotting, where we observed the effect of different doses of RNF125 on the expression of PD-L1. As the amount of Myc-RNF125 that was transfected increased, the expression level of HA-PD-L1 protein gradually decreased in a dose-dependent manner (Fig. 1B).

To further study the effect of RNF125 on the stability of PD-L1 in tumor, we first used IFN- γ to treat HEK293T cells, HL-7702 cells, MHCC-97H cells, SMCC-7721 cells and Hep-G2 cells. We selected human liver cancer Hep-G2 cells for the next experiment because RNF125 expression was obviously induced by IFN- γ (Fig. 1C). The same method was used to transfect HA-RNF125 into Hep-G2 cells. After IFN- γ was added to stimulate the cells, the expression of PD-L1 gradually was found to decrease in a dose-dependent manner (Fig. 1D). Then, we transfected an siRNA specific for RNF125 and a control siRNA into

Hep-G2 cells, added IFN- γ , and collected the cells 36 h later. The collected cells were equally divided into two parts: one part was used to detect the knockdown efficiency of RNF125 by QRT-PCR, and the other part was used in the western blot experiments, where the expression of PD-L1 was detected by PD-L1 antibody. The results showed that transfection with si-RNF125 significantly reduced the expression of RNF125 in Hep-G2 cells (Fig. 1E), and the PD-L1 expression level in the RNF125 knockdown group was significantly higher than it was in the non-knockdown control group (Fig. 1F). These results suggest that RNF125 can negatively regulate the expression of PD-L1, and show a negative correlation after IFN- γ stimulation.

E3 ligase RNF125 modifies the expression of PD-L1 through ubiquitination

To understand whether the ubiquitin E3 ligase RNF125 reduced the expression of PD-L1 through its ubiquitination enzyme activity, we first cotransfected the Flag-RNF125 and HA-PD-L1 expression plasmids and the ubiquitin expression plasmid Myc-Ub into HEK293T cells through transient transfection. Compared with that in the Flag-Vector group, the level of PD-L1 ubiquitination in the Flag-RNF125 group was significantly increased, indicating that RNF125 could promote the ubiquitination of PD-L1 (Fig. 2A). To further confirm the ability of the ubiquitin E3 ligase RNF125 to act through its Ring domain to ubiquitinate PD-L1, we transiently transfected Flag-RNF125 and Flag-RNF125 (C72/75A) and HA-PD-L1 plasmids. The HA-PD-L1 expression plasmid and the ubiquitin expression plasmid Myc-Ub were cotransfected into HEK293T cells for ubiquitination experiments. The results showed that compared with the Flag-Vector group, the ubiquitination level of PD-L1 increased significantly after adding Flag-RNF125, while the ubiquitination level of PD-L1 after adding Flag-RNF125 (C72/75A) was lower than it was in the group that received Flag-RNF125 (Fig. 2B). These results indicate that the E3 ligase RNF125 can promote the ubiquitination of PD-L1 through its Ring domain, which is in the of the active enzymatic region.

Studies had shown that glycosylation of PD-L1 could inhibit its ubiquitination, so we added tunicamycin (TM) and then studied the effect of RNF125 on the ubiquitination level of PD-L1. We cotransfected HEK293T cells with Flag-RNF125 and Flag-RNF125 (C72/75A) with the HA-PD-L1 expression plasmid and the ubiquitin expression plasmid Myc-Ub; then, 24 hours after plating the cells, TM was added at a final concentration of 5 μ M for 8 hours. Then, 5 μ mol/L MG132 was added to the supernatant to stimulate the cells, and the cells were collected for analysis after 8 hours. We determined that after adding TM, the overall ubiquitination level of PD-L1 was enhanced compared with of the group that was not treated with TM (Fig. 2C). The most common ubiquitination modifications in cell signal transduction were Lys48-linked ubiquitination and Lys63-linked ubiquitination. To study the ways that RNF125 ubiquitinated PD-L1, we transfected HEK293T cells with different forms of HA-Ub (WT, K48 only, or K63 only) with Flag-RNF125 and Myc-PD-L1 and then added MG132 (5 μ mol/L) 24 hours later. Eight hours later the cells were collected. The results showed that compared with the Flag-Vector group, the group with HA-Ub (WT) and HA-Ub (K48 only) could promote the ubiquitination of Myc-PD-L1, but after adding HA-Ub (K63 only), there was no significant difference in the ubiquitination of Myc-PD-L1 compared with the control group (Fig. 2D). Therefore, PD-L1 is mainly undergoes K48 ubiquitination .

Rnf125 Promotes Tumor Immunity In Mice

Finally, we analyzed the association of PD-L1 with CD8 and F4/80 using the database from TCGA to study the effect of RNF125 on tumor growth and immunity. A positive correlation between RNF125 expression and genes involved in tumor immunity in colorectal adenocarcinoma and liver hepatocellular carcinoma was identified (Fig. 3A-D).

Then, we used the RNF125 knockout mouse model to examine our data, and we prepared cell suspensions of MC-38 cells cultured in vitro and subcutaneously injected them into the backs of RNF125 KO mice and WT mice. The growth of tumor-bearing mice was observed every day, and the volume of tumor masses was calculated every other day beginning on the 6th day after tumor detection. We found that the tumors in KO mice grew significantly faster than they did in WT mice (Fig. 4F). On the 12th day after tumors were detected, we dissected the tumors from the mice and found that the tumor size in KO mice was visibly larger (Fig. 3E). The body weight of KO mice was also much greater than that of WT mice due to the larger tumor (Fig. 3F). In summary, RNF125 can promote tumor immunity in mice by fighting against tumor development.

Discussion

PD-L1 plays an important role in promoting liver immunity, and it is involved in the occurrence and development of liver diseases. Clinical studies suggest that whether the PD-1/PD-L1 antibody can effectively play a role in tumors is closely related to the expression of PD-L1, while ubiquitination of PD-L1 plays an important role in its regulation[12]. In view of the complexity of PD-1/PD-L1-mediated immune checkpoint-related signaling pathways and the diversity of kinds of ubiquitination regulation, the results we report here will provide a molecular basis for the study of different clinical response rates of immune inhibitors, enabling the search for new molecules that regulate PD-L1 in different tumor types and different backgrounds as well as enabling the identification of ubiquitination sites, ubiquitination forms and mechanisms of action. Our study reveals that RNF125 can interact with PD-L1 and negatively regulate PD-L1 expression.

Studies have shown that RNF125 may regulate the occurrence and development of liver diseases through innate immunity[15]. However, it is not clear whether RNF125 can regulate the tumor immune response and participate in tumor development. In this study, we show that the expression of RNF125 and PD-L1 are still negatively correlated following IFN- γ stimulation, indicating that RNF125 not only can participate in the progression of liver disease through innate immunity but also can directly participate in the tumor immune response through the role of tumor signaling pathways. Our analysis of the database from TCGA demonstrates positive correlations between RNF125 and several key genes, such as CD8 and F4/80, which are implicated in tumor immunity.

In addition to its role in immune regulation, recent studies have also found that RNF125 is also involved in tumor development and drug resistance[16, 17]. However, it is not clear whether RNF125 can modify

other substrates through ubiquitination to regulate tumor immunity. Here we show that RNF125 can downregulate the expression of PD-L1 in a dose-dependent manner, and the PD-L1 ubiquitination experiment suggest that RNF125 can promote PD-L1 ubiquitination, whereas RNF125 with mutations in the RING domain loses its enzymatic function toward PD-L1. This indicates that RNF125 can modify PD-L1 through ubiquitination to promote the degradation of PD-L1, so this study further illuminates how PD-L1 is posttranslationally modified. In the RNF125 KO tumor-bearing mouse model, we have found that WT mice survive better than KO mice because they have smaller tumor size.

In conclusion, we have established that RNF125 participates in tumor immunity by regulating PD-L1 and have further explored the molecular mechanism of this process. This study enriches the understanding of the tumor immune regulation mechanism for the occurrence and development of tumors, establishes diagnostic and prognostic indicators for tumor immunity, and provides a foundation for the development of therapeutic drugs targeting immune checkpoints.

Declarations

Authors' contributions Y.P. and H.Y. performed the experiments and wrote the paper. Z.L. and H.L. analyzed the data. F.W. and C.W. designed the project. T.B., X.W. and MW. provided the core facilities. Z.C., Z.T. and T.W. shared partial reagents.

Conflict of interest None.

Data Availability Statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement In vivo experiments were performed in accordance with protocols approved by the Ethics Committee of Affiliated Tumor Hospital of Guangxi Medical University.

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Figures

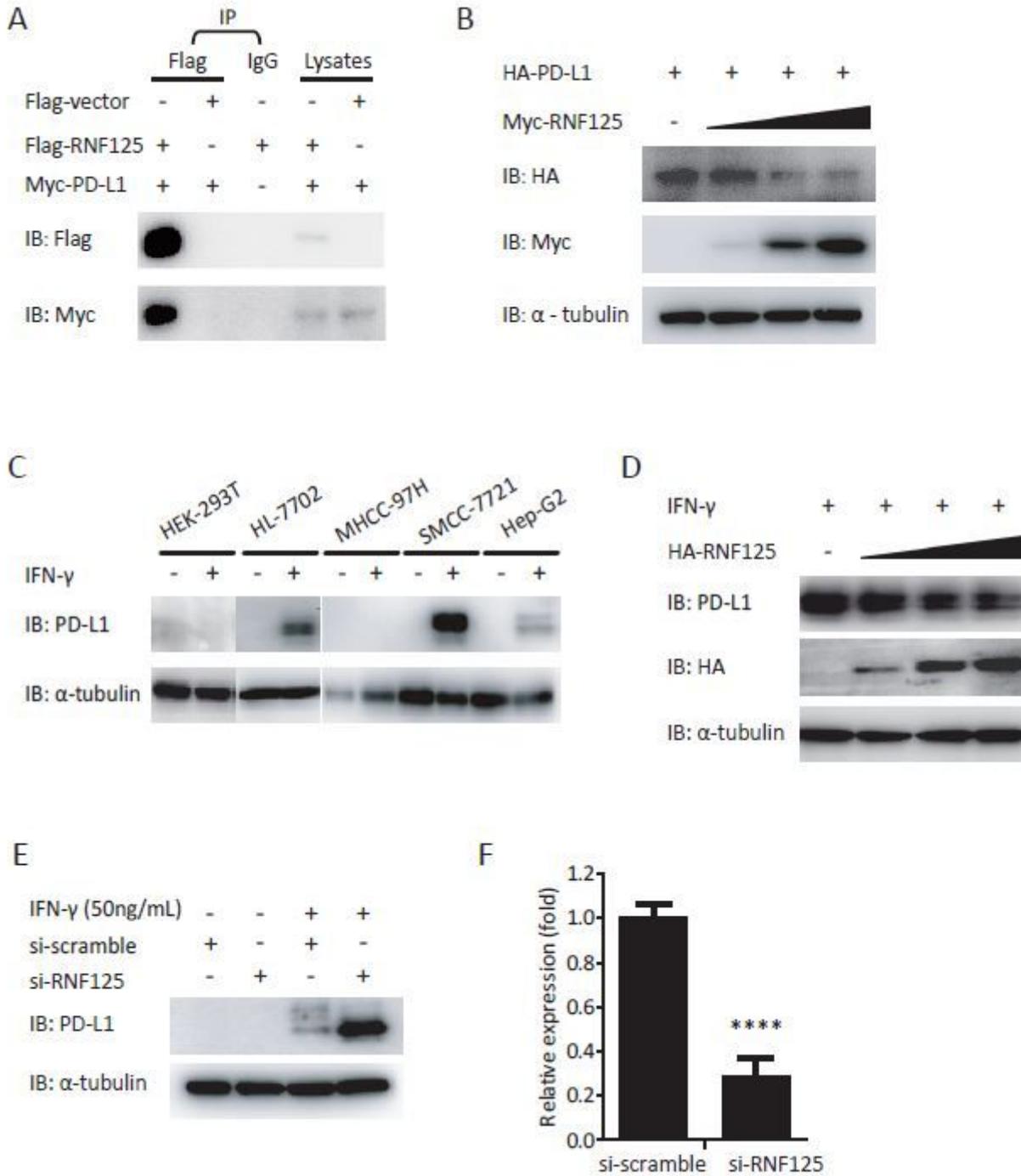


Figure 1

The effect of RNF125 on the expression of PD-L1 (A) Immunoprecipitation analysis in HEK293T cells transfected with Flag-vector or Flag-RNF125 in the presence of Myc-PD-L1. (B) Increasing amounts of Myc-RNF125 and HA-PD-L1 were cotransfected into HEK293T cells. Then, whole cell lysate was used for western blotting. (C) IFN- γ (50 ng/mL) was added to HEK293T, HL-7702, MHCC-97H, SMCC-772 and Hep-G2 cells. Western blotting was used to determine how the amount of PD-L1 changed in a liver cell line following treatment with IFN- γ and a PD-L1 antibody. (D) HA-RNF125 was added at increasing amounts to human liver cancer Hep-G2 cells, and after adding 50 ng/mL IFN- γ , the expression of RNF125 and PD-L1 was detected. (E) RNF125 was knocked down in Hep-G2 cells with an siRNA, IFN- γ was added to stimulate cells for 24 hours, and western blotting was used to detect PD-L1 expression. (F) The knockdown efficiency of RNF125 was determined using q-PCR.

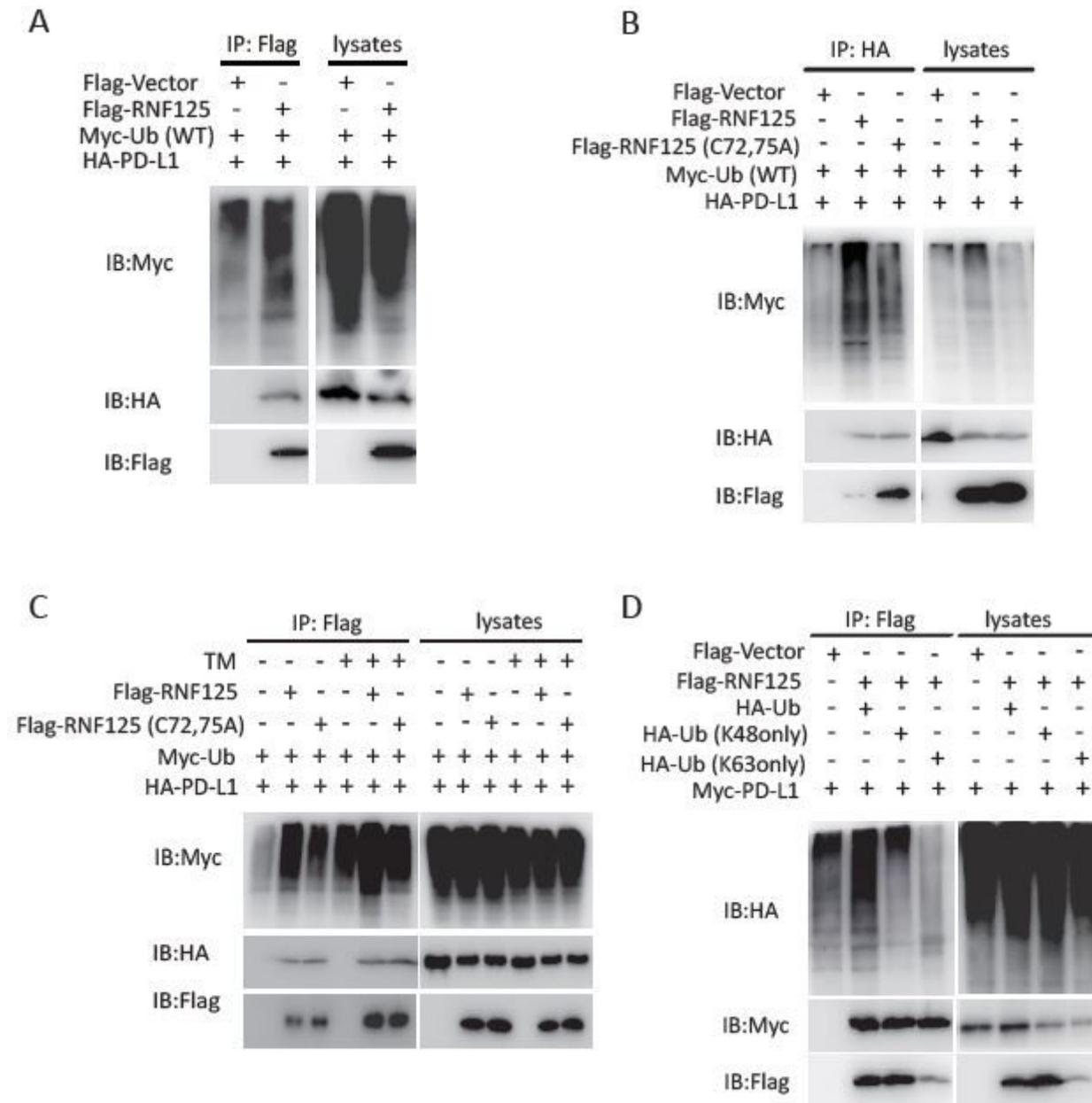


Figure 2

Modulation of PD-L1 ubiquitination by RNF125 (A) Flag-RNF125, Myc-Ub and HA-PD-L1 were cotransfected into HEK293T cells. After immunoprecipitation with anti-HA magnetic beads, the level of PD-L1 ubiquitination was detected. (B) Flag-RNF125 and Flag-RNF125 (C72/75A) expression plasmids were cotransfected into HEK293T cells with an HA-PD-L1 expression plasmid and a ubiquitin expression plasmid (Myc-Ub (WT)) and then were tested by to determine the ubiquitination level of PD-L1. (C) Flag-RNF125 and Flag-RNF125 (C72/75A) were cotransfected into HEK293T cells with an HA-PD-L1 expression plasmid and a ubiquitin expression plasmid Myc-Ub (WT). One group was treated with TM, and the other group was not treated. The level of PD-L1 ubiquitination was detected through a ubiquitination experiment. (D) Flag-RNF125, different forms of Ub (WT, K48 only, and K63 only) and HA-PD-L1 were cotransfected into HEK293T cells, and the level of PD-L1 ubiquitination was detected through ubiquitination experiments.

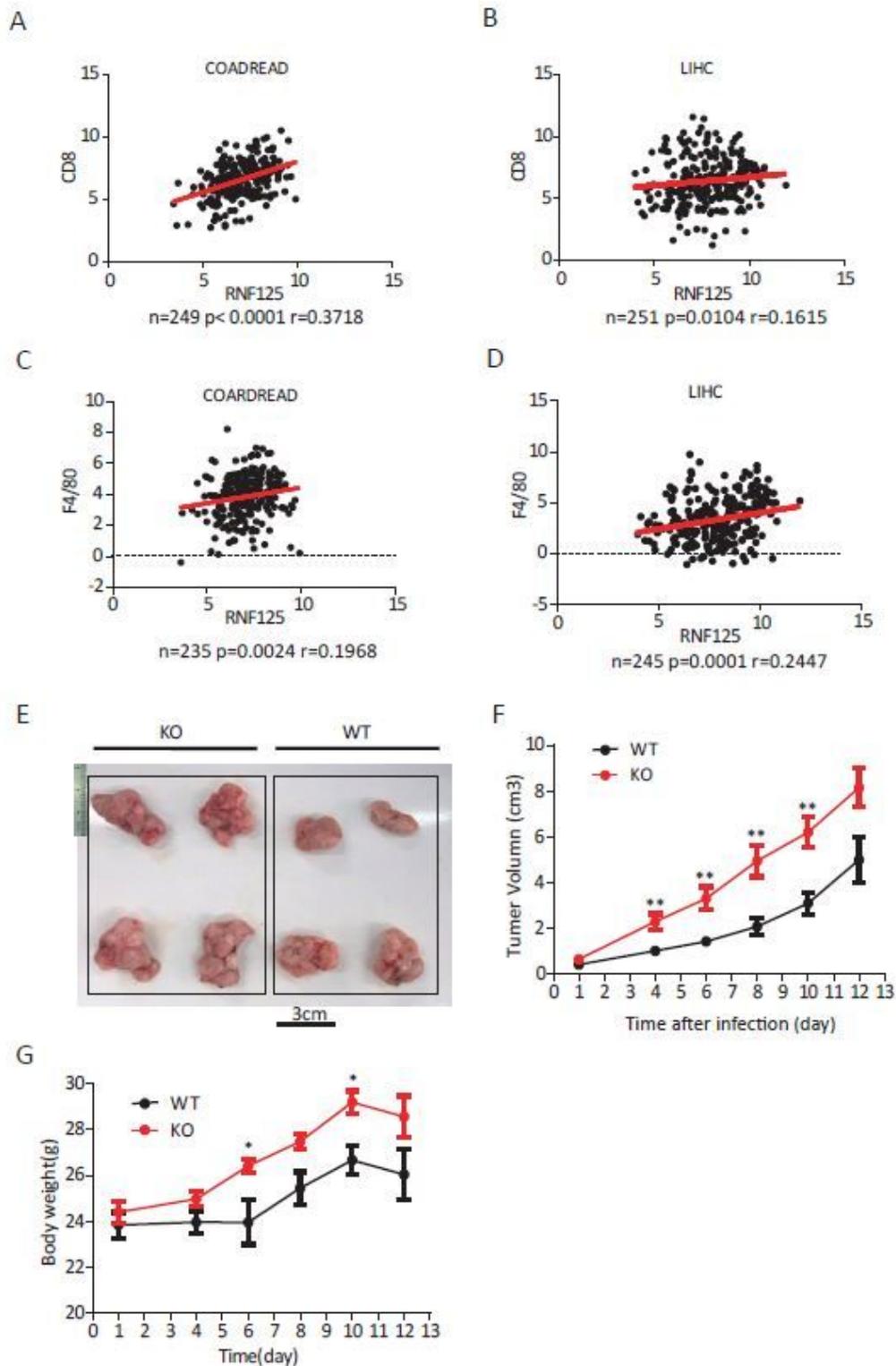


Figure 3

The effect of RNF125 on tumor immunity in mice (A-D) The correlation between RNF125 expression and CD8 and F4/80 in colorectal adenocarcinoma and liver hepatocellular carcinoma samples from the data set from TCGA. A RNF125 KO tumor-bearing mouse model was constructed, the body weight (G) was recorded every other day beginning at the 6th day of tumor observation, and the volume of the tumor

mass was calculated (F). Mice were dissected on the 12th day after tumor observation, and tumor size was measured (E).

Supplementary Files

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