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CDK5 destabilizes PD-L1 via chaperon-mediated autophagy to control cancer immune surveillance in hepatocellular carcinoma

Ruonan Zhang,[1,2] Jie Wang,[1] Yu Du,[3] Ze Yu,[1] Yihan Wang,[4] Yixiao Jiang,[5] Yixin Wu,[1] Ting Le,[1] Ziqi Li,[1] Guoqiang Zhang,[5] Lei Lv ,[2] Haijie Ma[1]

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RZ, JW and YD contributed equally.

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For numbered affiliations see end of article.

Correspondence to
Dr Haijie Ma;
haijie215@163.com

Professor Lei Lv;
lvlei@fudan.edu.cn

ABSTRACT

Background In the past few years, immunotherapies of hepatocellular carcinoma (HCC) targeting programmed cell death protein 1 (PD-1) and its ligand programmed cell death ligand 1 (PD-L1), have achieved durable clinical benefits. However, only a fraction of HCC patients showed objective clinical response to PD-1/PD-L1 blockade alone. Despite the impact on post-translational modifications of PD-L1 being substantial, its significance in resistance to HCC immunotherapy remains poorly defined.

Methods Cyclin-dependent kinase 5 (CDK5) expression was knocked down in HCC cells, CDK5 and PD-L1 protein levels were examined by Western blot. Coimmunoprecipitation was conducted to evaluate the interaction between proteins. Preclinical HCC mice model was constructed to evaluate the effect of CDK5 inhibitor alone or in combination with PD-1 antibody. Clinical HCC samples were used to elucidate the clinical relevance of CDK5, PD-L1, and PD-L1 T290 phosphorylation in HCC.

Results We find that CDK5 deficiency upregulates PD-L1 protein expression in HCC cells and decipher a novel molecular mechanism under which PD-L1 is downregulated by CDK5, that is, CDK5 mediated PD-L1 phosphorylation at T290 promotes its binding with chaperon protein heat-shock cognate protein 70 (HSC70) and degradation through chaperon-mediated autophagy. Notably, treatment of CDK5 inhibitor, PNU112455A, effectively upregulates the tumorous PD-L1 level, promotes the response to anti-PD-1 immunotherapy,and prolongs the survival time of mice bearing HCC tumors. What is more, the T290 phosphorylation status of PD-L1 correlates with the prognosis of HCC.

Conclusions Targeting CDK5 can synergize with PD-1 blockade to suppress HCC growth, which may have clinical benefits. Our study reveals a unique regulation of the degradation of PD-L1 in HCC, and provides an attractive therapeutic target, a potential drug, and a new prognostic marker for the clinical treatment of HCC.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Only a fraction of hepatocellular carcinoma (HCC) patients showed objective clinical response to PD-1/PD-L1 blockade alone, highlighting the urgent need for developing novel therapeutic strategies.

WHAT THIS STUDY ADDS

⇒ Cyclin-dependent kinase (CDK5) phosphorylates PD-L1 at T290 and promotes the interaction between PD-L1 and HSC70, which facilitates PD-L1 degradation through chaperon-mediated autophagy. Upregulation of PD-L1 by CDK5 inhibitor PNU112455A improves the efficacy of anti-PD-1 therapy in preclinical hepatocellular carcinoma models.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The inhibitory effects of CDK5 synergizing with PD-1 blockade represents a potential novel strategy for HCC immunotherapy.

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is the third-leading cause of cancer-related death worldwide, mainly due to limited treatment options and late diagnosis.[1 2] In the past few years, immunotherapies of HCC targeting programmed cell death protein 1(PD-1) and its ligand programmed cell death ligand 1(PD-L1), have achieved durable clinical benefits.[3–5] However, only a fraction of HCC patients showed objective clinical response to PD-1/PD-L1 blockade alone.[6] Therefore, a comprehensive study to understand the mechanism of PD-1/PD-L1-based combinational therapy may significantly contribute to developing novel therapeutic strategies.

Accumulating evidence has indicated that post-translational modifications (PTMs) of PD-L1, mainly including N-glycosylation,[7 8] phosphorylation,[9 10] ubiquitination,[11 12] acetylation[13] and palmitoylation,[14 15] play crucial roles in modulating the translocation, recycling and stability of PD-L1, as well as its interaction with PD-1.[7–15] For instance, PD-L1 is phosphorylated by glycogen synthase kinase 3β (GSK3β) at T180 and S184 sites, leading to

its polyubiquitination by β-transducin repeat-containing protein (β-TRCP). Epidermal growth factor receptor (EGFR) governs GSK3β-mediated PD-L1 phosphorylation and degradation, and inhibition of EGFR increases the efficacy of anti-PD-1 therapy in mouse models.[7] Due to the wide use of EGFR inhibitors in the treatment of cancer, combination with PD-1/PD-L1 blockade provides a promising avenue to enhance the efficacy of immunotherapy.[16] P300-mediated acetylation and HDAC2-dependent deacetylation of PD-L1 modulate the nuclear translocation of PD-L1, which governs the expression of multiple immune response-related genes. Blocking PD-L1 nuclear translocation by HDAC2 inhibitor largely improves the efficacy of PD-1 blockade.[13] These findings suggest that targeting PTMs of PD-L1 has been emerging as an effective strategy for improving the efficacy of PD-1/PD-L1 blockade. Thus, the identification of crucial regulators of PD-L1 PTMs is urgently required to boost anti-PD-1/PD-L1 therapy for cancer.

Cell cycle proteins that are frequently dysregulated in tumors, such as cyclin-dependent kinases (CDKs), have attracted considerable interest as potential targets for cancer therapy.[17–19] Thus, beginning with the demonstration that CDK4/CDK6 inhibitors, including palbociclib (PD-0332991), ribociclib (LEE-011) and abemaciclib (LY2835219), can be used for treating hormone receptor-positive breast cancer.[20 21] The safety and efficacy of these and other CDK4/CDK6 inhibitors in various hematological malignancies and solid tumors are now being evaluated (ClinicalTrials.gov). Strikingly, a novel function of CDK4 to regulate PD-1/PD-L1-mediated tumor immunity has been recently identified. The protein level of PD-L1 is modulated by cyclin D-CDK4 and the Cullin3^{SPOP} E3 ligase by ubiquitination-dependent degradation. Notably, the combination of CDK4/6 inhibitor and anti-PD-1 immunotherapy dramatically enhances tumor regression and prolongs the overall survival of tumor-bearing mice.[11] CDK5, a unique CDK, is activated on binding to p35 and p39 proteins, but not cyclins.[22] Importantly, CDK5 is a multifunctional kinase physiologically involved in proliferation, invasion, angiogenesis, genome instability and metabolism in the development of cancers.[23–26] However, the potential role of CDK5 in the modulation of PD-L1 PTMs involved in cancer immune surveillance is still unclear.

In this study, we found that CDK5 phosphorylated PD-L1 at T290 to promote its interaction with chaperon protein heat-shock cognate protein 70 (HSC70) and degradation through chaperon-mediated autophagy (CMA). Furthermore, inhibition of CDK5 significantly upregulated the tumorous protein level of PD-L1 and improved the efficacy of PD-1 blockade in preclinical HCC models. Our study strongly highlights the significance of CDK5 in the regulation of PD-L1 PTMs and therefore provides a potential strategy for improving the efficacy of HCC immunotherapy.

MATERIALS AND METHODS

Cell culture and transfection

The human HCC cell lines BEL-7402 and SMMC-7721, the mouse HCC cell line Hepa1-6, as well as the 293T cells, were cultured in the Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Transfection of the plasmid was conducted using the EZ-Trans reagent. Transfection of small interfering RNA (siRNA) was conducted using the Lipofectamine RNAiMAX Transfection Reagent. All experimental procedures were performed according to the manufacturer's protocols. For lentivirus packaging, the packaging plasmids psPAX2 and pMD2.G, together with the LentiCRISPRv2 KO constructs were transfected into the 293T cells. After replenishing with fresh culture media 8 hours post transfection, the cell culture supernatant was collected twice with 24 hours intervals and filtered using a 0.45 µm filter followed by aliquoting and freezing. For lentivirus infection, the lentivirus-containing medium with polybrene (10 µg/mL) supplementation was used to culture the target cells for 48 hours, after which the cells were subjected to puromycin selection.

Coimmunoprecipitation

The cell lysates were centrifuged at 12 000 rpm for 15 min in 4°C condition to collect the supernatants. Then, the supernatants were incubated with anti-FLAG beads overnight at 4°C. After incubation, the beads were washed with NP-40 buffer for three times and heated with SDS-PAGE sample loading buffer at 95°C for 15 min to prepare for Western blot analysis.

Immunohistochemistry

Mouse tumors were dissected and stored in 4% polyformaldehyde. For immunohistochemistry (IHC) staining, the tumor samples were subjected to fixation, sectioning, and staining according to the standard protocol. Antibodies used for IHC staining are shown above. The numbers of $CD3^+$, $CD8^+$ T cells and granzyme B expression were examined under a microscopic field at 200×magnification. The HCC tumor specimens were obtained from Outdo BioTech (Shanghai, China). The following IHC steps were according to the standard protocol. For the results, the proportion of PD-L1 and p-PD-L1(T290) in HCC samples was graded as follows: ≤5%, grade 0; 6%–19%, grade 1; 20%–49%, grade 2; 50%–74%, grade 3; ≥75%, grade 4. Staining grades included: 0, no staining; 1, weakly positive staining; 2, medium staining; 3, strong staining. Based on the product of the two scores, a total score of ≤3 represented low expression, and >3 indicated high expression. The criteria for the CDK5 staining was scored as follows: the intensity score was counted as the same as PD-L1 and the proportion of stained cells was classified as ≤25%, grade 1; 26%–74%, grade 2; 75%–89%, grade 3; ≥90%, grade 4. Based on the product of the two scores, we defined a final staining score of ≤4 as the low expression group, and >4 indicated the high expression group.

Animal experiments

C57BL/6 mice (6–8 weeks old) were purchased from Qizhen Laboratory Animals. Hepa1-6 (1×10^6 cells) were implanted subcutaneously into the right flank of mice. The PD-1 antibody (10 mg/kg)[27] and PNU (150 mg/kg[28]; solvent: PBS/DMSO/Solutol 8:1:1) were injected intraperitoneally on days 9, 12, 15, and 18. Tumors were measured every 3 days with a caliper, and tumor volume was calculated using the formula: $\pi/6\times\text{length}\times\text{width}^2$. Mice were sacrificed when tumors reached 1500 mm^3.

Statistical analysis

GraphPad Prism V.8 software was used to perform statistical analysis. Data of bar graphs represent as fold change or percentage relative to control with SD of three independent experiments. Normally distributed data were analyzed using Student's t-test. One-way analysis of variance with Tukey's multiple comparisons was used when more than two groups were analyzed. Survival was estimated by the Kaplan-Meier method and compared by the log-rank test. Statistical significance was defined as a $p<0.05$. Levels of significance were indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Supplemental methods

Chemicals and reagents, DNA construction and mutagenesis, quantitative reverse transcriptase PCR, RNA interference, Western blot, immunofluorescence staining and flow cytometry are shown in online supplemental materials.

RESULTS

CDK5 negatively regulates the protein level of PD-L1

To assess the effect of CDK5 on PD-L1 expression, we first overexpressed CDK5 in 293 T cells stably expressing Flag-PD-L1, and found that Flag-PD-L1 was significantly downregulated by CDK5 overexpression (figure 1A). As shown in figure 1B, the expression of PD-L1 was also decreased in CDK5 overexpressing HCC cells. In line with

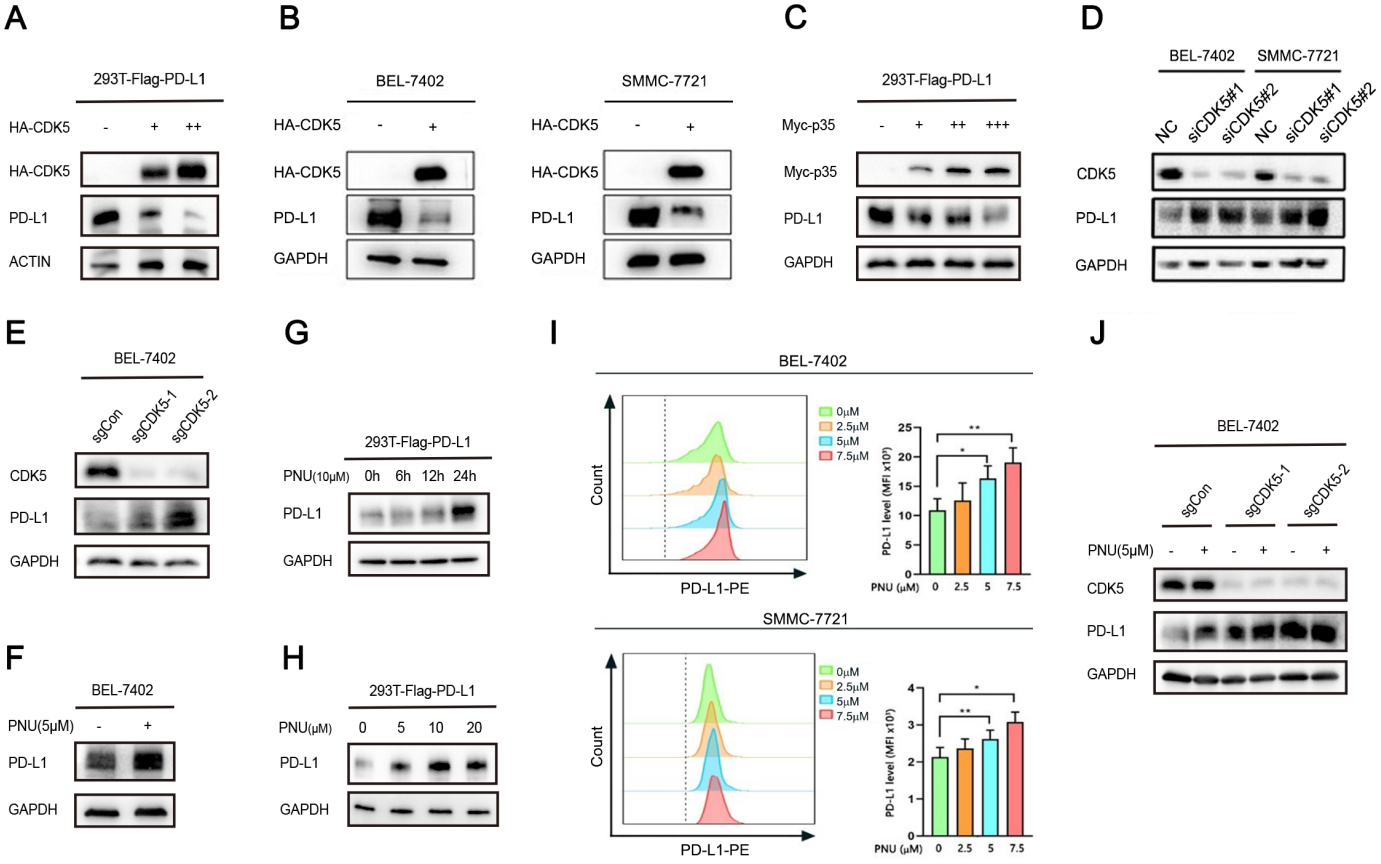

Figure 1 CDK5 negatively regulates PD-L1 protein level. (A) Western blot analysis of Flag-PD-L1 protein levels under overexpression of HA-CDK5 in 293 T cells stably expressing Flag-PD-L1. (B) Western blot analysis of PD-L1 protein levels under overexpression of HA-CDK5 in HCC cells. (C) Western blot analysis of Flag-PD-L1 protein levels under overexpression of Myc-p35 in 293 T cells stably expressing Flag-PD-L1. (D) Western blot analysis of PD-L1 protein levels under CDK5 knockdown in BEL-7402 and SMMC-7721 cells. (E) Western blot analysis of PD-L1 protein levels under CDK5 knockout in BEL-7402 cells. (F) Western blot analysis of PD-L1 protein levels under the treatment of PNU in BEL-7402 cells. (G, H) Western blot analysis of Flag-PD-L1 protein levels under the indicated concentration or time of PNU treatment in 293 T cells stably expressing Flag-PD-L1. (I) Flow cytometry analysis of PD-L1 protein levels on surfaces of HCC cells treated with PNU (n=3). (J) Western blot analysis of PD-L1 protein levels under the treatment of PNU in control and CDK5 knockout BEL-7402 cells. CDK, cyclin-dependent kinase; HCC, hepatocellular carcinoma.Levels of significance were indicated as *$p<0.05$, **$p<0.01$.

this, overexpression of p35, a CDK5 activator, dramatically decreased the Flag-PD-L1 level in a dose-dependent manner (figure 1C). In contrast to CDK5 overexpression and activation, knockdown of CDK5 considerably increased the protein expression of PD-L1 in BEL-7402 and SMMC-7721 cells (figure 1D), but not the mRNA level (online supplemental figure 1). Consistently, genetic depletion of CDK5 with sgRNAs or pharmacological inhibition of CDK5 with PNU112455A (PNU) also upregulated protein levels of PD-L1 (figure 1E,F). In addition, elevated Flag-PD-L1 levels were observed by the treatment of PNU in 293T cells, in both time-dependent and dose-dependent manners (figure 1G,H). Also, upregulation of PD-L1 on the surface of BEL-7402 and SMMC-7721 cells was found in a dose-dependent manner (figure 1I). Moreover, this effect of PNU was dramatically reversed by knockout of CDK5 (figure 1J), suggesting that the function of PNU in the upregulation of PD-L1 protein was dependent on CDK5. Taken together, these findings strongly indicate that CDK5 negatively regulates the protein level of PD-L1.

CDK5 promotes PD-L1 degradation through lysosome

To determine how CDK5 regulates PD-L1, we examined the half-life of Flag-PD-L1 in 293T cells under the treatment of protein synthesis inhibitor cycloheximide (CHX) and CDK5 inhibitor PNU, and found that inhibition of CDK5 dramatically suppressed Flag-PD-L1 degradation (figure 2A). Consistently, knockdown of CDK5 or inhibition of CDK5 with PNU also significantly prolonged the half-life of endogenous PD-L1 in both BEL-7402 and SMMC-7721 cell lines (figure 2B–E). To figure out the pathway through which CDK5 downregulates PD-L1, CDK5 was overexpressed into 293T cells stably overexpressing Flag-PD-L1. As shown in figure 2F,G, supplementation with lysosome inhibitor NH_4Cl, but not proteasome inhibitor MG132, could rescue the PD-L1 level decreased by CDK5 overexpression, indicating that lysosome was involved in CDK5 mediated PD-L1 degradation.

CDK5 binds to and phosphorylates PD-L1 at T290

Next, we further investigated the mechanisms by which CDK5 destabilized PD-L1 in HCC cells. Given that CDK5 was a serine/threonine kinase, we wondered whether CDK5 could phosphorylate PD-L1 and decrease its stability. As expected, we found that CDK5 interacted with Flag-PD-L1 by coimmunoprecipitation (co-IP) assay (figure 3A,B). In line with these, PD-L1 colocalized with CDK5 on cell membrane and in cytoplasm in 293T cells stably expressing Flag-PD-L1 (figure 3C). Furthermore, both full-length and intracellular domain of PD-L1 could bind with CDK5, but not the extracellular domain truncation (figure 3D). In addition, inhibition of CDK5 decreased the pan-threonine phosphorylation levels of PD-L1 in both dose-dependent and time-dependent manners (figure 3E,F). Then, we tried to determine the phosphorylation site on PD-L1 by CDK5. According to the mass spectrometry data provided by the PhosphoSite Plus database (https://www.phosphosite.org/), nine potential serine/threonine phosphorylation sites were identified on PD-L1 protein. Notably, T290, which mainly mutates from T to A (T290A) in tumors, has been reported to be the most frequent mutation site in all nine sites (figure 3G).[29] We, thus, examined the pan-threonine phosphorylation levels of wild-type and phosphoresistant T290A mutant PD-L1. Intriguingly, PD-L1 T290A mutant showed a lower level of threonine phosphorylation (figure 3H). Furthermore, PNU treatment upregulated wild-type PD-L1 protein level, but had no effect on T290 mutant PD-L1, and markedly, the expression level of PD-L1 T290A mutant was much higher than wild-type PD-L1 (figure 3I). Therefore, we assumed that CDK5 might impair the stability of PD-L1 via T290 phosphorylation. To test this hypothesis, we designed a site-specific antibody targeting PD-L1 T290 phosphorylation (figure 3J). Dot blot using T290 non-phosphorylation and phosphorylation peptides was conducted to verify the specificity of p-T290 antibody. As shown in figure 3K,L, the p-T290 antibody specifically recognized T290 phosphorylation peptides and wild-type PD-L1, but not T290A mutant. Then, we used this antibody to check whether CDK5 mediated T290 phosphorylation of PD-L1. Indeed, overexpression of CDK5 or CDK5 activator p35 significantly boosted the T290 phosphorylation of PD-L1 (figure 3M,N), while treatment with CDK5 inhibitor PNU markedly reduced the T290 phosphorylation level of PD-L1 (figure 3O). Collectively, these results demonstrate that CDK5 binds to and phosphorylates PD-L1 at T290.

CDK5 mediated PD-L1 T290 phosphorylation promotes PD-L1 degradation through CMA

Next, we investigated the mechanism underlying CDK5 mediated lysosome-dependent degradation of PD-L1. As shown in figure 4A, inhibition of autophagy initiation by 3-methyladenine (3-MA), an autophagosome formation inhibitor, could not block the CDK5 mediated PD-L1 lysosomal degradation, suggesting other degradative pathways involved in this process. CMA is one of the protein degradation pathways mediated by lysosome, and importantly, CMA is selective and specific to the target proteins compared with microautophagy and macroautophagy.[30] In CMA process, HSC70 is responsible for recruiting substrates to lysosome for degradation via lysosomal membrane receptor lysosome-associated membrane protein two isoform A (LAMP2A). First, we verified whether HSC70 physically binds with PD-L1 by co-IP. The results showed an interaction between PD-L1 and HSC70 (figure 4B,C). Moreover, overexpression of CDK5 enhanced this interaction (figure 4D), while PNU treatment weakened it (figure 4E), demonstrating CDK5 promoted PD-L1 degradation via CMA, which prompted us to examine whether T290 phosphorylation was involved in this process. As shown in figure 4F, in comparison with wild-type PD-L1 protein, the T290A mutant showed a compromised ability to bind with HSC70, indicating T290 phosphorylation might promote HSC70-PD-L1

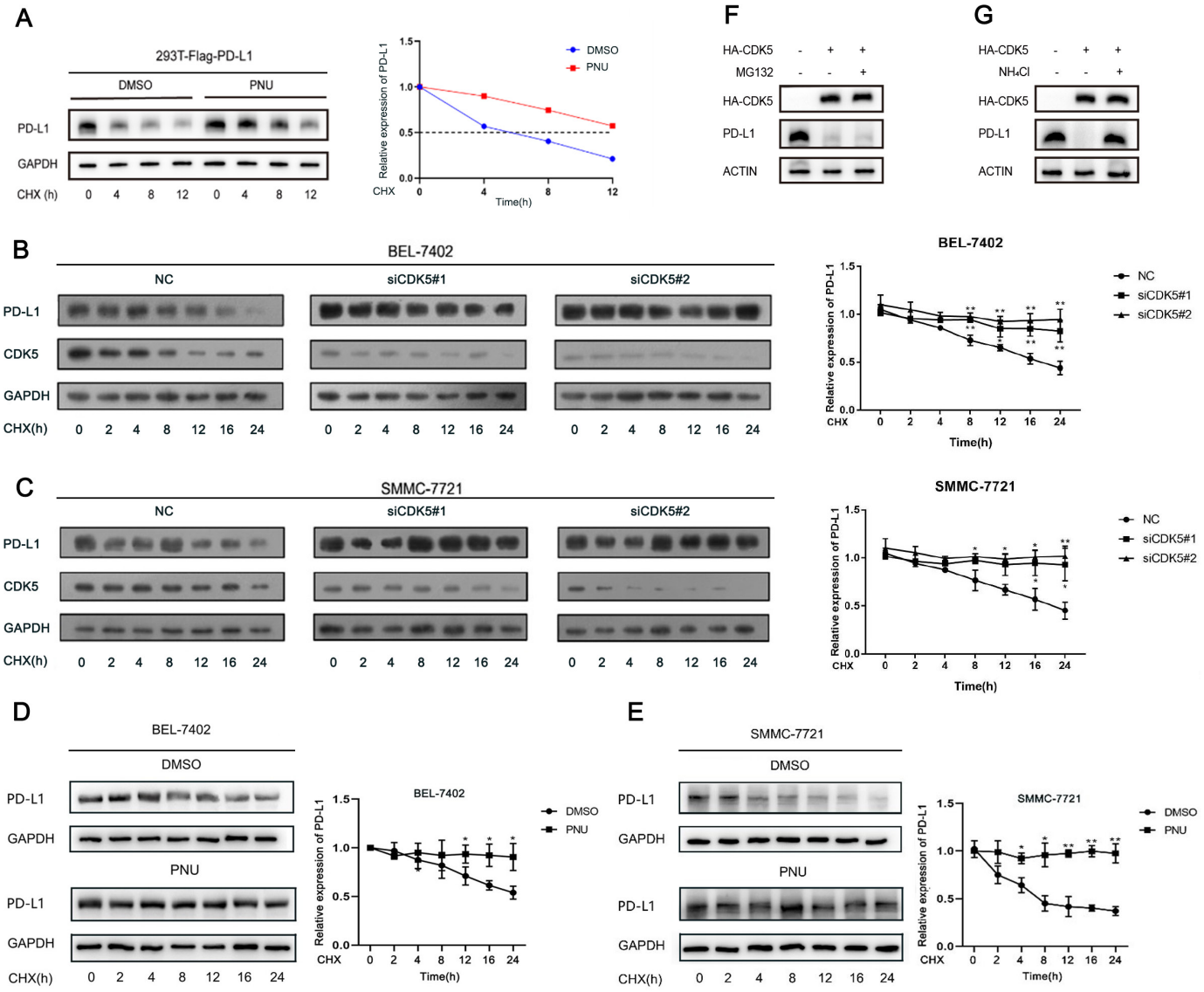

Figure 2 CDK5 promotes the lysosomal degradation of PD-L1 protein. (A) The half-life of Flag-PD-L1 in control and PNU treatment groups was determined by CHX-chase assay in 293T cells stably expressing Flag-PD-L1. The quantifications were shown on the right. (B, C) The half-life of PD-L1 in control and CDK5 knockdown groups was determined by CHX-chase assay in BEL-7402 cells (B) and SMMC-7721 cells (C). The quantifications were shown on the right (n=3). (D, E) The half-life of PD-L1 in control and CDK5 inhibition groups was determined by CHX-chase assay in BEL-7402 cells (D) and SMMC-7721 cells (E). The quantifications were shown on the right (n=3). (F, G) 293T cells stably expressing Flag-PD-L1 were transfected with HA-CDK5 and treated with MG132 (F) or NH_4Cl (G), the Flag-PD-L1 levels were measured by Western blot. CDK, cyclin-dependent kinase.

interaction. To further validate this finding, we checked the PD-L1 phosphorylation level both in input and HSC70 immunoprecipitated samples. Consistently, the T290 phosphorylated PD-L1 was enriched in the HSC70 immunoprecipitated sample (figure 4G). And the T290 mutation markedly prolonged the half-life of Flag-PD-L1 in 293T cells (figure 4H). Furthermore, under overexpression of CDK5, either knockdown of HSC70 or LAMP2A, significantly restored the decreased PD-L1 protein levels (figure 4I,J). Together, our findings unveil a novel mechanism by which CDK5 mediated PD-L1 T290 phosphorylation promotes PD-L1 degradation through CMA.

CDK5 inhibition synergizes with PD-1 blockade to suppress HCC growth

In light of our observation that CDK5 inhibition upregulated PD-L1 expression in HCC cells, we determined whether the inhibitor of CDK5 could synergize with PD-1/PD-L1 blockade to affect the therapeutic efficacy of HCC. As shown in figure 5A,B, treatment of immunoproficient mice bearing Hepa1-6 tumors with CDK5 inhibitor PNU plus anti-PD-1 antibody dramatically retarded tumor progression and resulted in seven complete responses out of eight treated mice, while mice body weight showed no significant difference across the four experiment groups

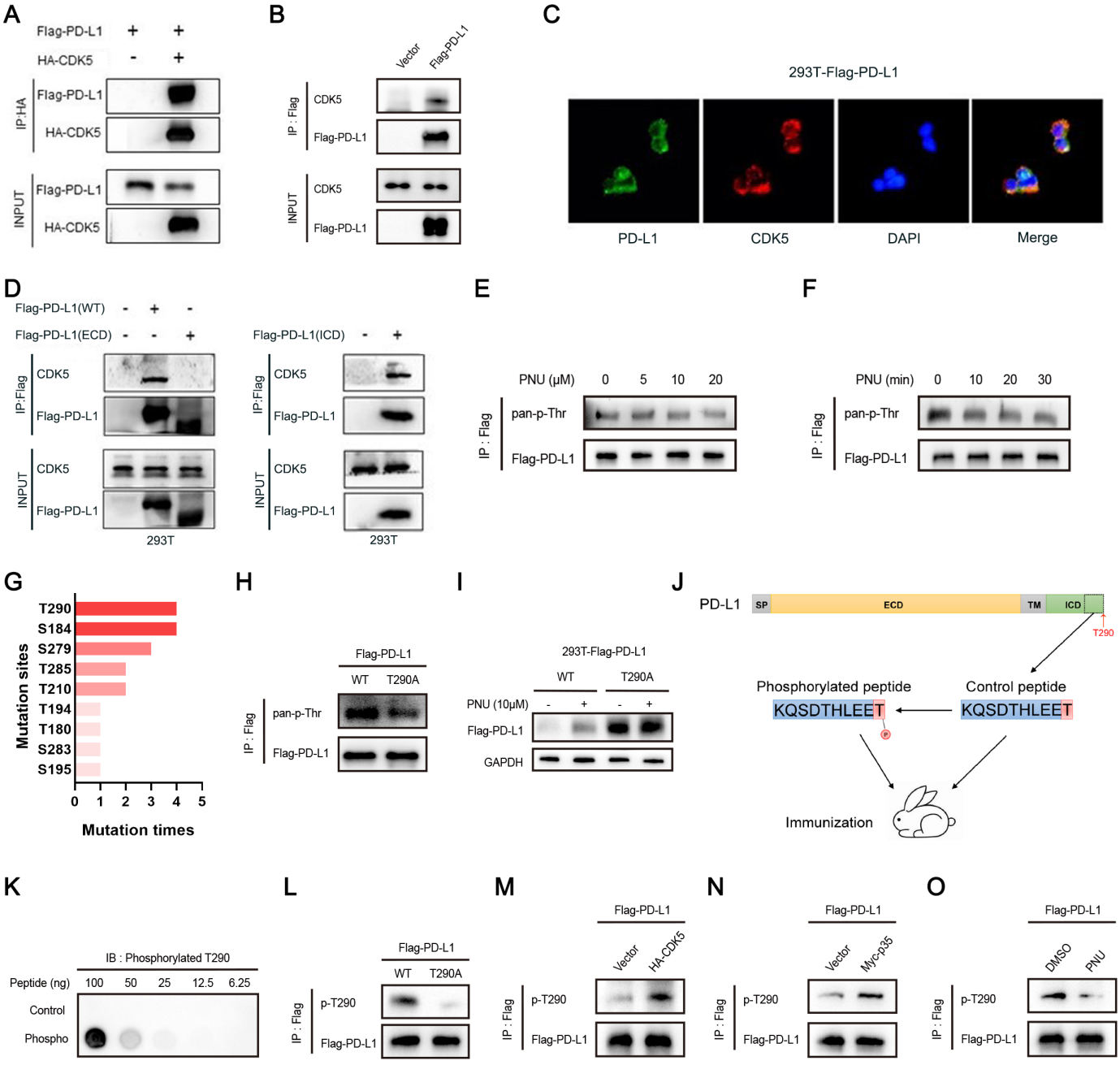

Figure 3 CDK5 binds with and phosphorylates PD-L1 at T290. (A) Co-IP analysis showed the binding of HA-CDK5 and Flag-PD-L1 in 293T cells stably expressing Flag-PD-L1 (B) Co-IP analysis showed the binding of Flag-PD-L1 and CDK5 in 293T cells stably expressing Flag-PD-L1. (C) Immunofluorescence analysis showed the colocalization of Flag-PD-L1 and CDK5 in 293T cells stably expressing Flag-PD-L1. (D) Co-IP analysis showed the binding between 293T cells overexpressing Flag-PD-L1 (WT), Flag-PD-L1 (ECD), and Flag-PD-L1 (ICD) with CDK5, respectively. (E, F) Western blot analysis of the pan-threonine phosphorylation levels of Flag-PD-L1 under the treatment of PNU with indicated concentrations (E) and time (F). (G) Mutation frequency of the indicated S/T sites of PD-L1 in tumors previously reported. (H) Western blot analysis of the pan-threonine phosphorylation levels of wild-type (WT) and T290A mutant PD-L1. (I) Western blot analysis of the expression levels of WT and T290A mutant PD-L1 under PNU treatment. (J) Schematic representation of the process of developing PD-L1 T290 phosphorylation antibody. (K) Dot blot analysis of the specificity of PD-L1 T290 phosphorylation antibody. (L) Western blot analysis of T290 phosphorylation levels of WT and T290A mutant PD-L1 using p-T290- PD-L1 antibody. (M–O) Western blot analysis of PD-L1 T290 phosphorylation levels under overexpression of HA-CDK5 (M) or Myc-p35 (N) or PNU treatment (O) as indicated. CDK, cyclin-dependent kinase; Co-IP, coimmunoprecipitation; ECD, extracellular domain.

(online supplemental figure 2). Furthermore, compared with single-agent treated groups, combining PNU with anti-PD-1 antibody significantly improved the overall survival of mice (figure 5C). Consistent with previous results, the tumorous expression of PD-L1 was significantly upregulated in PNU treated mice (figure 5D,E). Importantly, addition of PNU to anti-PD-1 antibody treatment markedly increased the numbers of tumor infiltrating

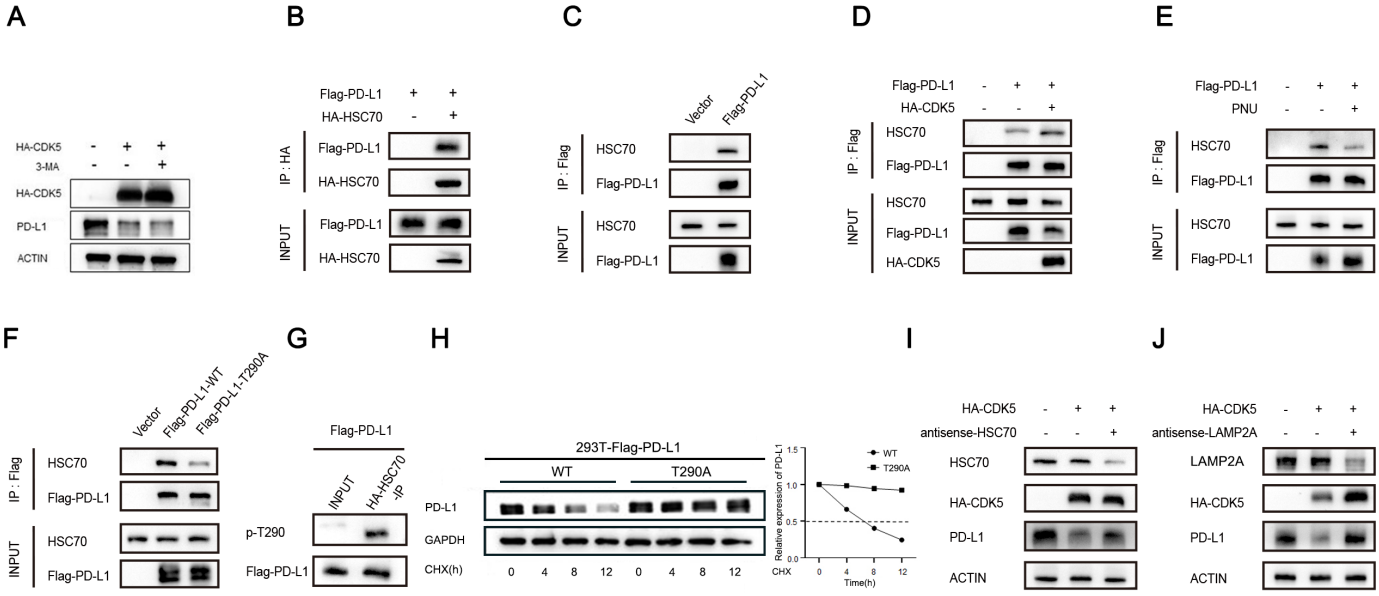

Figure 4 PD-L1 T290 phosphorylation by CDK5 promotes its degradation via chaperone mediated autophagy. (A) 293T cells stably expressing Flag-PD-L1 were transfected with HA-CDK5 and treated with 3-MA, the Flag-PD-L1 levels were measured by Western blot. (B, C) Co-IP analysis of the binding of HA-HSC70 and Flag-PD-L1(B) or HSC70 and Flag-PD-L1 (C) in 293T cells stably expressing Flag-PD-L1. (D) The effect of overexpression of HA-CDK5 on the binding between Flag-PD-L1 and HSC70 was determined by co-IP followed by Western blot. (E, F) The effect of PNU treatment (E) or T290A mutation (F) on the binding between Flag-PD-L1 and HSC70 was determined by co-IP followed by Western blot. (G) Western blot analysis of PD-L1 T290 phosphorylation levels in input and HA-HSC70 co-IP sample. (H) The half-life of PD-L1 was determined by CHX-chase assay in 293T cells transfected with Flag-PD-L1 (WT) and Flag-PD-L1 (T290A). The quantifications were shown on the right. (I, J) HSC70 (I) or LAMP2A (J) antisense plasmid was transfected into 293T cells stably expressing Flag-PD-L1. The effects of CDK5 overexpression on PD-L1 levels were examined by Western blot. CDK, cyclin-dependent kinase; Co-IP, coimmunoprecipitation.

lymphocytes (TILs), including $CD3^+$, $CD8^+$cells and elevated the expression of granzyme B (figure 5D,E). Taken together, these findings demonstrate that CDK5 inhibitor PNU upregulates PD-L1 protein levels in vivo, and more importantly, improves the efficacy of anti-PD-1 therapy.

CDK5/PD-L1 signaling pathway is clinically prognostic

To elucidate the clinical relevance of CDK5, PD-L1, and PD-L1 T290 phosphorylation in HCC, immunohistochemical staining was performed on 88 HCC samples to determine their expression. As shown in figure 6A,B, the level of p-T290-PD-L1 was positively associated with CDK5 expression, but negatively associated with PD-L1 protein level. Specifically, approximately 63% of HCC samples with high CDK5 expression exhibited strong p-T290-PD-L1 staining, and 60% of those with low CDK5 expression showed weak or no p-T290-PD-L1 staining (figure 6A). In addition, 61% of HCC tissues with low PD-L1 expression displayed strong p-T290-PD-L1 staining, while 69% of those with high PD-L1 expression exhibited weak or no p-T290-PD-L1 staining (figure 6B). In addition, there was also a tendency of coupling between poorer survival in patients and lower expression of p-T290-PD-L1 (figure 6C). Moreover, low PD-L1 expression in HCCs showed more $CD8^+$ T cell infiltration than those of high PD-L1 expression, while high levels of PD-L1 T290 phosphorylation in HCCs showed more $CD8^+$ T cell infiltration than those of low levels of PD-L1 T290 phosphorylation, suggesting a suppressive immune microenvironment in HCC with high PD-L1 expression or low levels of PD-L1 T290 phosphorylation (figure 6D). These findings strongly indicate that T290 phosphorylation status of PD-L1 might serve as a potential prognostic biomarker for HCC.

DISCUSSION

PD-L1 expression on tumors correlates with a durable objective response rate to anti-PD-1/PD-L1 therapy,[31 32] highlighting the importance of having an in-depth understanding of the regulation of PD-L1 expression. Phosphorylation, as a common type of PTM, exerts critical regulatory functions on PD-L1 stability.[9 10 33] For instance, PD-L1 is phosphorylated by Janus kinase 1 (JAK1) in response to IL-6, which recruits the endoplasmic reticulum N-glycosyltransferase STT3A to facilitate its glycosylation and maintain its stability.[10] Never in mitosis gene A-related kinase 2 (NEK2) maintains PD-L1 stability by phosphorylation at T194 and T210.[33] As mentioned above, kinase-mediated PD-L1 phosphorylation followed by other molecular interactions is a common pattern of regulation on PD-L1 stability. Notably, CDK5 has been identified as a crucial regulator of PD-L1 expression. CDK5 disruption results in the persistent expression of the PD-L1 transcriptional repressors, interferon regulatory factor 2 binding protein 2 (IRF2BP2) and interferon

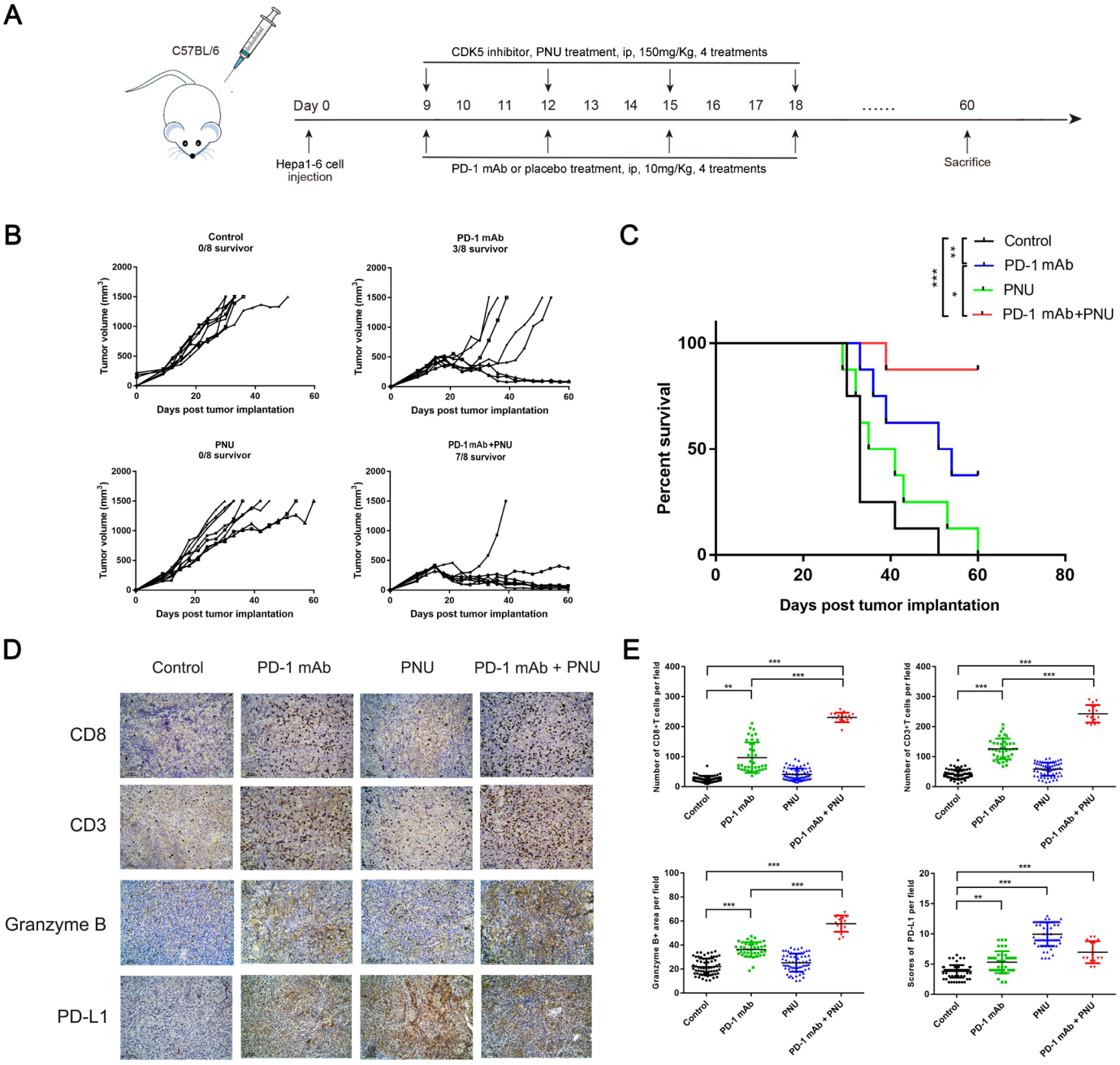

Figure 5 CDK5 inhibitor synergizes with PD-1 antibody to suppress HCC growth. (A) Schematic representation of the animal experiment process. (B) Mice bearing Hepa1-6 tumors were enrolled in four treatment groups as indicated. Tumor volumes of mice treated with control antibody, anti-PD-1 mAb, CDK5 inhibitor (PNU) or combined therapy were measured every 3 days and plotted individually. (C) Kaplan-Meier survival curves for mice bearing Hepa1-6 tumors treated with PNU or/and anti-PD-1 antibody. The p value, comparing every two groups, was determined by log-rank test. (D) IHC showing $CD3^+$, $CD8^+$ T cell infiltration and the expression of granzyme B and PD-L1 in the Hepa1-6 tumor tissues as indicated (scale bars, 20 µm). (E) Data represent mean±SD from six independent samples of each group. Statistical differences were determined by Student's t-test. CDK, cyclin-dependent kinase; HCC, hepatocellular carcinoma.Levels of significance were indicated as *p<0.05, **p<0.01, ***p<0.001.

regulatory factor 2 (IRF2), which in turn declines the expression of PD-L1 and promotes antitumor immunity in medulloblastoma.[34] Additionally, CDK5 inhibition indirectly reduces PD-L1 protein level through increasing the expression of E3 ligase FBXO22 F-box only 22(FBXO22) in non-small cell lung cancer.[35] These findings have shown that CDK5, a proline-directed serine-threonine kinase that is aberrantly activated in several tumor types, plays a vital role in the positive regulation of PD-L1. Interestingly, in this study, we found that CDK5 negatively modulated PD-L1 expression in HCC. Mechanistically, CDK5 directly phosphorylates PD-L1 at T290, which promotes its binding with HSC70 and degradation via CMA, inhibition of CDK5 upregulates tumorous PD-L1 levels and

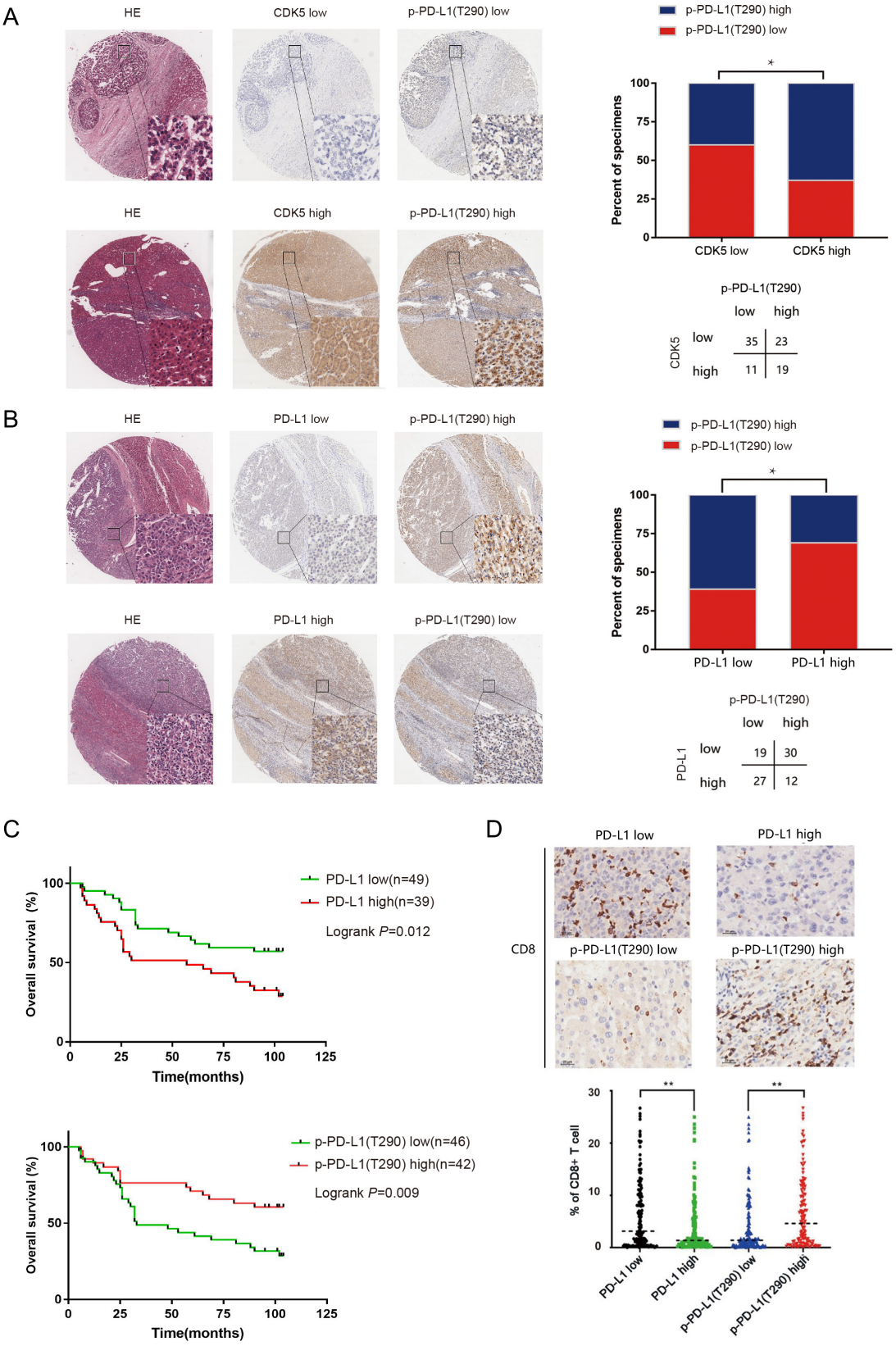

Figure 6 CDK5/PD-L1 signaling pathway is clinically prognostic. (A) Representative images of CDK5/p-T290-PD-L1 IHC and statistical analysis of IHC results in 88 cases of HCC tissues. (B) Representative images of PD-L1/p-T290-PD-L1 IHC and statistical analysis of IHC results in 88 cases of HCC tissues. (C) Kaplan-Meier analysis of overall survival with low or high PD-L1 and p-T290-PD-L1 IHC staining in 88 patients with HCCs. (D) Representative images of CD8 IHC and statistical analysis of IHC results in 88 cases of HCC tissues (scale bars, 20 µm). Data represent mean±SD from three independent samples of each group. CDK, cyclin-dependent kinase; HCC, hepatocellular carcinoma; IHC, immunohistochemistry. Levels of significance were indicated as *p<0.05, **p<0.01.

facilitated the PD-1 antibody-mediated immunotherapy in preclinical HCC models. More importantly, a negative correlation between the level of PD-L1 T290 phosphorylation and PD-L1 expression was detected in HCC specimens, and higher PD-L1 T290 phosphorylation level was found to be positively correlated with better prognosis in HCC. In line with our findings, a pan-cancer analysis of PD-L1 mutations in 314 631 patient samples has shown that the PD-L1 T290 site frequently mutates to A or M, alluding to its potential role in maintaining PD-L1 stability and contributing to tumor immune evasion.[29] Collectively, our findings illustrate a potential mechanism under which cancer cells escape from immune surveillance through T290 mutation and targeting phosphorylation of PD-L1 at T290 might be a promising strategy to facilitate anticancer immunotherapy.

Another significant observation that stems from our work is the role of CMA in regulating PD-L1 protein level, which demonstrates a new pathway for PD-L1 degradation. Emerging evidence has demonstrated that PD-L1 stabilization is regulated by the ubiquitin-proteasome pathway and lysosomal proteolysis.[7 9 11 12 15 36–38] Ubiquitination-mediated PD-L1 degradation is modulated by different E3 ubiquitin ligases, such as β-TRCP,[7] HMG-CoA reductase degradation protein 1 (HRD1),[9] speckle-type POZ protein (SPOP),[11] and STIP1 homology and U-Box containing protein 1 (STUB1).[37] In addition, accumulating studies showed that lysosomes control the fate of PD-L1. CKLF-like MARVEL transmembrane domain-containing protein 6 (CMTM6) facilitates the endocytic recycling of PD-L1 to cell surface and prevents PD-L1 from lysosomal degradation.[36 37] Huntingtin-interacting protein 1-related (HIP1R) binding with PD-L1 depletes PD-L1 through lysosome-dependent proteolysis and enhances T cell-mediated cytotoxicity.[38] In this study, we demonstrated that CMA actively participates in the degradation of PD-L1. Specifically, the T290 phosphorylation of PD-L1, mediated by CDK5, promotes its binding with HSC70, which is responsible for recruiting substrates in CMA process. Indeed, until now, it has been recognized that PTMs of CMA targeted proteins outside the canonical motif undergo conformational changes, which lead to masking or exposing the binding motif.[39 40] In line with this, our findings indicate that a novel PTM of PD-L1 controls its level by CMA, although the effect of T290 phosphorylation on the structure of PD-L1 requires further exploration.

The functions of inhibitory immune checkpoints are tightly modulated by ligand-receptor interactions, their surface expression levels, and complex intracellular signal transduction pathways.[41] Currently, the development of immune checkpoint blockades in the treatment of cancer mainly focuses on targeting the ligand-receptor interaction.[42] However, evidence has mounted that targeting the regulators of PD-L1 expression might open another exciting avenue to reinvigorate efficacious antitumor immune responses.[9 43–45] Numerous small molecule agents have been reported to directly or indirectly downregulate PD-L1 expression and facilitate the immunotherapy of cancer. For instance, Metformin enhances T cell-mediated cytotoxicity against cancer cells via promoting endoplasmic-reticulum-associated degradation of PD-L1.[9] Silvestrol, an eukaryotic initiation factor 4A (eIF4A) helicase inhibitor, elicits a powerful antitumor immune response via downregulation of PD-L1.[43] The COP9 signalosome 5 (CSN5) inhibitor, curcumin, diminishes PD-L1 expression and sensitizes cancer cells to anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) therapy.[44] D-mannose significantly improves the efficacy of PD-1 blockade and radiotherapy in triple-negative breast cancer through degradation of PD-L1.[45] What is more, PD-L1 upregulation has been considered to turn cold tumors into hot tumors, which means tumors are more vulnerable to immunotherapy,[46] and small-molecule drugs which boost PD-L1 expression have also been developed to improve the efficacy of cancer immunotherapy.[47 48] For instance, olaparib, a poly (ADP-ribose) polymerase inhibitor (PARPi), upregulates PD-L1 expression by activating the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway to augment antitumor immune response in Brca1-deficient ovarian cancer.[47] MET inhibitors can stabilize PD-L1 and improve antitumor activity in HCC models.[48] Given our observation that inhibition of CDK5 elevated PD-L1 levels, we speculated that inhibitors of CDK5 might synergize with anti-PD-1/PD-L1 therapy to elicit an enhanced therapeutic effect. Notably, the addition of CDK5 inhibitor to anti-PD-1 therapy dramatically suppressed HCC growth and prolonged the survival of preclinical HCC models. These exciting findings open new opportunities for CDK5 inhibitors as immunotherapeutic drugs, in addition to their direct antitumor effects. On the one hand, upregulation of PD-L1 expression by inhibition of CDK5 in HCC cells increases their sensitivity to PD-1/PD-L1 immune checkpoint blockade. On the other hand, it might also provide a safe and potentially effective strategy to overcome the adverse effect of CDK5 inhibitor by combining with anti-PD-1/PD-L1 therapy. In this regard, it is warranted that the strategy be tested in future clinical trials for HCC based on the encouraging preclinical data.

In summary, our study demonstrates that CDK5 mediated PD-L1 T290 phosphorylation promotes PD-L1 degradation through CMA, and inhibition of CDK5 enhances the efficacy of anti-PD-1 therapy in preclinical HCC models, providing a new potential strategy for HCC treatment (online supplemental figure 3).

Author affiliations
[1]Cellular and Molecular Biology Laboratory, Affiliated Zhoushan Hospital of Wenzhou Medical University, Zhoushan, Zhejiang, China
[2]MOE Key Laboratory of Metabolism and Molecular Medicine, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai, China
[3]Nourse Centre for Pet Nutrition, Wuhu, Anhui, China
[4]School of Management, Xi'an Jiaotong University, Xi'an, Shanxi, China
[5]Department of General Surgery, Zhoushan Hospital, Zhoushan, Zhejiang, China

Contributors RZ, JW and YD played a major role in designing and performing experiments; ZY and YW helped to analyze the data; YJ, YW, TL, ZL and GZ contributed to technical assistance; HM and RZ wrote the manuscript; HM and LL conceived and designed the study and HM supervised the study. HM was responsible for the overall content as guarantor.

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ORCID iD

Lei Lv http://orcid.org/0000-0003-4820-3125

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