Inhibition of DCLK1 down-regulates PD-L1 expression through Hippo pathway in human pancreatic cancer

Rui Yan[a], JianJian Li[b], Ying Zhou[a], Li Yao[c], Ruya Sun[b], Ying Xu[b], Yang Ge[a], Guangyu An[a]

[a]Beijing Chao-Yang Hospital Department of Oncology, Capital Medical University, Beijing, 100020, China

[b]Institute for Immunology, Department of Basic Medical Sciences, School of Medicine, Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing, 100084, China

[c]Department of Computational Biology, Cornell University, Ithaca, NY 14853, USA

Address Correspondence to:

Guangyu An PhD and Yang Ge PhD
8 Gongren Tiyuchang Nanlu Road, Chaoyang Dist. Beijing, China, 100020
Phone: 86-010-85231407; Fax: 86-010-85231570; E-mail: agybjcy@163.com and interna-1@163.com

Abstract

Immunotherapy is one of the most promising strategies for cancer, compared with traditional treatments. As one of the key emerging immunotherapies, anti-PD-1/PD-L1 treatment has brought survival benefits to many advanced cancer patients. However, in pancreatic cancer, immunotherapy-based approaches have not achieved a favorable clinical effect because of mismatch repair deficiencies. Therefore, the majority of pancreatic tumors are regarded as immune-quiescent tumors and non-responsive to single-checkpoint blockade therapies. Many preclinical and clinical studies suggest that it is still important to clarify the regulatory mechanism of the PD-1/PD-L1 pathway in pancreatic cancer. As a marker of cancer stem cells, DCLK1 has been found to play an important role in the occurrence and development of a plethora of human cancers. Recent researches have revealed that DCLK1 is closely related to EMT process of tumor cells, meanwhile, it could also be used as a biomarker in gastrointestinal tumors to predict the prognoses of patients. However, the role that DCLK1 plays in the immune regulation of tumor microenvironments remains unknown. Therefore, we sought to understand if DCLK1 could positively regulate the expression of PD-L1 in pancreatic cancer cells. Furthermore, we examined if DCLK1 highly correlated with the Hippo

pathway through TCGA database analysis. We found that DCLK1 helped regulate the level of PD-L1 expression by affecting the corresponding expression level of yes-associated protein in the Hippo pathway. Collectively, our study identifies DCLK1 as an important regulator of PD-L1 expression in pancreatic tumor and highlights a central role of DCLK1 in the regulation of tumor immunity.

Key words：DCLK1, PD-L1, Hippo pathway, YAP, tumor immunity

1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease characterized by an aggressive nature, high recurrence rate, resistance to chemotherapy, and the lowest 5-year survival rate (9 %) among all kinds of cancers[1]. However, despite the realization of high mortality and low survival rates, incidence rates for PDAC continue to increase worldwide[1]. The unfavorable prognosis for patients afflicted with PDAC is associated with late diagnosis, early metastasis, and low positive response rates for treatment[2]. Therefore, having a better understanding of the biological characteristics of PDAC would help researchers in developing more effective targeted therapies for patients afflicted with pancreatic cancer. Currently, treatments using immunotherapies have shown promise and have resulted in durable responses in many kinds of tumors. The discovery of numerous immunotherapy checkpoint blockades, including programmed death-1/programmed death-ligand 1 (PD-1/PD-L1), cytotoxic T lymphocyte associated antigen-4 (CTLA-4), and B7 superfamily member 1 (B7S1) have provided increased treatment opportunities for patients. The PD-1/PD-L1 axis has proven to be main signals that inhibit activation of T cells, ultimately resulting in tumor cell immune evasion processes[3]. The strategy, that targeting the checkpoints by using PD-L1 and its receptor PD-1 blockade, has opened a new era for treating cancers. However, the clinical benefit rate was still limited in patients with lung cancer and melanoma, which proved to respond to checkpoint blockade immunotherapy[4]. Immunotherapy used for treatment of pancreatic cancer has thus far showed few breakthroughs, however, a large number of clinical trials of anti-PD-1/PD-L1 therapy in pancreatic cancer are presently ongoing[5]. Therefore, a better understanding of the regulatory mechanisms of PD-1 and PD-L1 will help to further improve the efficacy of the PD-1/PD-L1 for other cancer patients and as a part of the ongoing development of combined treatment strategies.

Doublecortin-like kinase 1 (DCLK1) is a member of the protein kinase superfamily and was first identified for its function in neurogenesis[6-8]. However, in recent years, many researchers have discovered critical functions of DCLK1 in the dynamics of tumorigenesis. High-expression of DCLK1 has been reported in many tumors, including colorectal cancer[9,10], pancreatic tumors[11,12], and renal cell carcinoma tumors[13]. Growing evidences suggest that DCLK1 is a stem cell marker and associates with epithelial-mesenchymal transformation (EMT) progression in gastrointestinal tumors[14, 15]. Moreover, research by Weygant et al. (2016) revealed that the expression level of DCLK1 had significant value as a predictor of the survival time for patients with gastrointestinal tumors[16]. Meanwhile, Westphalen et al. (2016), reported that DCLK1+ cells can initiate Kras mutant pancreatic tumors in the context of pancreatitis after injury[17]. Recent research has also found that IL-17 was able to induce DCLK1 expression, which in turn could induce pancreatic cancer and help cancer cells to avoid immune surveillance[18]. The regulatory function of DCLK1 in tumor immunity is being gradually revealed. But, whether DCLK1 is a key factor in regulating the expression of PD-L1, remains unknown.

In this study, we aimed to investigate the role of DCLK1 in the regulation of PD-L1 expression. We induced overexpression of DCLK1 and inhibited DCLK1 (using inhibitors LRRK2-IN-1, XMD8-92) in pancreatic tumor cell lines, and sought to examine if DCLK1 and PD-L1 were strongly correlated. Mechanistically, we also explored if DCLK1 may regulate PD-L1 through affecting the Hippo pathway and ultimately whether DCLK1 may play an immunoregulatory role in the tumor microenvironment of pancreatic cancer.

2 Materials and methods

2.1. Cell culture

AsPC-1, BxPC-3, and PANC-1 cells were purchased from American Type Culture Collections (ATCC). PANC-1 cells were grown in DMEM. Whereas AsPC-1 and BxPC-3 cells were both maintained in RPMI-1640 medium. All mediums were supplemented with 10 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. All cell lines were incubated at 37 °C in a humid chamber with a controlled atmosphere of 5 % CO2 and stored in liquid nitrogen.

2.2. Reagents and transfection

LRRK2-IN-1 were purchased from TargetMol (catalog T2246), XMD8-92 from Selleck (catalog S7525), and verteporfin from MedChemExpress (catalog HY-B0146). Human DCLK1 (NM_001195416.2) cDNA was cloned into pLVX-IRES-Zsgreen vector. We transfected cells with Neofect reagent according to manufacturer's protocols. After 24-48 hours (hrs) of transfection, the transfection efficiency was observed by using fluorescence-based microscopy.

2.3. Protein extraction and Western blotting

Total protein was extracted from cell lines in RIPA lysis Buffer. Samples were separated on 10 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in 8 % non-fat milk for one hour and incubated with primary antibodies at 4 °C overnight. The membranes were washed three times with TBST, and probed with specific secondary antibody for one hour at room temperature. Then, we measured the level of protein expression using an ECL blotting analysis system. The primary antibodies used were rabbit anti-DCLK1 (Abcam, ab31704; 1:1000), rabbit anti-YAP (CST, 14064; 1:1000), rabbit anti-PD-L1 antibody (Abcam, ab205921; 1:1000), and mouse anti-GAPDH (CST, 5174; 1:1000).

2.4. RNA extraction and Quantitative RT-PCR

Total RNA was isolated in TRIzol according to the manufacturer's protocols. RNA was transcribed to cDNA using PrimeScript™ RT reagent Kit (TaKaRa, RR047A), then cDNA was used as the template to perform real-time PCR, using SYBR Green for molecular probes. GAPDH was used as a control to normalize the threshold value. Relative gene expression levels were determined by the $2^{-\Delta\Delta C(t)}$ method. The following primers were used: GAPDH- forward: 5'- GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'- GGCTGTTGTCATACTTCTCATGG-3'; DCLK1-forward: 5'-ACACTAAGACTGTGTCCATGTTAGAACTC-3', reverse: 5'-AAGCCTTCCTCCGACACTTCT-3'; YAP- forward: 5'-CAGAACCGTTTCCCAGACTAC-3', reverse: 5'-ATCAGCTCCTCTCCTTCTATGT-3'; and PD-L1-forward: 5'-GGACAAGCAGTGACCATCAAG-3', reverse: 5'-CCCAGAATTACCAAGTGAGTCCT-3'.

2.5. PBMC separation

Peripheral blood samples were obtained from healthy donors, and performed density gradient centrifugation by Ficoll-Paque Plus (GE Healthcare, USA). The peripheral blood mononuclear cell (PBMC) layer was collected and subjected to CD4 T cell isolation using Human CD4+ T cell kit (Stem cell, Canada).

2.6. Coculture and stimulation

Isolated CD4 T cells were labelled with CFSE following manufacturer's instructions (Cell Trace CFSE Cell Proliferation Kit, catalog #C34554, Life Technologies). CFSE labelled human CD4+ T cells were co-cultured with Aspc-1 cells for aforementioned DCLK1 overexpression assay and DCLK1 inhibition assay for 3 days, with the presence of 1ug/mL Anti-CD3 (eBioscience, clone number) and 5ug/mL Anti-CD28(eBioscience, clone). The proliferation of CD4 T cell were detected by flow cytometry on Fortessa (BD) Flow cytometry detection.

2.7. Flow cytometry

AsPC-1 and BxPC-3 cells were cultured in 6-well plates. After 24 hrs of cell adherence, cells were treated with LRRK2-IN-1(10 um), XMD8-92 (15 um), or were treated with DMSO as a control. After 3 days, cells were trypsinized and harvested. To analyze the levels of surface expression of PD-L1, cells were washed by using PBS and were incubated with anti-PD-L1 antibody (Biolegend, clone: 29E.2A3) for 15 min at room temperature. All flow cytometry analyses were performed using BD Biosciences FACS Aria III. Data were analyzed using FlowJo software and all parameters were set to manufacturer defaults.

2.8. Datasets and Statistical analysis

RNA-seq results were obtained from The Cancer Genome Atlas (TCGA) runs for pancreatic adenocarcinoma (PAAD). Data are presented as mean ± standard error of the mean (SEM), and all statistical analyses were performed using GraphPad Prism (version 7.0) with all parameters set to manufacturer defaults. Statistical significance was analyzed using a Student t test, $p < 0.05$ was considered to be statistically significant.

3 Results

3.1. PD-L1 (CD274) expression in pancreatic cancer cell lines

We found that the expression level of PD-L1 were higher in BxPC-3 cells than the other pancreatic tumor cell lines (AsPC-1, PANC-1). Real-time PCR results indicated that BxPC-3 cells had a 60-fold higher level of PD-L1 mRNA expression than the levels were for PANC-1 cells, and western blotting results were consistent with mRNA analyses (Fig. 1A). Next, we confirmed the cell surfaces expression level of PD-L by flow cytometry and we found that the expression level of PD-L1 reached up to 90 % on the BxPC-3 cell line, and in contrast found that PD-L1 expression was a rare occurrence in examinations of PANC-1 cells (Fig. 1B).

3.2. DCLK1 involved in the PD-L1 expression in pancreatic tumor cells

To better understand the influence of DCLK1 on the expression level of PD-L1, we established the transient transfection cell line upon the AsPC-1 cell line, and detected the expression level of DCLK1 by real-time PCR and western blotting (Figure 2A). We then performed flow cytometry experiments to investigate the degree of change in PD-L1 expression. As expected, we found that overexpression of DCLK1 can dramatically affect PD-L1 mRNA expression as well as its surface protein expression in the cell line (Fig. 2B). In contrast, we treated AsPC-1 and BxPC-3 cells with LRRK2-IN-1 and XMD8-92 which are inhibitors can suppress the expression of DCLK1 on mRNA and protein level (Fig. 2C, E), and measured PD-L1 expression. Results indicated that down-regulation of DCLK1 lead to suppression of both PD-L1 transcription and cell surface expression in the two cell lines (Fig. 2D, F). In totality, these results revealed that the expression of DCLK1 is related with PD-L1 expression in pancreatic cancer cell lines.

3.3. The TCGA database analysis revealed that DCLK1 is correlated with Hippo pathway

DCLK1 has been proposed as a biomarker of stem cells of gastrointestinal tumors. Previous research from authors in our group revealed that DCLK1+ tumor stem cells are critical to the development and progression of pancreatic cancer. Likewise, a variety of recent studies have shown the Hippo pathway plays important roles in controlling tumor initiation, metastases, and cancer stem cell homeostasis[19]. Interestingly, Yes-

associated protein (YAP) as an effector molecule of the Hippo pathway, when interacting with its co-activator TAZ, can initiate downstream transcription of PD-L1[20]. Analysis of TCGA pancreatic ductal adenocarcinoma data indicated that there was a strong correlation between DCLK1 mRNA expression and Hippo signaling pathway genes across 183 TCGA PAAD samples, including AMOTL1, AMOTL2, LATS1, and LATS2 (Fig. 3A). Results for Spearman's correlation coefficients and corresponding p-values (obtained from TCGA PAAD datasets) suggested that genes in the Hippo pathway are correlated with DCLK1 (Fig. 3B). We used sources including experiments, co-expression, neighborhood, gene fusion, and co-occurrence from the STRING[21] analysis to predict the active interactions between DCLK1 and the Hippo-pathway signaling in the network (Fig. 3C).

3.4. DCLK1 promotes the expression of Yes-associated protein

Recent studies have focused on an interesting relationship between the Hippo pathway and tumor immunity. As previously described, dysregulation of the Hippo pathway is correlated with the hallmarks of oncogenesis and immune cell evasion. Yes- associated protein (YAP), as the downstream effector proteins of Hippo pathway, can promote PD-L1 expression and induce PD-L1 derived immune evasion. Therefore, we sought to investigate whether DCLK1 regulates Yes-associated protein (YAP) at the transcriptional and protein level in AsPC-1 and BxPC-3 cells. We examined the expression of YAP via real-time PCR and western blotting assays. The results were consistent with the results from TCGA analysis, the expression level of YAP was significantly increased after forced overexpression of DCLK1 compared to the control group cells (Fig. 4A). Further, we investigated whether suppression of DCLK1 expression decreased the expression of YAP in the two cell lines. We treated the cells with DCLK1 inhibitor LRRK2-IN-1 and XMD8-92 for 72 hrs, and the YAP expression was detected through determination on mRNA and proteins level. As expected, after DCLK1 was antagonized by the inhibitors in both cell lines, the expression level of YAP was significantly decreased in AsPC-1 and BxPC-3 cells (Fig. 4B, C).

3.5. DCLK1 regulate PD-L1 expression through Hippo pathway

To investigate whether DCLK1 played an important role in modulating PD-L1 expression through impacts upon the Hippo pathway, we treated cells with YAP

inhibitor verteporfin. Verteporfin is a small molecule that can prevent the interaction between YAP/TAZ and TEAD, and allows for use to examine the expression level of PD-L1. After we used 2.0 uM and 10.0 uM of verteporfin as treatments upon AsPC-1 and BxPC-3 cells, the results from real-time PCR indicated that the PD-L1 mRNA expression were significantly decreased. Accordingly, flow cytometry also indicated that the cell surface expression of PD-L1 were downregulated (Fig. 5A-D). Next, we attempted to verify whether DCLK1 expression was able to regulate PD-L1 through the Hippo pathway. We analyzed PD-L1 expression after DCLK1 overexpression with or without YAP inhibition. The results indicated that the elevated PD-L1 expression in DCLK1 overexpression cells could be suppressed by the YAP inhibitor verteporfin (Fig. 5E). In addition, we performed T cell suppression assay in presence of DCKL1 over-expressed and inhibited cancer cells. The results indicate that DCLK1 can affect the expression of PD-L1, which can directly suppress the proliferation of T cell (5F). Collectively, these findings suggest that DCLK1-mediated PD-L1 expression requires the Hippo pathway and confirm that the association between DCKL1 and PD-L1 can directly affect T cell function in tumor microenvironment.

4、Discussion

Immune escape is one of the most prominent characteristics of cancer cells. Accumulating evidences show that the interaction between tumor cells and the tumor microenvironment plays an important role in disease progression. The expression of PD-L1 in cancer cells is an important mechanism for avoiding immune surveillance. Therefore, it is of great significance to study the regulatory mechanisms of PD-1/PD-L1 expression. In recent years, studies have shown that the Hippo pathway plays an important role in tumorigenesis and development. The Hippo pathway can regulate a variety of biological functions and behaviors of cells, including cell determination, cell polarity, cell proliferation, cell apoptosis, and others[19, 22]. The main core of molecules of this pathway includes sterile 20-like kinase 1/2 (MST1/2), large tumor suppressor 1/2 (LATS1/2), the downstream effector molecule yes associated protein (YAP), and its co-activator TAZ, and in sum these molecules compose a kinase cascade.

When the Hippo pathway is activated by upstream signals, YAP proteins become phosphorylated and are retained in the cytoplasm. Once the Hippo pathway dysregulated, uninhibited YAP localized to the nucleus and activated downstream molecules by binding to transcription factor TEA-domain family member (TEAD). YAP is the most important functional protein in this pathway, and has proven to be highly expressed in many types of tumors, including ovarian cancer, hepatocellular carcinoma, non- small-cell lung cancer, and gastric cancer[23-26]. Hippo pathway has been proven to be closely related to Kras status, while Kras mutations play an important role in the occurrence and development of pancreatic cancer. For the above reasons, the Hippo pathway has attracted particular attention for its role in the pancreatic cancer[27]. Accordingly, recent studies have revealed a close relationship between the Hippo pathway and tumor immunity[28]. Likewise, some studies have indicated that YAP is able to inhibit anti-tumor immunity by regulating regulatory T cells, an immunosuppressive cell in tumor microenvironments, thus ultimately promoting the escape of tumor cells from detection by immune system[29]. In the study of prostate cancer, it has been suggested that YAP protein can inhibit immune regulation by up-regulating the expression of Cxcl5 and recruiting Cxcr2+MDSC cells[30]. Interestingly, the Hippo pathway is also thought to be involved in the regulation of immune checkpoint PD-L1[20, 31], but no studies have focused on it in pancreatic cancer. These results suggest that it is necessary to clarify the potential effects of the Hippo pathway on PD-L1 expression in pancreatic cancer.

Tumor stem cells are a small subset of cancer cells and are resistant to radiotherapy and chemotherapy. In recent years, researchers have found that this population of cells also plays an important role in tumor immune escape. In order to carry out an immunotherapy-based treatment approach targeting cancer stem cells, we must first clearly recognize and define the specific markers of cancer stem cells, must also be able to distinguish between cancer stem cells and non-stem cells[32, 33]. Over the years, as a marker of cancer stem cells, the important role of DCLK1 in tumorigenesis, development and epithelial-mesenchymal transformation of cancer cells has been gradually recognized. For example, Ge et al. (2018) reported that DCLK1 was highly expressed in renal clear cell carcinoma. In vivo experiments have shown that monoclonal antibodies against DCLK1 have therapeutic value for renal cell carcinoma[13]. Liu et al. (2018) found that short variants of DCLK1 were able to regulate the EMT process of cancer cells through the NF-kB pathway in colorectal

cancer[10]. In breast cancer, DCLK1 has been found to be a carcinogenic gene that can drive invasion and metastasis of breast cancer cells, and can also be used as an indicator of poor prognosis and outcomes for afflicted patients[34]. However, at present, the regulatory role of DCLK1 in tumor immunity still remains unclear.

We used two inhibitors, LRRK2-IN-1 and XMD8-92, to inhibit the expression of DCLK1 in AsPC-1 and BxPC-3 pancreatic cancer cell lines, as well as inducing the overexpression of DCLK1 in AsPC-1 cells, in order to study whether DCLK1 regulated the expression of PD-L1 in pancreatic cancer. Results indicated that DCLK1 was important in regulating the expression of PD-L1 and showed a positive correlation. Next, we continued to explore the mechanisms behind how DCLK1 works to regulate PD-L1.

The Hippo pathway plays an important role in the development of pancreatic cancer, as well as in the regulation of cancer stem cells[19]. Knowing this, we explored whether DCLK1 was able to regulate the expression of PD-L1 through the Hippo pathway. Through TCGA database analysis, we confirmed that DCLK1 is related to many molecules of Hippo pathway. As mentioned above, many recent studies have confirmed that YAP protein in the Hippo pathway binds to downstream transcription factors as the mechanisms by which it regulates the expression of PD-L1, thus promoting the immune escape of tumors. Therefore, we sought to further confirm whether DCLK1 was able to regulate PD-L1 through influence of the YAP molecule in vitro. The results showed that DCLK1 was able to regulate the expression of YAP, both when analysis of mRNA and total amounts of protein. In addition, we found that the use of YAP inhibitors inhibited the increase of PD-L1 expression induced by DCLK1. Therefore, we concluded that DCLK1 regulates PD-L1 through the Hippo pathway.

These results suggest that DCLK1 may play a role in tumor immune microenvironment. The findings lay an important foundation for follow-up research such as to determine if DCLK1 may be used as a specific antigen of cancer stem cells, which has clinical significance in the immunotherapy of pancreatic cancer.

Fig. 1. PD-L1 expression in human pancreatic tumor cell lines.
(A) Real-time PCR and western blotting analysis of the basic expression level of PD-L1 in 3 pancreatic cancer cell lines (AsPC-1, BxPC-3, and PANC-1). (B) Flow cytometry was used to detect the expression level of PD-L1 in cell surfaces.

Fig. 2. DCLK1 induces PD-L1 expression in Pancreatic ductal adenocarcinoma cells.
(A) Real-time PCR and western blotting confirm DCLK1 expression in AsPC-1 wild type and DCLK1 overexpression cells. (B) Real-time PCR analysis of PD-L1 expression in mRNA level. Flow cytometry analysis of PD-L1 cell surface expression in wild type and DCLK1 overexpression cells. The display is indicative of statistical results for values of mean fluorescence intensity. (C) and (E) Real-time PCR and western blotting analysis of DCLK1 expression in inhibitor (LRRK2-IN-1, XMD8-92) treated cells (AsPC-1, BxPC-3). (D) and (F) Real-time PCR and flow cytometry analysis of PD-L1 expression in terms of the levels of mRNA and of protein. Results are exhibited as mean ±SEM (standard error of the mean). *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's t-test.

Fig. 3. The guilty-by-association analysis revealed that the expression of DCLK1 is strongly correlated with expression of genes belonging to the Hippo pathway.
(A) Heatmap showing that the expression patterns, of representative Hippo signaling pathway genes, are associated with the levels of expression of DCLK1. A green entry represents a gene with a low rank; while a red rectangle indicates a gene is highly expressed in tumor samples. The 25th and 75th percentiles were used to classify samples into low and high DCLK1 groups (46 samples for each group). (B) Spearman's correlation coefficients and corresponding p-values suggest that genes in the Hippo pathway are correlated with DCLK1. Red points indicate positive correlation, while blue points indicate negative correlation. Points above the dashed line have p-values < 0.05. (C) Functional protein association network analysis between DCLK1 and genes in the Hippo pathway. Line thickness indicates the strength of support for the data.

Fig. 4. DCLK1 regulates YAP at the transcriptional and protein level.
(A) After forced overexpression of DCLK1 in AsPC-1 cells, YAP mRNA and protein expression increased significantly, as shown by Real-time PCR and western blotting. (B) Real-time PCR and western blotting analysis of YAP mRNA and protein levels in DMSO control or LRRK2-IN-1 or XMD8-92 treated AsPC-1 cells. Cells were harvested 72 hours after incubation with inhibitor. (C) Real-time PCR, western blotting analysis of YAP mRNA and protein levels in DMSO control or LRRK2-IN-1, XMD8-

92 treated BxPC-3 cells. Cells were harvested 4 days after initiation of incubation with the inhibitor.

Fig. 5. DCLK1 regulates cell surface expression of PD-L1 through the Hippo pathway.

(A) and (B) After treated AsPC-1 cells with YAP inhibitor Verteporfin, the expression of PD-L1 dramatically decreased in mRNA level as well as in protein level, the results are shown by Real-time PCR and flow cytometry. (C) and (D) The expression of PD-L1 in BxPC-3 cells treated with YAP inhibitors decreased significantly, as shown by Real-time PCR and flow cytometry. (E) After induction of overexpression of DCLK1 in AsPC-1 cells were treated with YAP inhibitors, and the increase of PD-L1 was inhibited, as shown by flow cytometry. (F) CD4+ T cells were isolated from blood. CFSE-labeled CD4+ T cells were co-cultured with Aspc-1 cells treated with inhibitor or DCLK1-overexpressed cell line. CD4+ T-cell proliferation was quantified by FACS analysis. (G) The schematic diagram shows that DCLK1 can promote the expression of YAP protein, thus up-regulating PD-L1 and helping cancer cells avoid immune surveillance.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81802738 to Yang Ge)

References

[1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69(1):7-34.

[2] Singhi AD, Koay EJ, Chari ST, Maitra A. Early Detection of Pancreatic Cancer: Opportunities and Challenges. Gastroenterology 2019; 156(7):2024-2040.

[3] Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. Nat Immunol 2013; 14(12):1212-1218.
[4] Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. Sci Transl Med 2016; 8(328):328rv324.
[5] Feng M, Xiong G, Cao Z, Yang G, Zheng S, Song X, et al. PD-1/PD-L1 and immunotherapy for pancreatic cancer. Cancer Lett 2017; 407:57-65.
[6] Joseph C. Corbo, Thomas A. Deuel, Jeffrey M. Long. Doublecortin Is Required in Mice for Lamination of the Hippocampus But Not the Neocortex. The Journal of Neuroscience 2002;22(17): 7548–7557
[7] Hiroyuki Koizumi, Hiromi Fujioka, Kazuya Togashi. DCLK1 Phosphorylates the Microtubule Associated Protein MAP7D1 to Promote Axon Elongation in Cortical Neurons. Dev Neurobiol. 2017 Apr;77(4):493-510.
[8] Koizumi H, Tanaka T, Gleeson JG. doublecortin like kinase Functions with doublecortin to Mediate Fiber Tract Decussation and Neuronal Migration. Neuron. 2006 Jan 5;49(1):55-66.
[9] Parthasarathy Chandrakesan, Jiannan Yao, Dongfeng Qu. Dclk1 a tumor stem cell marker regulates pro survival signaling and self renewal of intestinal tumor cells. Mol Cancer. 2017 Feb 1;16(1):30.
[10] Liu W, Wang S, Sun Q, Yang Z, Liu M, Tang H. DCLK1 promotes epithelial-mesenchymal transition via the PI3K/Akt/NF-kappaB pathway in colorectal cancer. Int J Cancer 2018; 142(10):2068-2079.
[11] Li J, Wang Y, Ge J, Li W, Yin L, Zhao Z, et al. Doublecortin-Like Kinase 1 (DCLK1) Regulates B Cell-Specific Moloney Murine Leukemia Virus Insertion Site 1 (Bmi-1) and is Associated with Metastasis and Prognosis in Pancreatic Cancer. Cell Physiol Biochem 2018; 51(1):262-277.
[12] Weygant N, Qu D, Berry WL. Small molecule kinase inhibitor LRRK2-IN-1 demonstrates potent activity against colorectal and pancreatic cancer through inhibition of doublecortin-like kinase 1. Mol Cancer. 2014 May 6; 13:103.
[13] Yang Ge, Nathaniel Weygant, Dongfeng Qu. Alternative splice variants of DCLK1 mark cancer stem cells, promote self-renewal and drug-resistance, and can be targeted to inhibit tumorigenesis in kidney cancer. Int J Cancer. 2018 Sep 1;143(5):1162-1175.
[14] P Chandrakesan, J Panneerselvam, D Qu. Regulatory Roles of Dclk1 in Epithelial Mesenchymal Transition and Cancer Stem Cells. J Carcinog Mutagen. 2016 Apr;7(2).
[15] Westphalen CB, Asfaha S, Hayakawa Y. Long-lived intestinal tuft cells serve as colon cancer initiating cells. J Clin Invest. 2014;124(3):1283-1295.
[16] Nathaniel Weygant, Yang Ge, Dongfeng Qu. Survival of Patients with Gastrointestinal Cancers Can Be Predicted by a Surrogate microRNA Signature for Cancer Stem like Cells Marked by DCLK1 Kinase. Cancer Res. 2016 Jul 15;76(14):4090-9.

[17] C. Benedikt Westphalen, Yoshihiro Takemoto, Takayuki Tanaka. Dclk1 Defines Quiescent Pancreatic Progenitors that Promote Injury-Induced Regeneration and Tumorigenesis. Cell Stem Cell. 2016 Apr 7;18(4):441-55.
[18] Yu Zhang, Michelle Zoltan, Erick Riquelme. Immune Cell Production of Interleukin 17 Induces Stem Cell Features of Pancreatic Intraepithelial Neoplasia Cells. Gastroenterology. 2018 Jul;155(1):210-223.
[19] Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. Nat Rev Cancer 2013; 13(4):246-257.
[20] Janse van Rensburg HJ, Azad T, Ling M, Hao Y, Snetsinger B, Khanal P, et al. The Hippo Pathway Component TAZ Promotes Immune Evasion in Human Cancer through PD-L1. Cancer Res 2018; 78(6):1457-1470.
[21] Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, et al. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Research 2012; 41(D1):D808-D815.
[22] Calses PC, Crawford JJ, Lill JR, Dey A. Hippo Pathway in Cancer: Aberrant Regulation and Therapeutic Opportunities. Trends in Cancer 2019; 5(5):297-307.
[23] Hall CA, Wang R, Miao J, Oliva E, Shen X, Wheeler T, et al. Hippo Pathway Effector Yap Is an Ovarian Cancer Oncogene. Cancer Research 2010; 70(21):8517-8525.
[24] Xu MZ, Yao T-J, Lee NPY, Ng IOL, Chan Y-T, Zender L, et al. Yes-associated protein is an independent prognostic marker in hepatocellular carcinoma. Cancer 2009; 115(19):4576-4585.
[25] Wang Y, Dong Q, Zhang Q, Li Z, Wang E, Qiu X. Overexpression of yes-associated protein contributes to progression and poor prognosis of non-small-cell lung cancer. Cancer Sci 2010; 101(5):1279-1285.
[26] Da CL, Xin Y, Zhao J, Luo XD. Significance and relationship between Yes-associated protein and survivin expression in gastric carcinoma and precancerous lesions. World J Gastroenterol 2009; 15(32):4055-4061.
[27] Kapoor A, Yao W, Ying H, Hua S, Liewen A, Wang Q, et al. Yap1 activation enables bypass of oncogenic Kras addiction in pancreatic cancer. Cell 2014; 158(1):185-197.
[28] Moroishi T, Hayashi T, Pan WW, Fujita Y, Holt MV, Qin J, et al. The Hippo Pathway Kinases LATS1/2 Suppress Cancer Immunity. Cell 2016; 167(6):1525-1539 e1517.
[29] Ni X, Tao J, Barbi J, Chen Q, Park BV, Li Z, et al. YAP Is Essential for Treg-Mediated Suppression of Antitumor Immunity. Cancer Discov 2018; 8(8):1026-1043.
[30] Wang G, Lu X, Dey P, Deng P, Wu CC, Jiang S, et al. Targeting YAP-Dependent MDSC Infiltration Impairs Tumor Progression. Cancer Discov 2016; 6(1):80-95.
[31] Kim MH, Kim CG, Kim SK, Shin SJ, Choe EA, Park SH, et al. YAP-Induced PD-L1 Expression Drives Immune Evasion in BRAFi-Resistant Melanoma. Cancer Immunol Res 2018; 6(3):255-266.

[32] Maccalli C, Volontè A, Cimminiello C, Parmiani G. Immunology of cancer stem cells in solid tumours. A review. European Journal of Cancer 2014; 50(3):649-655.
[33] Zhang D, Tang DG, Rycaj K. Cancer stem cells: Regulation programs, immunological properties and immunotherapy. Seminars in Cancer Biology 2018; 52:94-106.
[34] Wang J, Wang S, Zhou J, Qian Q. miR-424-5p regulates cell proliferation, migration and invasion by targeting doublecortin-like kinase 1 in basal-like breast cancer. Biomed Pharmacother 2018; 102:147-152.

Manuscript (with changes highlighted) Click here to view linked References

Inhibition of DCLK1 down-regulates PD-L1 expression through Hippo pathway in human pancreatic cancer

Rui Yan[a], JianJian Li[b], Ying Zhou[a], Li Yao[c], Ruya Sun[b], Ying Xu[b], Yang Ge[a], Guangyu An[a]

[a]Beijing Chao-Yang Hospital Department of Oncology, Capital Medical University, Beijing, 100020, China

[b]Institute for Immunology, Department of Basic Medical Sciences, School of Medicine, Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing, 100084, China

[c]Department of Computational Biology, Cornell University, Ithaca, NY 14853, USA

Address Correspondence to:

Guangyu An PhD and Yang Ge PhD
8 Gongren Tiyuchang Nanlu Road, Chaoyang Dist. Beijing, China, 100020
Phone: 86-010-85231407; Fax: 86-010-85231570; E-mail: agybjcy@163.com and interna-1@163.com

Abstract

Immunotherapy is one of the most promising strategies for cancer, compared with traditional treatments. As one of the key emerging immunotherapies, anti-PD-1/PD-L1 treatment has brought survival benefits to many advanced cancer patients. However, in pancreatic cancer, immunotherapy-based approaches have not achieved a favorable clinical effect because of mismatch repair deficiencies. Therefore, the majority of pancreatic tumors are regarded as immune-quiescent tumors and non-responsive to single-checkpoint blockade therapies. Many preclinical and clinical studies suggest that it is still important to clarify the regulatory mechanism of the PD-1/PD-L1 pathway in pancreatic cancer. As a marker of cancer stem cells, DCLK1 has been found to play an important role in the occurrence and development of a plethora of human cancers. Recent researches have revealed that DCLK1 is closely related to EMT process of tumor cells, meanwhile, it could also be used as a biomarker in gastrointestinal tumors to predict the prognoses of patients. However, the role that DCLK1 plays in the immune regulation of tumor microenvironments remains unknown. Therefore, we sought to understand if DCLK1 could positively regulate the expression of PD-L1 in pancreatic cancer cells. Furthermore, we examined if DCLK1 highly correlated with the Hippo

pathway through TCGA database analysis. We found that DCLK1 helped regulate the level of PD-L1 expression by affecting the corresponding expression level of yes-associated protein in the Hippo pathway. Collectively, our study identifies DCLK1 as an important regulator of PD-L1 expression in pancreatic tumor and highlights a central role of DCLK1 in the regulation of tumor immunity.

Key words：DCLK1, PD-L1, Hippo pathway, YAP, tumor immunity

1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease characterized by an aggressive nature, high recurrence rate, resistance to chemotherapy, and the lowest 5-year survival rate (9 %) among all kinds of cancers[1]. However, despite the realization of high mortality and low survival rates, incidence rates for PDAC continue to increase worldwide[1]. The unfavorable prognosis for patients afflicted with PDAC is associated with late diagnosis, early metastasis, and low positive response rates for treatment[2]. Therefore, having a better understanding of the biological characteristics of PDAC would help researchers in developing more effective targeted therapies for patients afflicted with pancreatic cancer. Currently, treatments using immunotherapies have shown promise and have resulted in durable responses in many kinds of tumors. The discovery of numerous immunotherapy checkpoint blockades, including programmed death-1/programmed death-ligand 1 (PD-1/PD-L1), cytotoxic T lymphocyte associated antigen-4 (CTLA-4), and B7 superfamily member 1 (B7S1) have provided increased treatment opportunities for patients. The PD-1/PD-L1 axis has proven to be main signals that inhibit activation of T cells, ultimately resulting in tumor cell immune evasion processes[3]. The strategy, that targeting the checkpoints by using PD-L1 and its receptor PD-1 blockade, has opened a new era for treating cancers. However, the clinical benefit rate was still limited in patients with lung cancer and melanoma, which proved to respond to checkpoint blockade immunotherapy[4]. Immunotherapy used for treatment of pancreatic cancer has thus far showed few breakthroughs, however, a large number of clinical trials of anti-PD-1/PD-L1 therapy in pancreatic cancer are presently ongoing[5]. Therefore, a better understanding of the regulatory mechanisms of PD-1 and PD-L1 will help to further improve the efficacy of the PD-1/PD-L1 for other cancer patients and as a part of the ongoing development of combined treatment strategies.

Doublecortin-like kinase 1 (DCLK1) is a member of the protein kinase superfamily and was first identified for its function in neurogenesis[6-8]. However, in recent years, many researchers have discovered critical functions of DCLK1 in the dynamics of tumorigenesis. High-expression of DCLK1 has been reported in many tumors, including colorectal cancer[9,10], pancreatic tumors[11,12], and renal cell carcinoma tumors[13]. Growing evidences suggest that DCLK1 is a stem cell marker and associates with epithelial-mesenchymal transformation (EMT) progression in gastrointestinal tumors[14, 15]. Moreover, research by Weygant et al. (2016) revealed that the expression level of DCLK1 had significant value as a predictor of the survival time for patients with gastrointestinal tumors[16]. Meanwhile, Westphalen et al. (2016), reported that DCLK1+ cells can initiate Kras mutant pancreatic tumors in the context of pancreatitis after injury[17]. Recent research has also found that IL-17 was able to induce DCLK1 expression, which in turn could induce pancreatic cancer and help cancer cells to avoid immune surveillance[18]. The regulatory function of DCLK1 in tumor immunity is being gradually revealed. But, whether DCLK1 is a key factor in regulating the expression of PD-L1, remains unknown.

In this study, we aimed to investigate the role of DCLK1 in the regulation of PD-L1 expression. We induced overexpression of DCLK1 and inhibited DCLK1 (using inhibitors LRRK2-IN-1, XMD8-92) in pancreatic tumor cell lines, and sought to examine if DCLK1 and PD-L1 were strongly correlated. Mechanistically, we also explored if DCLK1 may regulate PD-L1 through affecting the Hippo pathway and ultimately whether DCLK1 may play an immunoregulatory role in the tumor microenvironment of pancreatic cancer.

2 Materials and methods

2.1. Cell culture

AsPC-1, BxPC-3, and PANC-1 cells were purchased from American Type Culture Collections (ATCC). PANC-1 cells were grown in DMEM. Whereas AsPC-1 and BxPC-3 cells were both maintained in RPMI-1640 medium. All mediums were supplemented with 10 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. All cell lines were incubated at 37 °C in a humid chamber with a controlled atmosphere of 5 % CO2 and stored in liquid nitrogen.

2.2. Reagents and transfection

LRRK2-IN-1 were purchased from TargetMol (catalog T2246), XMD8-92 from Selleck (catalog S7525), and verteporfin from MedChemExpress (catalog HY-B0146). Human DCLK1 (NM_001195416.2) cDNA was cloned into pLVX-IRES-Zsgreen vector. We transfected cells with Neofect reagent according to manufacturer's protocols. After 24-48 hours (hrs) of transfection, the transfection efficiency was observed by using fluorescence-based microscopy.

2.3. Protein extraction and Western blotting

Total protein was extracted from cell lines in RIPA lysis Buffer. Samples were separated on 10 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in 8 % non-fat milk for one hour and incubated with primary antibodies at 4 °C overnight. The membranes were washed three times with TBST, and probed with specific secondary antibody for one hour at room temperature. Then, we measured the level of protein expression using an ECL blotting analysis system. The primary antibodies used were rabbit anti-DCLK1 (Abcam, ab31704; 1:1000), rabbit anti-YAP (CST, 14064; 1:1000), rabbit anti-PD-L1 antibody (Abcam, ab205921; 1:1000), and mouse anti-GAPDH (CST, 5174; 1:1000).

2.4. RNA extraction and Quantitative RT-PCR

Total RNA was isolated in TRIzol according to the manufacturer's protocols. RNA was transcribed to cDNA using PrimeScript™ RT reagent Kit (TaKaRa, RR047A), then cDNA was used as the template to perform real-time PCR, using SYBR Green for molecular probes. GAPDH was used as a control to normalize the threshold value. Relative gene expression levels were determined by the $2^{-\Delta\Delta C(t)}$ method. The following primers were used: GAPDH- forward: 5'- GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'- GGCTGTTGTCATACTTCTCATGG-3'; DCLK1-forward: 5'-ACACTAAGACTGTGTCCATGTTAGAACTC-3', reverse: 5'-AAGCCTTCCTCCGACACTTCT-3'; YAP- forward: 5'-CAGAACCGTTTCCCAGACTAC-3', reverse: 5'-ATCAGCTCCTCTCCTTCTATGT-3'; and PD-L1-forward: 5'-GGACAAGCAGTGACCATCAAG-3', reverse: 5'-CCCAGAATTACCAAGTGAGTCCT-3'.

2.5. PBMC separation

Peripheral blood samples were obtained from healthy donors, and performed density gradient centrifugation by Ficoll-Paque Plus (GE Healthcare, USA). The peripheral blood mononuclear cell (PBMC) layer was collected and subjected to CD4 T cell isolation using Human CD4+ T cell kit (Stem cell, Canada).

2.6. Coculture and stimulation

Isolated CD4 T cells were labelled with CFSE following manufacturer's instructions (Cell Trace CFSE Cell Proliferation Kit, catalog #C34554, Life Technologies). CFSE labelled human CD4+ T cells were co-cultured with Aspc-1 cells for aforementioned DCLK1 overexpression assay and DCLK1 inhibition assay for 3 days, with the presence of 1ug/mL Anti-CD3 (eBioscience, clone number) and 5ug/mL Anti-CD28(eBioscience, clone). The proliferation of CD4 T cell were detected by flow cytometry on Fortessa (BD) Flow cytometry detection.

2.7. Flow cytometry

AsPC-1 and BxPC-3 cells were cultured in 6-well plates. After 24 hrs of cell adherence, cells were treated with LRRK2-IN-1(10 um), XMD8-92 (15 um), or were treated with DMSO as a control. After 3 days, cells were trypsinized and harvested. To analyze the levels of surface expression of PD-L1, cells were washed by using PBS and were incubated with anti-PD-L1 antibody (Biolegend, clone: 29E.2A3) for 15 min at room temperature. All flow cytometry analyses were performed using BD Biosciences FACS Aria III. Data were analyzed using FlowJo software and all parameters were set to manufacturer defaults.

2.8. Datasets and Statistical analysis

RNA-seq results were obtained from The Cancer Genome Atlas (TCGA) runs for pancreatic adenocarcinoma (PAAD). Data are presented as mean ± standard error of the mean (SEM), and all statistical analyses were performed using GraphPad Prism (version 7.0) with all parameters set to manufacturer defaults. Statistical significance was analyzed using a Student t test, $p < 0.05$ was considered to be statistically significant.

3 Results

3.1. PD-L1 (CD274) expression in pancreatic cancer cell lines

We found that the expression level of PD-L1 were higher in BxPC-3 cells than the other pancreatic tumor cell lines (AsPC-1, PANC-1). Real-time PCR results indicated that BxPC-3 cells had a 60-fold higher level of PD-L1 mRNA expression than the levels were for PANC-1 cells, and western blotting results were consistent with mRNA analyses (Fig. 1A). Next, we confirmed the cell surfaces expression level of PD-L by flow cytometry and we found that the expression level of PD-L1 reached up to 90 % on the BxPC-3 cell line, and in contrast found that PD-L1 expression was a rare occurrence in examinations of PANC-1 cells (Fig. 1B).

3.2. DCLK1 involved in the PD-L1 expression in pancreatic tumor cells

To better understand the influence of DCLK1 on the expression level of PD-L1, we established the transient transfection cell line upon the AsPC-1 cell line, and detected the expression level of DCLK1 by real-time PCR and western blotting (Figure 2A). We then performed flow cytometry experiments to investigate the degree of change in PD-L1 expression. As expected, we found that overexpression of DCLK1 can dramatically affect PD-L1 mRNA expression as well as its surface protein expression in the cell line (Fig. 2B). In contrast, we treated AsPC-1 and BxPC-3 cells with LRRK2-IN-1 and XMD8-92 which are inhibitors can suppress the expression of DCLK1 on mRNA and protein level (Fig. 2C, E), and measured PD-L1 expression. Results indicated that down-regulation of DCLK1 lead to suppression of both PD-L1 transcription and cell surface expression in the two cell lines (Fig. 2D, F). In totality, these results revealed that the expression of DCLK1 is related with PD-L1 expression in pancreatic cancer cell lines.

3.3. The TCGA database analysis revealed that DCLK1 is correlated with Hippo pathway

DCLK1 has been proposed as a biomarker of stem cells of gastrointestinal tumors. Previous research from authors in our group revealed that DCLK1+ tumor stem cells are critical to the development and progression of pancreatic cancer. Likewise, a variety of recent studies have shown the Hippo pathway plays important roles in controlling tumor initiation, metastases, and cancer stem cell homeostasis[19]. Interestingly, Yes-

associated protein (YAP) as an effector molecule of the Hippo pathway, when interacting with its co-activator TAZ, can initiate downstream transcription of PD-L1[20]. Analysis of TCGA pancreatic ductal adenocarcinoma data indicated that there was a strong correlation between DCLK1 mRNA expression and Hippo signaling pathway genes across 183 TCGA PAAD samples, including AMOTL1, AMOTL2, LATS1, and LATS2 (Fig. 3A). Results for Spearman's correlation coefficients and corresponding p-values (obtained from TCGA PAAD datasets) suggested that genes in the Hippo pathway are correlated with DCLK1 (Fig. 3B). We used sources including experiments, co-expression, neighborhood, gene fusion, and co-occurrence from the STRING[21] analysis to predict the active interactions between DCLK1 and the Hippo-pathway signaling in the network (Fig. 3C).

3.4. DCLK1 promotes the expression of Yes-associated protein

Recent studies have focused on an interesting relationship between the Hippo pathway and tumor immunity. As previously described, dysregulation of the Hippo pathway is correlated with the hallmarks of oncogenesis and immune cell evasion. Yes- associated protein (YAP), as the downstream effector proteins of Hippo pathway, can promote PD-L1 expression and induce PD-L1 derived immune evasion. Therefore, we sought to investigate whether DCLK1 regulates Yes-associated protein (YAP) at the transcriptional and protein level in AsPC-1 and BxPC-3 cells. We examined the expression of YAP via real-time PCR and western blotting assays. The results were consistent with the results from TCGA analysis, the expression level of YAP was significantly increased after forced overexpression of DCLK1 compared to the control group cells (Fig. 4A). Further, we investigated whether suppression of DCLK1 expression decreased the expression of YAP in the two cell lines. We treated the cells with DCLK1 inhibitor LRRK2-IN-1 and XMD8-92 for 72 hrs, and the YAP expression was detected through determination on mRNA and proteins level. As expected, after DCLK1 was antagonized by the inhibitors in both cell lines, the expression level of YAP was significantly decreased in AsPC-1 and BxPC-3 cells (Fig. 4B, C).

3.5. DCLK1 regulate PD-L1 expression through Hippo pathway

To investigate whether DCLK1 played an important role in modulating PD-L1 expression through impacts upon the Hippo pathway, we treated cells with YAP

inhibitor verteporfin. Verteporfin is a small molecule that can prevent the interaction between YAP/TAZ and TEAD, and allows for use to examine the expression level of PD-L1. After we used 2.0 uM and 10.0 uM of verteporfin as treatments upon AsPC-1 and BxPC-3 cells, the results from real-time PCR indicated that the PD-L1 mRNA expression were significantly decreased. Accordingly, flow cytometry also indicated that the cell surface expression of PD-L1 were downregulated (Fig. 5A-D). Next, we attempted to verify whether DCLK1 expression was able to regulate PD-L1 through the Hippo pathway. We analyzed PD-L1 expression after DCLK1 overexpression with or without YAP inhibition. The results indicated that the elevated PD-L1 expression in DCLK1 overexpression cells could be suppressed by the YAP inhibitor verteporfin (Fig. 5E). In addition, we performed T cell suppression assay in presence of DCKL1 over-expressed and inhibited cancer cells. The results indicate that DCLK1 can affect the expression of PD-L1, which can directly suppress the proliferation of T cell (5F). Collectively, these findings suggest that DCLK1-mediated PD-L1 expression requires the Hippo pathway and confirm that the association between DCKL1 and PD-L1 can directly affect T cell function in tumor microenvironment.

4、Discussion

Immune escape is one of the most prominent characteristics of cancer cells. Accumulating evidences show that the interaction between tumor cells and the tumor microenvironment plays an important role in disease progression. The expression of PD-L1 in cancer cells is an important mechanism for avoiding immune surveillance. Therefore, it is of great significance to study the regulatory mechanisms of PD-1/PD-L1 expression. In recent years, studies have shown that the Hippo pathway plays an important role in tumorigenesis and development. The Hippo pathway can regulate a variety of biological functions and behaviors of cells, including cell determination, cell polarity, cell proliferation, cell apoptosis, and others[19, 22]. The main core of molecules of this pathway includes sterile 20-like kinase 1/2 (MST1/2), large tumor suppressor 1/2 (LATS1/2), the downstream effector molecule yes associated protein (YAP), and its co-activator TAZ, and in sum these molecules compose a kinase cascade.

When the Hippo pathway is activated by upstream signals, YAP proteins become phosphorylated and are retained in the cytoplasm. Once the Hippo pathway dysregulated, uninhibited YAP localized to the nucleus and activated downstream molecules by binding to transcription factor TEA-domain family member (TEAD). YAP is the most important functional protein in this pathway, and has proven to be highly expressed in many types of tumors, including ovarian cancer, hepatocellular carcinoma, non- small-cell lung cancer, and gastric cancer[23-26]. Hippo pathway has been proven to be closely related to Kras status, while Kras mutations play an important role in the occurrence and development of pancreatic cancer. For the above reasons, the Hippo pathway has attracted particular attention for its role in the pancreatic cancer[27]. Accordingly, recent studies have revealed a close relationship between the Hippo pathway and tumor immunity[28]. Likewise, some studies have indicated that YAP is able to inhibit anti-tumor immunity by regulating regulatory T cells, an immunosuppressive cell in tumor microenvironments, thus ultimately promoting the escape of tumor cells from detection by immune system[29]. In the study of prostate cancer, it has been suggested that YAP protein can inhibit immune regulation by up-regulating the expression of Cxcl5 and recruiting Cxcr2+MDSC cells[30]. Interestingly, the Hippo pathway is also thought to be involved in the regulation of immune checkpoint PD-L1[20, 31], but no studies have focused on it in pancreatic cancer. These results suggest that it is necessary to clarify the potential effects of the Hippo pathway on PD-L1 expression in pancreatic cancer.

Tumor stem cells are a small subset of cancer cells and are resistant to radiotherapy and chemotherapy. In recent years, researchers have found that this population of cells also plays an important role in tumor immune escape. In order to carry out an immunotherapy-based treatment approach targeting cancer stem cells, we must first clearly recognize and define the specific markers of cancer stem cells, must also be able to distinguish between cancer stem cells and non-stem cells[32, 33]. Over the years, as a marker of cancer stem cells, the important role of DCLK1 in tumorigenesis, development and epithelial-mesenchymal transformation of cancer cells has been gradually recognized. For example, Ge et al. (2018) reported that DCLK1 was highly expressed in renal clear cell carcinoma. In vivo experiments have shown that monoclonal antibodies against DCLK1 have therapeutic value for renal cell carcinoma[13]. Liu et al. (2018) found that short variants of DCLK1 were able to regulate the EMT process of cancer cells through the NF-kB pathway in colorectal

cancer[10]. In breast cancer, DCLK1 has been found to be a carcinogenic gene that can drive invasion and metastasis of breast cancer cells, and can also be used as an indicator of poor prognosis and outcomes for afflicted patients[34]. However, at present, the regulatory role of DCLK1 in tumor immunity still remains unclear.

We used two inhibitors, LRRK2-IN-1 and XMD8-92, to inhibit the expression of DCLK1 in AsPC-1 and BxPC-3 pancreatic cancer cell lines, as well as inducing the overexpression of DCLK1 in AsPC-1 cells, in order to study whether DCLK1 regulated the expression of PD-L1 in pancreatic cancer. Results indicated that DCLK1 was important in regulating the expression of PD-L1 and showed a positive correlation. Next, we continued to explore the mechanisms behind how DCLK1 works to regulate PD-L1.

The Hippo pathway plays an important role in the development of pancreatic cancer, as well as in the regulation of cancer stem cells[19]. Knowing this, we explored whether DCLK1 was able to regulate the expression of PD-L1 through the Hippo pathway. Through TCGA database analysis, we confirmed that DCLK1 is related to many molecules of Hippo pathway. As mentioned above, many recent studies have confirmed that YAP protein in the Hippo pathway binds to downstream transcription factors as the mechanisms by which it regulates the expression of PD-L1, thus promoting the immune escape of tumors. Therefore, we sought to further confirm whether DCLK1 was able to regulate PD-L1 through influence of the YAP molecule in vitro. The results showed that DCLK1 was able to regulate the expression of YAP, both when analysis of mRNA and total amounts of protein. In addition, we found that the use of YAP inhibitors inhibited the increase of PD-L1 expression induced by DCLK1. Therefore, we concluded that DCLK1 regulates PD-L1 through the Hippo pathway.

These results suggest that DCLK1 may play a role in tumor immune microenvironment. The findings lay an important foundation for follow-up research such as to determine if DCLK1 may be used as a specific antigen of cancer stem cells, which has clinical significance in the immunotherapy of pancreatic cancer.

Fig. 1. PD-L1 expression in human pancreatic tumor cell lines.
(A) Real-time PCR and western blotting analysis of the basic expression level of PD-L1 in 3 pancreatic cancer cell lines (AsPC-1, BxPC-3, and PANC-1). (B) Flow cytometry was used to detect the expression level of PD-L1 in cell surfaces.

Fig. 2. DCLK1 induces PD-L1 expression in Pancreatic ductal adenocarcinoma cells.
(A) Real-time PCR and western blotting confirm DCLK1 expression in AsPC-1 wild type and DCLK1 overexpression cells. (B) Real-time PCR analysis of PD-L1 expression in mRNA level. Flow cytometry analysis of PD-L1 cell surface expression in wild type and DCLK1 overexpression cells. The display is indicative of statistical results for values of mean fluorescence intensity. (C) and (E) Real-time PCR and western blotting analysis of DCLK1 expression in inhibitor (LRRK2-IN-1, XMD8-92) treated cells (AsPC-1, BxPC-3). (D) and (F) Real-time PCR and flow cytometry analysis of PD-L1 expression in terms of the levels of mRNA and of protein. Results are exhibited as mean ±SEM (standard error of the mean). *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's t-test.

Fig. 3. The guilty-by-association analysis revealed that the expression of DCLK1 is strongly correlated with expression of genes belonging to the Hippo pathway.
(A) Heatmap showing that the expression patterns, of representative Hippo signaling pathway genes, are associated with the levels of expression of DCLK1. A green entry represents a gene with a low rank; while a red rectangle indicates a gene is highly expressed in tumor samples. The 25th and 75th percentiles were used to classify samples into low and high DCLK1 groups (46 samples for each group). (B) Spearman's correlation coefficients and corresponding p-values suggest that genes in the Hippo pathway are correlated with DCLK1. Red points indicate positive correlation, while blue points indicate negative correlation. Points above the dashed line have p-values < 0.05. (C) Functional protein association network analysis between DCLK1 and genes in the Hippo pathway. Line thickness indicates the strength of support for the data.

Fig. 4. DCLK1 regulates YAP at the transcriptional and protein level.
(A) After forced overexpression of DCLK1 in AsPC-1 cells, YAP mRNA and protein expression increased significantly, as shown by Real-time PCR and western blotting. (B) Real-time PCR and western blotting analysis of YAP mRNA and protein levels in DMSO control or LRRK2-IN-1 or XMD8-92 treated AsPC-1 cells. Cells were harvested 72 hours after incubation with inhibitor. (C) Real-time PCR, western blotting analysis of YAP mRNA and protein levels in DMSO control or LRRK2-IN-1, XMD8-

92 treated BxPC-3 cells. Cells were harvested 4 days after initiation of incubation with the inhibitor.

Fig. 5. DCLK1 regulates cell surface expression of PD-L1 through the Hippo pathway.

(A) and (B) After treated AsPC-1 cells with YAP inhibitor Verteporfin, the expression of PD-L1 dramatically decreased in mRNA level as well as in protein level, the results are shown by Real-time PCR and flow cytometry. (C) and (D) The expression of PD-L1 in BxPC-3 cells treated with YAP inhibitors decreased significantly, as shown by Real-time PCR and flow cytometry. (E) After induction of overexpression of DCLK1 in AsPC-1 cells were treated with YAP inhibitors, and the increase of PD-L1 was inhibited, as shown by flow cytometry. (F) CD4+ T cells were isolated from blood. CFSE-labeled CD4+ T cells were co-cultured with Aspc-1 cells treated with inhibitor or DCLK1-overexpressed cell line. CD4+ T-cell proliferation was quantified by FACS analysis. (G) The schematic diagram shows that DCLK1 can promote the expression of YAP protein, thus up-regulating PD-L1 and helping cancer cells avoid immune surveillance.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81802738 to Yang Ge)

References

[1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69(1):7-34.

[2] Singhi AD, Koay EJ, Chari ST, Maitra A. Early Detection of Pancreatic Cancer: Opportunities and Challenges. Gastroenterology 2019; 156(7):2024-2040.

[3] Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. Nat Immunol 2013; 14(12):1212-1218.
[4] Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. Sci Transl Med 2016; 8(328):328rv324.
[5] Feng M, Xiong G, Cao Z, Yang G, Zheng S, Song X, et al. PD-1/PD-L1 and immunotherapy for pancreatic cancer. Cancer Lett 2017; 407:57-65.
[6] Joseph C. Corbo, Thomas A. Deuel, Jeffrey M. Long. Doublecortin Is Required in Mice for Lamination of the Hippocampus But Not the Neocortex. The Journal of Neuroscience 2002;22(17): 7548–7557
[7] Hiroyuki Koizumi, Hiromi Fujioka, Kazuya Togashi. DCLK1 Phosphorylates the Microtubule Associated Protein MAP7D1 to Promote Axon Elongation in Cortical Neurons. Dev Neurobiol. 2017 Apr;77(4):493-510.
[8] Koizumi H, Tanaka T, Gleeson JG. doublecortin like kinase Functions with doublecortin to Mediate Fiber Tract Decussation and Neuronal Migration. Neuron. 2006 Jan 5;49(1):55-66.
[9] Parthasarathy Chandrakesan, Jiannan Yao, Dongfeng Qu. Dclk1 a tumor stem cell marker regulates pro survival signaling and self renewal of intestinal tumor cells. Mol Cancer. 2017 Feb 1;16(1):30.
[10] Liu W, Wang S, Sun Q, Yang Z, Liu M, Tang H. DCLK1 promotes epithelial-mesenchymal transition via the PI3K/Akt/NF-kappaB pathway in colorectal cancer. Int J Cancer 2018; 142(10):2068-2079.
[11] Li J, Wang Y, Ge J, Li W, Yin L, Zhao Z, et al. Doublecortin-Like Kinase 1 (DCLK1) Regulates B Cell-Specific Moloney Murine Leukemia Virus Insertion Site 1 (Bmi-1) and is Associated with Metastasis and Prognosis in Pancreatic Cancer. Cell Physiol Biochem 2018; 51(1):262-277.
[12] Weygant N, Qu D, Berry WL. Small molecule kinase inhibitor LRRK2-IN-1 demonstrates potent activity against colorectal and pancreatic cancer through inhibition of doublecortin-like kinase 1. Mol Cancer. 2014 May 6; 13:103.
[13] Yang Ge, Nathaniel Weygant, Dongfeng Qu. Alternative splice variants of DCLK1 mark cancer stem cells, promote self-renewal and drug-resistance, and can be targeted to inhibit tumorigenesis in kidney cancer. Int J Cancer. 2018 Sep 1;143(5):1162-1175.
[14] P Chandrakesan, J Panneerselvam, D Qu. Regulatory Roles of Dclk1 in Epithelial Mesenchymal Transition and Cancer Stem Cells. J Carcinog Mutagen. 2016 Apr;7(2).
[15] Westphalen CB, Asfaha S, Hayakawa Y. Long-lived intestinal tuft cells serve as colon cancer initiating cells. J Clin Invest. 2014;124(3):1283-1295.
[16] Nathaniel Weygant, Yang Ge, Dongfeng Qu. Survival of Patients with Gastrointestinal Cancers Can Be Predicted by a Surrogate microRNA Signature for Cancer Stem like Cells Marked by DCLK1 Kinase. Cancer Res. 2016 Jul 15;76(14):4090-9.

[17] C. Benedikt Westphalen, Yoshihiro Takemoto, Takayuki Tanaka. Dclk1 Defines Quiescent Pancreatic Progenitors that Promote Injury-Induced Regeneration and Tumorigenesis. Cell Stem Cell. 2016 Apr 7;18(4):441-55.
[18] Yu Zhang, Michelle Zoltan, Erick Riquelme. Immune Cell Production of Interleukin 17 Induces Stem Cell Features of Pancreatic Intraepithelial Neoplasia Cells. Gastroenterology. 2018 Jul;155(1):210-223.
[19] Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. Nat Rev Cancer 2013; 13(4):246-257.
[20] Janse van Rensburg HJ, Azad T, Ling M, Hao Y, Snetsinger B, Khanal P, et al. The Hippo Pathway Component TAZ Promotes Immune Evasion in Human Cancer through PD-L1. Cancer Res 2018; 78(6):1457-1470.
[21] Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, et al. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Research 2012; 41(D1):D808-D815.
[22] Calses PC, Crawford JJ, Lill JR, Dey A. Hippo Pathway in Cancer: Aberrant Regulation and Therapeutic Opportunities. Trends in Cancer 2019; 5(5):297-307.
[23] Hall CA, Wang R, Miao J, Oliva E, Shen X, Wheeler T, et al. Hippo Pathway Effector Yap Is an Ovarian Cancer Oncogene. Cancer Research 2010; 70(21):8517-8525.
[24] Xu MZ, Yao T-J, Lee NPY, Ng IOL, Chan Y-T, Zender L, et al. Yes-associated protein is an independent prognostic marker in hepatocellular carcinoma. Cancer 2009; 115(19):4576-4585.
[25] Wang Y, Dong Q, Zhang Q, Li Z, Wang E, Qiu X. Overexpression of yes-associated protein contributes to progression and poor prognosis of non-small-cell lung cancer. Cancer Sci 2010; 101(5):1279-1285.
[26] Da CL, Xin Y, Zhao J, Luo XD. Significance and relationship between Yes-associated protein and survivin expression in gastric carcinoma and precancerous lesions. World J Gastroenterol 2009; 15(32):4055-4061.
[27] Kapoor A, Yao W, Ying H, Hua S, Liewen A, Wang Q, et al. Yap1 activation enables bypass of oncogenic Kras addiction in pancreatic cancer. Cell 2014; 158(1):185-197.
[28] Moroishi T, Hayashi T, Pan WW, Fujita Y, Holt MV, Qin J, et al. The Hippo Pathway Kinases LATS1/2 Suppress Cancer Immunity. Cell 2016; 167(6):1525-1539 e1517.
[29] Ni X, Tao J, Barbi J, Chen Q, Park BV, Li Z, et al. YAP Is Essential for Treg-Mediated Suppression of Antitumor Immunity. Cancer Discov 2018; 8(8):1026-1043.
[30] Wang G, Lu X, Dey P, Deng P, Wu CC, Jiang S, et al. Targeting YAP-Dependent MDSC Infiltration Impairs Tumor Progression. Cancer Discov 2016; 6(1):80-95.
[31] Kim MH, Kim CG, Kim SK, Shin SJ, Choe EA, Park SH, et al. YAP-Induced PD-L1 Expression Drives Immune Evasion in BRAFi-Resistant Melanoma. Cancer Immunol Res 2018; 6(3):255-266.

[32] Maccalli C, Volontè A, Cimminiello C, Parmiani G. Immunology of cancer stem cells in solid tumours. A review. European Journal of Cancer 2014; 50(3):649-655.
[33] Zhang D, Tang DG, Rycaj K. Cancer stem cells: Regulation programs, immunological properties and immunotherapy. Seminars in Cancer Biology 2018; 52:94-106.
[34] Wang J, Wang S, Zhou J, Qian Q. miR-424-5p regulates cell proliferation, migration and invasion by targeting doublecortin-like kinase 1 in basal-like breast cancer. Biomed Pharmacother 2018; 102:147-152.

Authors' contributions

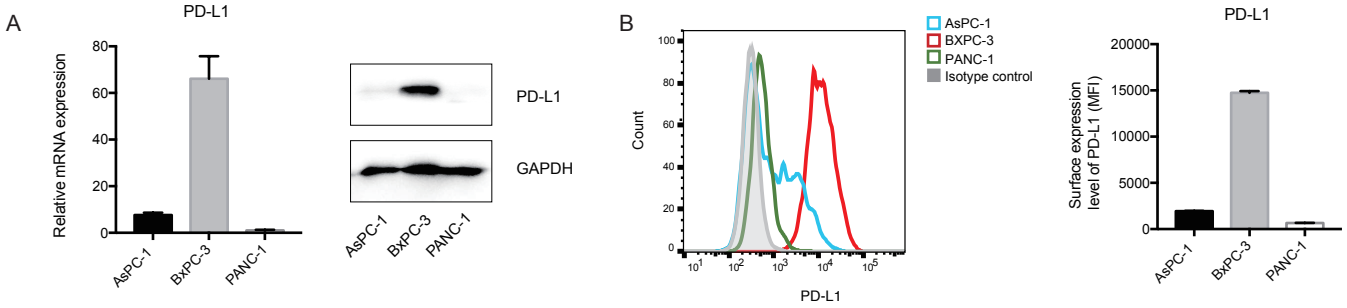
Guangyu An and Yang Ge designed the study. Rui Yan and JianJian Li generated the majority of the data. Ying Zhou helped the western blot analysis. Li Yao helped analyzed the TCGA database. Ying Xu helped constructed the plasmids and performed the transfection. Ruya Sun helped extracted the RNA and protein. Rui Yan and Guangyu An was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

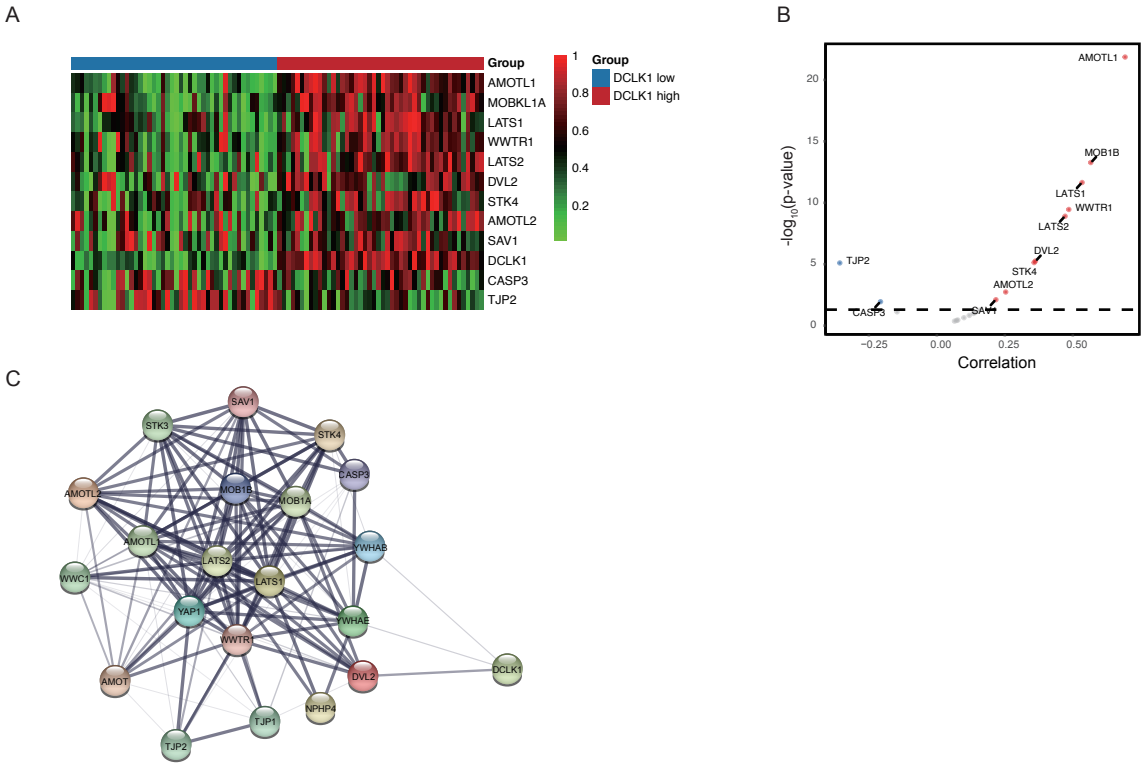
Figure

Figure 1



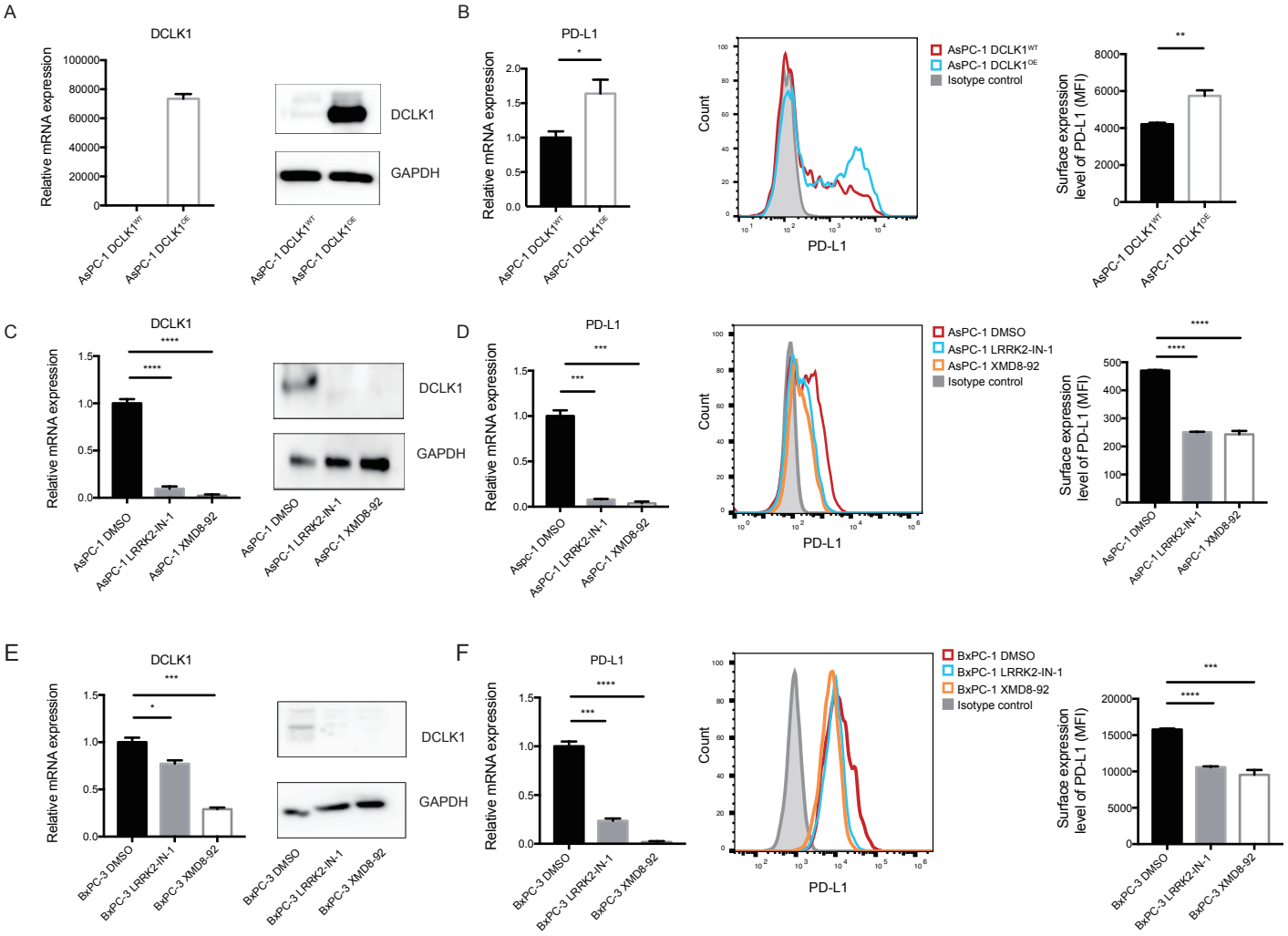
Figure

Figure 3



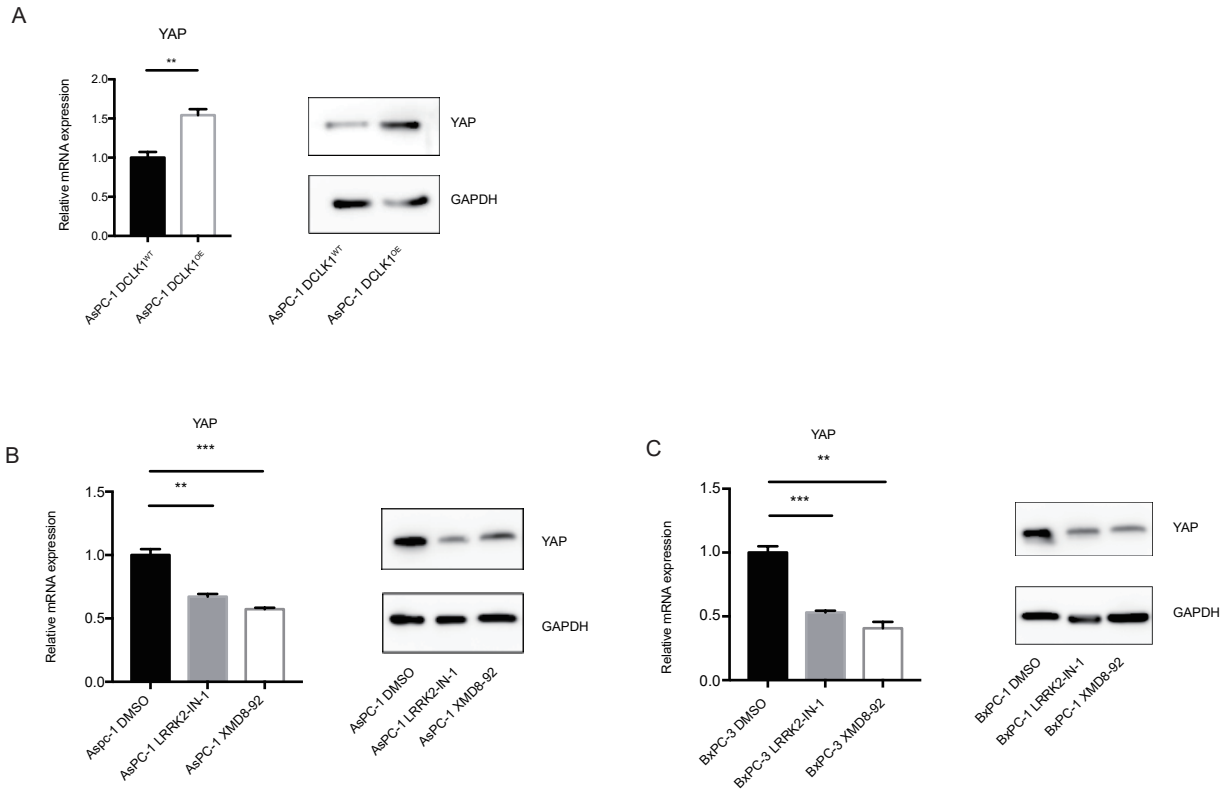
Figure

Figure 2



Figure

Figure 4



Figure

Figure 5