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Formin protein DAAM1 positively regulates PD-L1 expression via mediating the JAK1/STAT1 axis in pancreatic cancer

Rui Xu[1,2†], Mengyun Wan[3†], Jiadong Pan[4†], Jie Mei[1,2], Ji Zhou[3], Yan Shen[1,2], Jiayue Yang[5*], Yichao Zhu[3,6*] and Jing Sun[1*]

Abstract

Background Dishevelled-associated activator of morphogenesis1 (DAAM1) is a member of the evolutionarily conserved Formin family and plays a significant role in the malignant progression of various human cancers. This study aims to explore the clinical and biological significance of DAAM1 in pancreatic cancer.

Methods Multiple public datasets and an in-house cohort were utilized to assess the clinical relevance of DAAM1 in pancreatic cancer. The LinkedOmics platform was employed to perform enrichment analysis of DAAM1-associated molecular pathways in pancreatic cancer. Subsequently, a series of in vitro and in vivo experiments were conducted to evaluate the biological roles of DAAM1 in pancreatic cancer cells and its effects on intratumoral T cells.

Results DAAM1 was found to be upregulated in pancreatic cancer tissues, with higher expression levels observed in tumor cells. Additionally, high expression of DAAM1 was associated with poor prognosis. DAAM1 acted as an oncogene in pancreatic cancer, and its inhibition suppressed tumor cell proliferation, migration, and invasion, while promoted apoptosis. Furthermore, DAAM1 was involved in the JAK1/STAT1 signaling pathway and regulated PD-L1 expression in pancreatic cancer cells. The inhibition of DAAM1 also significantly reduced the exhaustion levels of CD8+ T cells.

Conclusion In conclusion, DAAM1 functions as an oncogene and is immunologically implicated in pancreatic cancer, these findings suggest that DAAM1 may serve as a promising therapeutic target for the clinical management of pancreatic cancer.

Keywords DAAM1, PD-L1, JAK1/STAT1, Pancreatic cancer, T cells

†Rui Xu, Mengyun Wan, and Jiadong Pan contributed equally to this work.

*Correspondence:
Jiayue Yang
yang_jiayue123@163.com
Yichao Zhu
zhuyichao@njmu.edu.cn
Jing Sun
sunj@njmu.edu.cn
[1] Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China
[2] The First Clinical Medicine College, Nanjing Medical University, Nanjing, Jiangsu, China
[3] Department of Physiology, School of Basic Medical Sciences, Nanjing Medical University, Nanjing 211166, China
[4] Departments of Gastroenterology, The Third People's Hospital of Kunshan, Suzhou 215300, China
[5] Departments of Endocrinology, The Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi Medical Center, Nanjing Medical University, Wuxi, Jiangsu, China
[6] Department of General Surgery, The Affiliated Taizhou People's Hospital of Nanjing Medical University, Taizhou, Jiangsu, China

Background

Pancreatic cancer is one of the most prevalent cancers worldwide, particularly in developed countries, with risk factors such as obesity, diabetes, and alcohol consumption contributing to its incidence [1]. It is characterized by an insidious onset, early invasion, metastasis, and resistance to adjuvant therapies [2]. Pancreatic cancer is one of the deadliest solid tumors, with a 5-year survival rate of less than 10%. Despite advances in molecular-targeted therapies that have improved outcomes in other cancers, pancreatic cancer still has a poor prognosis and limited treatment options. Chemotherapies such as FOLFIRINOX and gemcitabine/nab paclitaxel improve median survival by only 2–4 months and associated with significant toxic side effects [3]. Pancreatic cancer, classified as an immune-cold tumor, shows limited response to immunotherapy in clinical trials. A major challenge in treating pancreatic cancer is the lack of specific diagnosis biomarkers and therapeutic targets due to its high molecular heterogeneity. Therefore, understanding the mechanisms of tumor progression and immunotherapeutic resistance is critical.

Dishevelled-associated activator of morphogenesis1 (DAAM1), a formin family protein, regulates filopodia formation and phagocytosis by mediating cytoskeletal rearrangement through non-canonical Wnt signaling [4]. Previous studies have demonstrated that DAAM1 plays a critical role in tumor metastasis and migration by promoting actin filament elongation [5, 6]. DAAM1 is overexpressed in various tumor tissues, including breast cancer [7], gastric cancer [8], and colorectal cancer [9]. DAAM1 has been identified as a potential immunotherapy biomarker in renal cell carcinoma [10]. DAAM1 is strongly corrected with the levels of immune cells and immune checkpoints. Data from public cohorts show that DAAM1 is positively associated with PD-L1, suggesting that DAAM1 may serve as a biomarker for PD-1/PD-L1 blockade therapy [10]. However, the role of DAAM1 in regulating PD-L1 expression in pancreatic cancer remains largely unknown.

In current years, immunotherapy, which aims to reactivate weakened immune systems, has become an increasingly important approach in cancer treatment. [11]. Among various immunotherapy approaches, immune checkpoint blockade (ICB) therapy has been the most widely applied in clinical practice [12, 13]. However, not all patients with advanced tumors respond to ICB therapy, and some may experience hyperprogression or pseudoprogression following ICB treatment. One critical factor response to ICB therapy is the expression level of immune checkpoint inhibitors [14, 15]. PD-L1 expression status is a key determinant of whether a patient will respond to immunotherapy [16]. Therefore, there is an urgent need to investigate the molecular regulatory mechanisms of PD-L1 expression in malignant cells, especially in pancreatic cancer.

In this research, we examined the expression levels and prognostic significance of DAAM1 in pancreatic cancer. We discovered that DAAM1 was strongly associated with poor prognosis in pancreatic cancer and promoted malignant phenotypes in tumor cells. Furthermore, we demonstrated that DAAM1 regulates PD-L1 expression by modulating the JAK1/STAT1 signaling pathway in pancreatic cancer cells. Overall, our findings suggest that DAAM1 may serve as a potential biomarker for the management of pancreatic cancer.

Methods

Clinical samples

Pancreatic cancer tissue microarray (TMA, catalog HPanA120Su02) containing 66 paraffin-embedded tumor and 54 para-tumor samples was obtained from Outdo BioTech (Shanghai, China), and ethical approval was granted by the Clinical Research Ethics Committee in Outdo Biotech. All clinic-pathological and follow-up data were provided by Outdo Biotech. In addition, another pancreatic cancer TMA (catalog HPanA120CS01) containing 69 tumor samples was used to assess the correlation between DAAM1 and PD-L1 as well as CD8 using multiplexed immunohistochemistry (mIHC). Ethical approval was granted by the Clinical Research Ethics Committee in Outdo Biotech as well.

Immunohistochemistry and mIHC

Human paraffin-embedded TMA (catalog HPanA120Su02) underwent immunohistochemistry (IHC) staining of DAAM1, and paraffin-embedded TMA (catalog HPanA120CS01) underwent mIHC staining of DAAM1, PD-L1, and CD8. Standard operating procedures were employed for IHC and mIHC staining [17, 18]. The primary antibodies used were as follows: anti-DAAM1 (1:200 dilution, catalog 14876-1-AP, ProteinTech, Wuhan, China), anti-PD-L1 (1:1000 dilution, catalog 13684, Cell Signaling Technology), and anti-CD8 (1:1000 dilution, catalog 85336, Cell Signaling Technology). Antibody staining was visualized with DAB and hematoxylin counterstain, and stained sections were scanned using Aperio Digital Pathology Slide Scanners. The IHC staining was independently assessed by two pathologists. For semi-quantitative assessment of DAAM1 IHC staining, the H-score criterion was used. In addition, mIHC staining was evaluated using HALO software to determine cells positive with various markers.

Cell culture and treatment

Human pancreatic cancer cell lines PANC1 (catalog KGG3372-1) and BXPC3 (catalog KGG3244-1) were purchased from KeyGEN (Nanjing, China). PANC1 cells were cultured in RPMI-1640 media added with 10% fetal bovine serum and 10 μg/ml insulin at 37 °C with 5% CO_2 and BXPC3 cells were cultured in RPMI-1640 media added with 10% calf bovine serum at 37 °C with 5% CO_2. All human cell lines were authenticated using short tandem repeat profiling and all assays were conducted with mycoplasma-free. For in vitro assays, cells were transfected with control siRNA or siRNA for DAAM1 [19] using Lipofectamine 3000 according to the manufacturer's manual. Recombined active IFN-γ protein (catalog KGD1211) was purchased from KeyGEN (Nanjing, China), and the concentration of (10 ng/mL) was used for cell culture for 24 h.

Quantitative real-time PCR, Western blot, and flow cytometry analysis

The total RNA of cells was extracted utilizing Trizol reagent (catalog KGF5101, KeyGEN, Nanjing, China). The primers for DAAM1, PD-L1 and GAPDH mRNA reverse transcription were synthesized in KeyGEN. Quantitative real-time PCR (qRT-PCR) was performed utilizing the One-Step TB GreenTM PrimeScriptTM RT-PCR Kit II (catalog RR086B, TaKaRa, Kyoto, Japan). Primers utilized for gene amplification were as follows: DAAM1: (forward) 5′-AGCCAGTTCATCACAGTAGCC-3′ and (reverse) 5′-TATTTTGCCAGCCTCTTCCCC-3′, PD-L1: (forward) 5′-CCTCTGGCACATCCTCCAAAT-3′ and (reverse) 5′-GTCAGTGCTACACCAAGGCAT-3′, and GAPDH (forward) 5′-AGATCATCAGCAATGCCT CCT-3′ and (reverse) 5′-TGAGTCCTTCCACGATAC CAA-3′.

Pancreatic cancer cells were plated in maintained in 6-well plates. The total proteins of cells were harvested using a lysis buffer. Then, SDS-PAGE and Western blotting analysis were conducted referring to standardized protocols. The primary antibodies used as follows: DAAM1 (1:1000 dilution, catalog 14876-1-AP, ProteinTech), PD-L1 (1:1000 dilution, catalog 17952-1-AP, ProteinTech), JAK1 (1:1000 dilution, catalog ab133666, Abcam), p-JAK1 (1:1000 dilution, catalog ab138005, Abcam), STAT1 (1:1000 dilution, catalog 10144-2-AP, ProteinTech), p-STAT1 (1:1000 dilution, catalog 7649T, Cell Signaling Technology), BAX (1:1000 dilution, catalog 60267-1-Ig, Proteintech), Caspase9 (1:1000 dilution, catalog 10380-1-AP, Proteintech), E-cadherin (1: 20,000 dilution, catalog 20874-1-AP, Proteintech), N-cadherin (1: 1000 dilution, catalog 22018-1-AP, Proteintech), and GAPDH (1:2000 dilution, catalog 60004-1-Ig, ProteinTech). Protein levels were standardized to GAPDH.

Moreover, considering that membrane-expressed PD-L1 is essential for tumor immune escape, the membrane-expressed PD-L1 in pancreatic cancer cells was also checked by the flow cytometry analysis. The primary antibody used was PD-L1 flow cytometry antibody (catalog APC-65081, ProteinTech).

In vitro assays for cellular functions

Suspended cancer cells were sown at a density of 5×10^3 cells/mL (100 μL/well) into a 96-well plate and incubated at 37 °C to measure the levels of cell proliferation. Subsequently, each well received 10 μL of CCK-8 reagent (catalog KGA9305, KeyGEN), and the plate was incubated for 2 h. Next, using a microplate reader, the optical density of each well was determined at 450 nm. Furthermore, 500 cancer cells were planted per well in six-well plates, and the cells were grown for one to two weeks at 37 °C. Colonies were stained with crystal violet solution for 30 min after the incubation period. The colonies were counted after the wells were cleaned with water and let to air dry. Each measurement was performed in triplicate. In addition, Transwell chambers were utilized, either with or without Matrigel (Corning) coating, depending on the intended test, to measure the amounts of cell migration and invasion. In the upper compartment, 500 μL of media containing 10% FBS was added, and cancer cells (5×10^4) were sown in 200 μL of serum-free medium. The cells that had moved or infiltrated the membrane's lower surface were seen and counted after a 24-h period. The apoptosis of cancer cells was measured using the Annexin V-FITC/PI Kit (catalog KGA1102, KeyGEN) and flow cytometry analysis in accordance with the manufacturer's instructions in order to determine the levels of cell apoptosis.

In vitro cytotoxicity assay

Peripheral blood mononuclear cells (PBMC) from a healthy control were collected with the ethical approval from the Clinical Research Ethics Committee of The Affiliated Wuxi People's Hospital of Nanjing Medical University. Using the Dynabeads™ human CD8 selection Kit (catalog 11333D, Invitrogen), $CD8^+$T cells were extracted, and they were then cultivated in ImmunoCult™-XF T cell expansion medium (catalog 10981, STEMCELL Technologies). After activating T cells with ImmunoCult human CD3/CD28 T cell activator (catalog 10971, STEMCELL Technologies), T cells were co-cultured with tumor cells at a 5:1 effector-to-target ratio at 37 °C for 48 h. The levels of T cell exhaustion were determined based on membrane PD-1 expression

using the corresponding antibody (catalog 379210, BioLegend, California, USA) by flow cytometry analysis.

Bioinformatics analysis

The expression of DAAM1 in pan-cancer was investigated by the GEPIA platform (http://gepia.cancer-pku.cn/) [20], the expression of DAAM1 in various cell types in pancreatic cancer was explored by the TISCH2 tool (http://tisch.comp-genomics.org/home/) [21], and the enrichment analysis of DAAM1 in pancreatic cancer was conducted using the LinkedOmics tool (https://www.linkedomics.org/login.php) [22]. Default options were used for all parameters in the above public databases. In addition, transcriptome dataset and clinical annotations of pancreatic cancer patients in the Cancer Genome Atlas (TCGA) dataset were acquired through the University of California Santa Cruz (UCSC) Xena platform (https://xenabrowser.net/datapages/). Samples with available complete clinical data were specifically chosen for further analysis.

Tumor-bearing mouse model

C57BL/6 mice (4–5 weeks old) were purchased from Suzhou SiBeiFu Co., Ltd. The mice were raised in SPF-grade experimental animal centers and provided with free access to food and water. To establish the syngeneic mouse model, PANC02 mouse pancreatic cancer cells (catalog KGG2235-1, KeyGEN) maintained in RPMI-1640 media supplemented with 10% FBS were subcutaneously injected into the flanks of these male mice (5×10^6 cells). For DAAM1 knockdown in mouse tumor cells, the DAAM1 shRNA lentivirus (catalog sc-62191-V, Santa Cruz) was used. Upon tumors in the control group reaching an average size of approximately 100 mm^3 (day 1), tumors were monitored and regularly measured with calipers every two days. The tumors were removed from the unconscious animals at day 14, which was subsequently documented and weighed. Paraffin-embedded tumor samples from the above model were submitted for DAAM1 (1:200 dilution, catalog 14876-1-AP, ProteinTech), PD-L1 (1:1000 dilution, catalog 17952-1-AP, ProteinTech), CD8 (1:2000 dilution, catalog ab217344, Abcam), N-cadherin (1: 2000 dilution, catalog 22018-1-AP, Proteintech), Ki-67 (1:1000 dilution, catalog 28074-1-AP, Proteintech), and Caspase9 (1:500 dilution, catalog 10380-1-AP, Proteintech). All animal experiments were approved by the Research Ethics Committee in The Affiliated Wuxi People's Hospital of Nanjing Medical University.

Statistical analysis

All statistical analyses were conducted using SPSS software (version 23.0) and GraphPad Prism 6. Experimental results are presented as the mean ± SD based on the results from three independent experiments. Unpaired two-tailed Students t test was used for the calculation of P values in most cases. Prognostic values of DAAM1 in pancreatic cancer were checked using the log-rank test. Correlation analysis was performed using the Pearson correlation analysis. P values < 0.05 were considered statistically significant.

Results

DAAM1 was highly expressed in pancreatic cancer and predicted poor prognosis

We first analyzed the expression of DAAM1 across a spectrum of cancers, observing that in pancreatic cancer, the expression of DAAM1 within tumors is markedly higher than in normal tissues (Fig. 1A, B). Further we performed IHC staining on a pancreatic cancer TMA to verify DAAM1 expression in tumor and para-tumor tissues. We found that compared with para-tumor tissues, the expression level of DAAM1 was significantly upregulated in tumor tissues in pancreatic cancer (Fig. 1C, D). After that, we tried to assess the relationship between DAAM1 and prognosis in pancreatic cancer via public and in-house cohorts. The results exhibited that patients with high DAAM1 expression showed poorer prognosis compared with those with low expression in the TCGA cohort (Fig. 1E). In addition, the high expression of DAAM1 was notably associated with increased dead cases and also predicted

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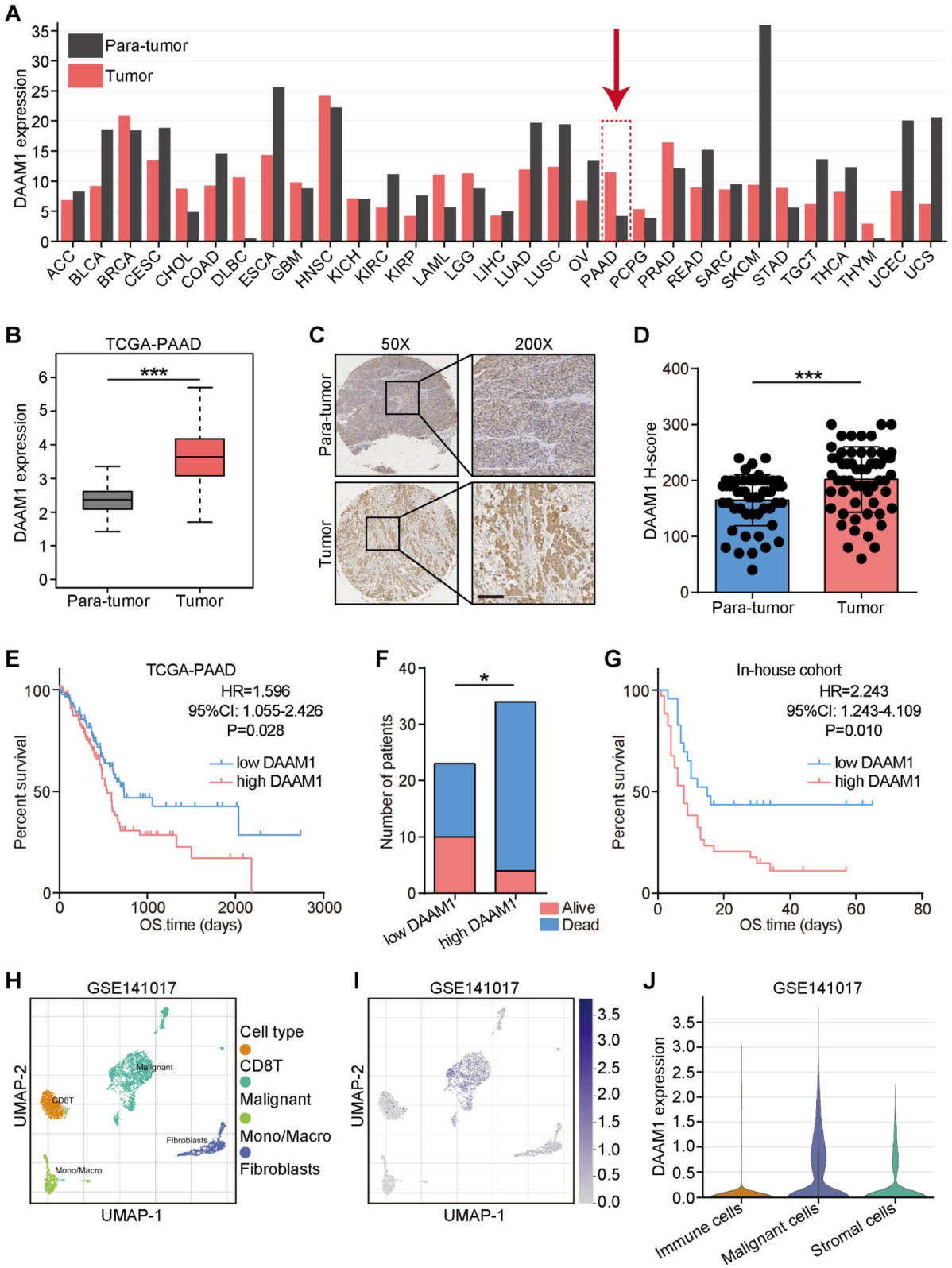
Fig. 1 DAAM1 was upregulated in pancreatic tumor tissues and enriched in tumor cells. **A** Pan-cancer analysis of DAAM1 expression in tumor and para-tumor samples. The data was obtained from the GEPIA database. **B** DAAM1 expression in tumor and para-tumor samples in pancreatic cancer. The data was obtained from the GEPIA database. Difference was checked by Student's t test. ***P < 0.001. **C** Representative images uncovering the expression of DAAM1 in tumor and para-tumor samples in the HPanA120Su02 pancreatic cancer cohort. Bar = 100 μm. **D** Quantitative analysis of DAAM1 expression in tumor and para-tumor samples in the in-house pancreatic cancer cohort. Difference was checked by Student's t test. ***P < 0.001. **E** Prognostic value of DAAM1 in pancreatic cancer in the TCGA cohort. Difference was checked by log-rank test. **F** Association between DAAM1 expression and survival status in the HPanA120Su02 cohort. Difference was checked by Chi-square test. **G** Prognostic value of DAAM1 in pancreatic cancer in the HPanA120Su02 cohort. Difference was checked by log-rank test. **H**, **I** UMAP-1 analysis displaying the expression of DAAM1 in various cell subtypes in the GSE141017 dataset. **J** Difference in DAAM1 expression in immune cells, malignant cells, and stromal cells in the GSE141017 dataset



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unfavorable prognosis in the in-house cohort (Fig. 1F, G). Next, the expression of DAAM1 in various cell types in pancreatic tumor tissues was also checked by the public single-cell sequencing data. The results found that DAAM1 mainly expressed on cancer cells, rather than immune and stromal cells in tumor microenvironment (Fig. 1H–J, Supplementary Fig. 1A–C) Taken together, DAAM1 is highly upregulated in pancreatic cancer tissues, especially in tumor cells, and associated with poor prognosis.

DAAM1 stimulated pancreatic cancer cell aggressiveness

Given the oncogenic role of DAAM1 in several cancer types [7–9], we guessed that DAAM1 might act as oncogene in pancreatic cancer as well. To explore potential effects of DAAM1 on pancreatic cancer cells, we determined the relationship between DAAM1 expression and gene markers for cell proliferation, invasion, and apoptosis in the TCGA dataset, and the results showed that DAAM1 was positively correlated with markers for proliferation and invasion, but negatively correlated with apoptosis markers (Fig. 2A, B). To confirm this conclusion, cell experiments were conducted. We performed DAAM1 siRNA knockdown in PANC1 and BXPC3 cell lines respectively. Transfection efficiency was confirmed by qPCR and Western blot assays (Fig. 2C, Supplementary Fig. 2). Apoptosis assays showed that DAAM1 depletion promoted apoptosis levels of pancreatic cancer cells, compared with the controlled group (Fig. 2D). CCK-8 and colony formation assays demonstrated that DAAM1 knockdown decreased pancreatic cancer cell proliferation ability (Fig. 2E, F). In addition, Transwell assays indicated that the migration and invasion abilities of tumor cells markedly declined upon DAAM1 knockdown (Fig. 2G, H). Moreover, the classical epithelial-mesenchymal transition (EMT) markers E-cadherin and N-cadherin, and apoptosis markers BAX and Caspase9 were detected by Western blot experiments that after knocking down DAAM1 in PANC1 and BXPC3 cells. The expression of E-cadherin was increased, and the expression of N-cadherin was increased, and the expression of apoptotic factors BAX and Caspase9 were increased (Supplementary Fig. 3A–D). Thus, these findings confirm that DAAM1 may exert an oncogenic role in pancreatic cancer.

DAAM1 modulated the JAK1/STAT1 pathway in pancreatic cancer

Next, DAAM1-related molecular pathways in pancreatic cancer were further explored using the LinkedOmics tool. KEGG pathway analysis suggested that DAAM1 was positively interacted with the JAK/STAT signaling pathway, microRNAs in cancer, and Prolaction signaling pathway in pancreatic cancer (Fig. 3A). Based on the ranking of FDR values, we chose the JAK/STAT signaling pathway for further investigation (Fig. 3B). Then, we used transcriptional data from the TCGA dataset to evaluate the correlation between individual members in JAK family and DAAM1. We found that the expression of JAK1 was positively related with DAAM1 (Fig. 3C). STAT1, the downstream of JAK1 [23, 24], was confirmed to have positive correlation with DAAM1 (Fig. 3D). Therefore, we hypothesized that DAAM1 regulated pancreatic cancer progression by modulating the JAK1/STAT1 signaling. We assessed the levels of total and phosphorylated JAK1 and STAT1 in DAAM1 knockdown cancer cells and control groups. The diminished p-STAT1 and p-JAK1 levels were observed in cancer cells transfected with DAAM1 siRNA (Fig. 3E). Therefore, we conclude that DAAM1 positively regulates the JAK1/STAT1 pathway in pancreatic cancer.

DAAM1 was highly correlated with and regulated PD-L1 in pancreatic cancer

Previous studies have confirmed that PD-L1 was a direct target of the JAK1/STAT1 pathway [25, 26]. We after analysis of the relationship between JAK1/STAT1 and PD-L1 in the TCGA database, we discovered that the expression of JAK1/STAT1 was positively related to PD-L1 in pancreatic cancer (Fig. 4A, B). Thus, we hypothesized that DAAM1 could regulated PD-L1 via the JAK1/STAT1 pathway. The analysis of the TCGA

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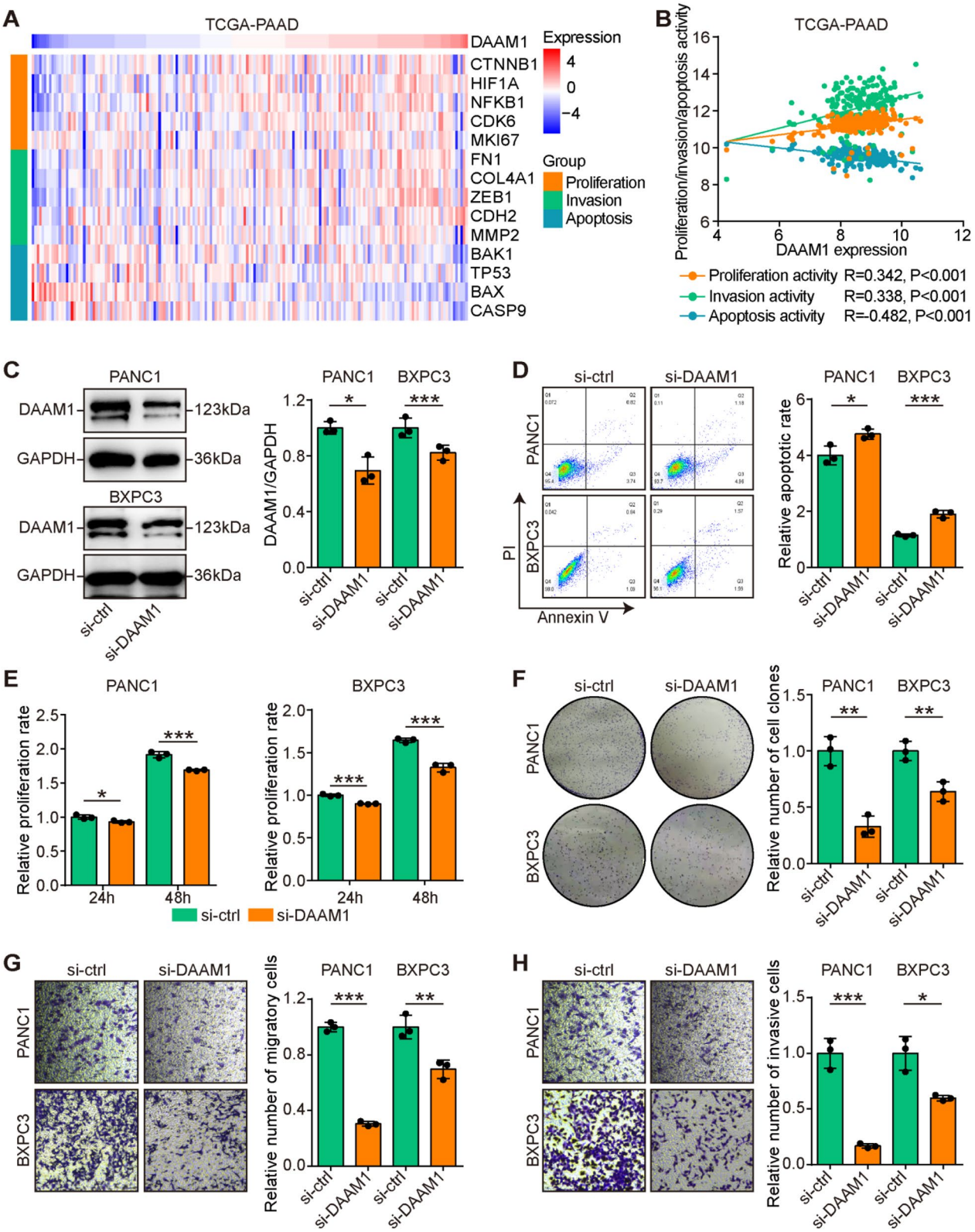
Fig. 2 DAAM1 promoted tumor cells aggressiveness in pancreatic cancer. **A** Heatmap displaying the correlation between DAAM1 and gene markers for proliferation, invasion, and apoptosis. The data was obtained from the TCGA database. **B** Correlation analysis of DAAM1 and median value of gene markers for proliferation, invasion, and apoptosis. The data was obtained from the TCGA database. Difference was checked by Pearson test. **C** The silencing efficiency of DAAM1 in pancreatic cancer cells was assessed by Western blotting. Difference was checked by Student's t test. *P < 0.05, ***P < 0.001. **D** The apoptotic level of control and DAAM1-silencing pancreatic cancer cells was examined by flow cytometry. Difference was checked by Student's t test. *P < 0.05, ***P < 0.001. **E**, **F** The proliferative capacity of control and DAAM1-silencing pancreatic cancer cells was examined by CCK-8 and clone formation assays. Difference was checked by Student's t test. *P < 0.05, **P < 0.01, ***P < 0.001. **G**, **H** The migratory and invasive capacity of control and DAAM1-silencing pancreatic cancer cells were examined by Boyden chamber assay. Difference was checked by Student's t test. *P < 0.05, **P < 0.01, ***P < 0.001



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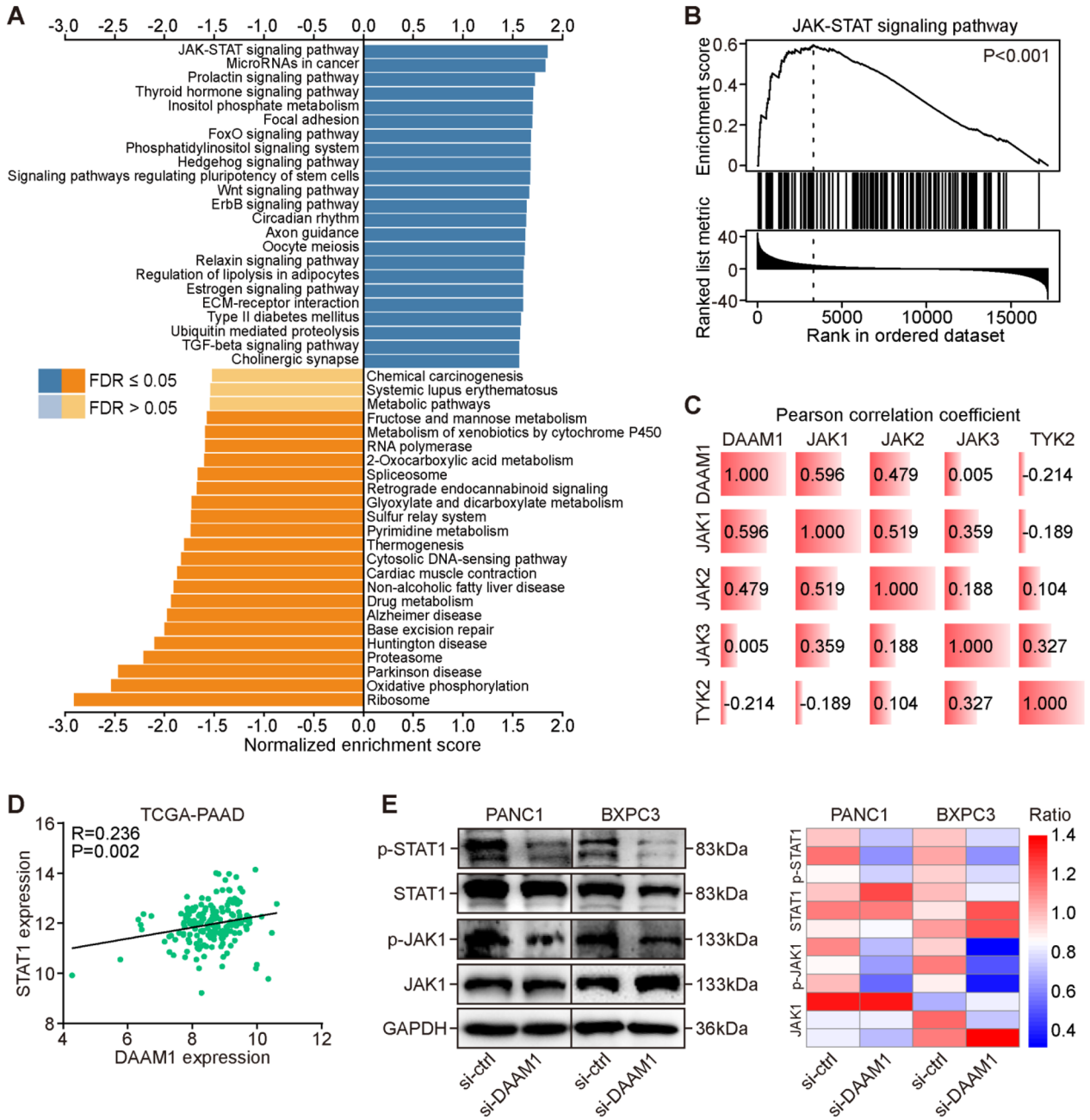

Fig. 3 DAAM1 positively correlated with the JAK1/STAT1 pathway. **A** Significantly enriched KEGG pathways of DAAM1 in pancreatic cancer. The data was obtained from the LinkedOmics database. **B** GSEA analysis of the correlation between DAAM1 expression and the JAK/STAT pathway in pancreatic cancer. The data was obtained from the LinkedOmics database. **C** Correlation between DAAM1 and four members of JAK family in pancreatic cancer. The data was obtained from the TCGA database. Difference was checked by Pearson test. **D** Correlation between DAAM1 and STAT1 in pancreatic cancer. The data was obtained from the TCGA database. Difference was checked by Pearson test. **E** Western blotting analysis of expression of total and phosphorylated JAK1 and STAT1 in control and DAAM1-silencing pancreatic cancer cells

dataset demonstrated that DAAM1 was positively correlated with PD-L1 expression (Fig. 4C). For further investigation, we transfected PANC1 and BXPC3 cells with DAAM1 siRNA or control siRNA. Results showed that both mRNA and protein levels of PD-L1 were markedly downregulated in DAAM1 knockdown tumor cells, and the inhibition of PD-L1 could be reversed by JAK1/STAT1 pathway activator IFN-γ (Fig. 4D, Supplementary Fig. 4). Moreover, considering that membrane-expressed PD-L1 is essential for tumor immune escape,

the membrane-expressed PD-L1 in pancreatic cancer cells was also checked by the flow cytometry analysis, and the results showed that DAAM1 inhibition notably decreased membrane-expressed PD-L1, and the inhibition of PD-L1 could be reversed by JAK1/STAT1 pathway activator IFN-γ (Fig. 5). Overall, these results suggest that DAAM1 positively regulates the expression of PD-L1 in pancreatic cancer.

DAAM1 was associated with increased T cell infiltration but promoted its exhaustion

Given that PD-L1 expression was induced by $CD8^+$T cells via IFN-γ in tumor microenvironment [27] and DAAM1 was positively correlated with PD-L1 in the above studies, we next examined whether DAAM1 was associated with $CD8^+$T cells. Based on the TCGA dataset, including $CD8^+$T cell, dendritic cell, macrophage, NK cell, and Th1 cell and the results showed that most immune cells were positively correlated with DAAM1 expression (Fig. 4E). Moreover, the TIMER algorithm was used to estimate various immune cells abundance and we found that DAAM1 showed the highest correlation with $CD8^+$T cell abundance (Fig. 4F). In addition, the in vitro co-culture model was used to evaluate the impact of DAAM1 knockdown in tumor cells on T cell exhaustion. As shown in Fig. 4G, DAAM1 knockdown in pancreatic cancer cells notably decreased the exhaustion levels of $CD8^+$T cells, and IFN-γ pre-treated pancreatic cancer cells with DAAM1 knockdown enhanced the exhaustion levels of $CD8^+$T cells (Fig. 4G). Due to the important role of PD-L1 and $CD8^+$T cell in tumors, we used the in-house cohort to validate the association between DAAM1 and PD-L1 expression as well as $CD8^+$T cells, and the results confirmed the positive correlation (Fig. 4H). Collectively, DAAM1 is a negative regulator for T cell activation and DAAM1 inhibition restores the anti-tumor activity of T cells.

DAAM1 promoted pancreatic cancer progression in vivo

To assess the effect of DAAM1 on pancreatic cancer progression in vivo, we established a subcutaneous tumor bearing model of C57BL/6 mice using PANC02 cells infected with the control and DAAM1 shRNA lentivirus (Fig. 5A). DAAM1 knockdown did not change mouse weight but significantly inhibited tumor growth in vivo (Fig. 5B–E). In addition, immunofluorescence also confirmed that tumor tissues with DAAM1 knockdown exhibited low expression of PD-L1, N-cadherin, and Ki-67, high expression of Caspase9, and high infiltration of CD8+ T cells (Fig. 5F). The above data confirm that DAAM1 accelerated tumor growth and immune escape of pancreatic cancer.

Discussion

DAAM1, a member of the Formin family, has been extensively studied for its role in regulating the actin cytoskeleton [4, 28, 29]. Studies have shown that DAAM1 expression is upregulated in various cancers, and DAAM1, a member of the Formin family, has been extensively studied for its role in regulating the actin cytoskeleton [7–9, 19, 30, 31]. While the oncogenic roles of DAAM1 are primarily attributed to its regulation of the actin cytoskeleton, it may also involve other regulatory mechanisms. In lung cancer, Tyr652 of DAAM1 is phosphorylated through Src-mediated signaling [32]. Our current study uncovered a novel role for DAAM1 in pancreatic cancer. Our comprehensive analyses indicated that high DAAM1 expression is associated with poor prognosis in pancreatic cancer patients, and that DAAM1 acts as a critical oncogene by regulating the JAK1/STAT1 pathway.

In exploring the molecular mechanisms underlying the effects of DAAM1 on pancreatic cancer, enrichment analysis identified the JAK1/STAT1 pathway as one of the key pathways influenced by DAAM1. The JAK1/STAT1 pathway is a classical inflammatory signaling and highly conserved in mammals, which can efficiently transmit extracellular signals from transmembrane receptors to the nucleus. It is involved in cell differentiation, metabolism, survival, homeostasis, and immune response [33, 34]. After cytokine bind to the receptor, JAK1 is activated and phosphorylated, creating docking sites that recruit cytoplasmic STAT proteins. Activated phosphorylated

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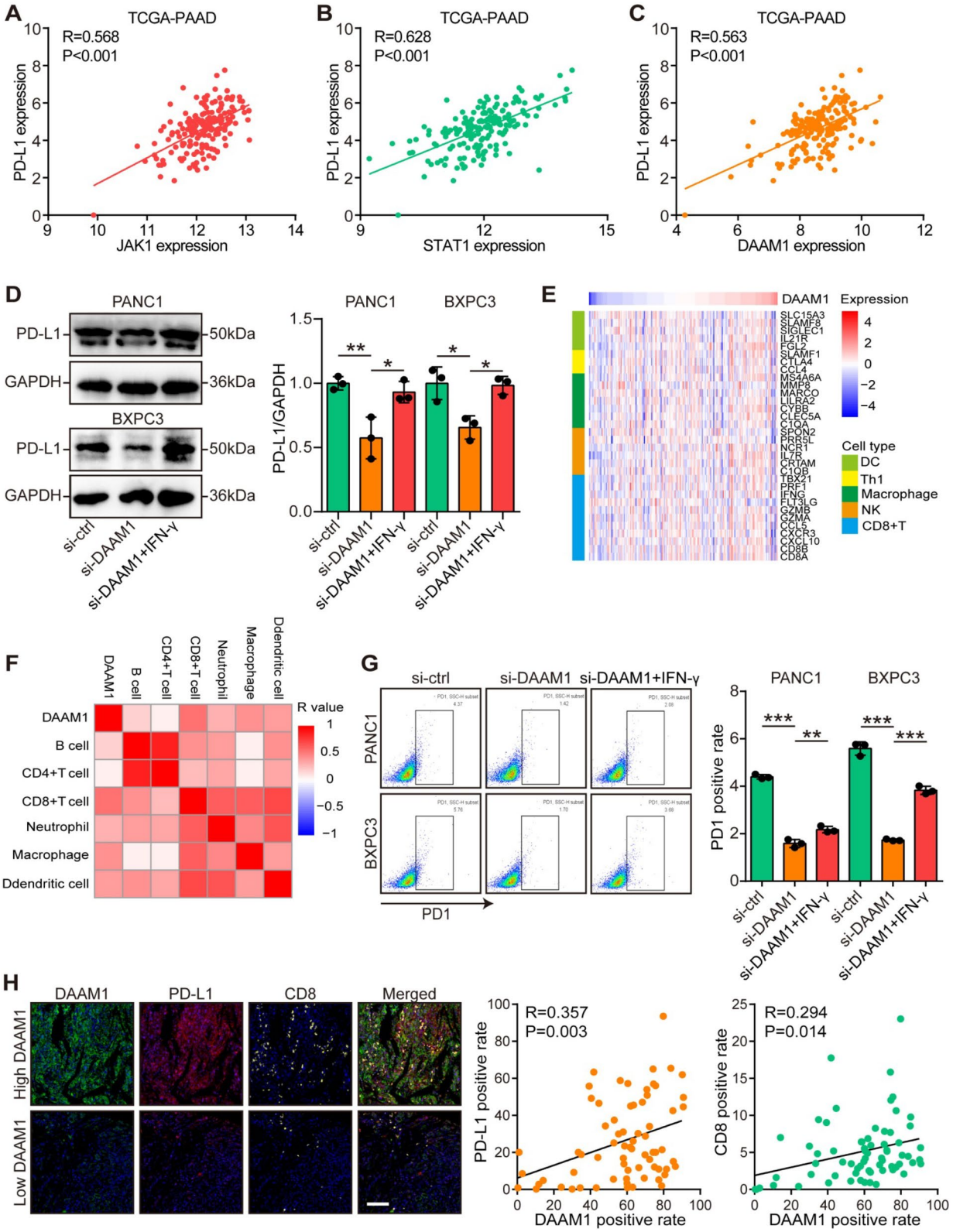
Fig. 4 DAAM1 regulated PD-L1 expression T cells exhaustion in pancreatic cancer. **A**–**C** Correlations between JAK1, STAT1, DAAM1, and PD-L1 in pancreatic cancer. The data was obtained from the TCGA database. Difference was checked by Pearson test. **D** Western blotting analysis of expression of PD-L1 in control, DAAM1-silencing, and IFN-γ-treated pancreatic cancer cells. Difference was checked by Student's t test. *P < 0.05, **P < 0.01. **E** Heatmap displaying the correlation between DAAM1 and various gene markers for intratumoral immune cells. The data was obtained from the TCGA database. **F** Correlation analysis of DAAM1 and the abundance of various intratumoral immune cells estimated by the TIMER algorithm. The data was obtained from the TCGA database. Difference was checked by Pearson test. **G** The exhaustion of T cells co-cultured with control, DAAM1-silencing, and IFN-γ-treated pancreatic cancer cells were examined by flow cytometry assay. Difference was checked by Student's t test. **P < 0.01, ***P < 0.001. **H** Representative images uncovering the expression of CD8 and PD-L1 in tumor tissues with low and high DAAM1 expression in the HPanA120CS01 pancreatic cancer cohort and correlation analysis. Bar = 100 μm. Difference was checked by Pearson test



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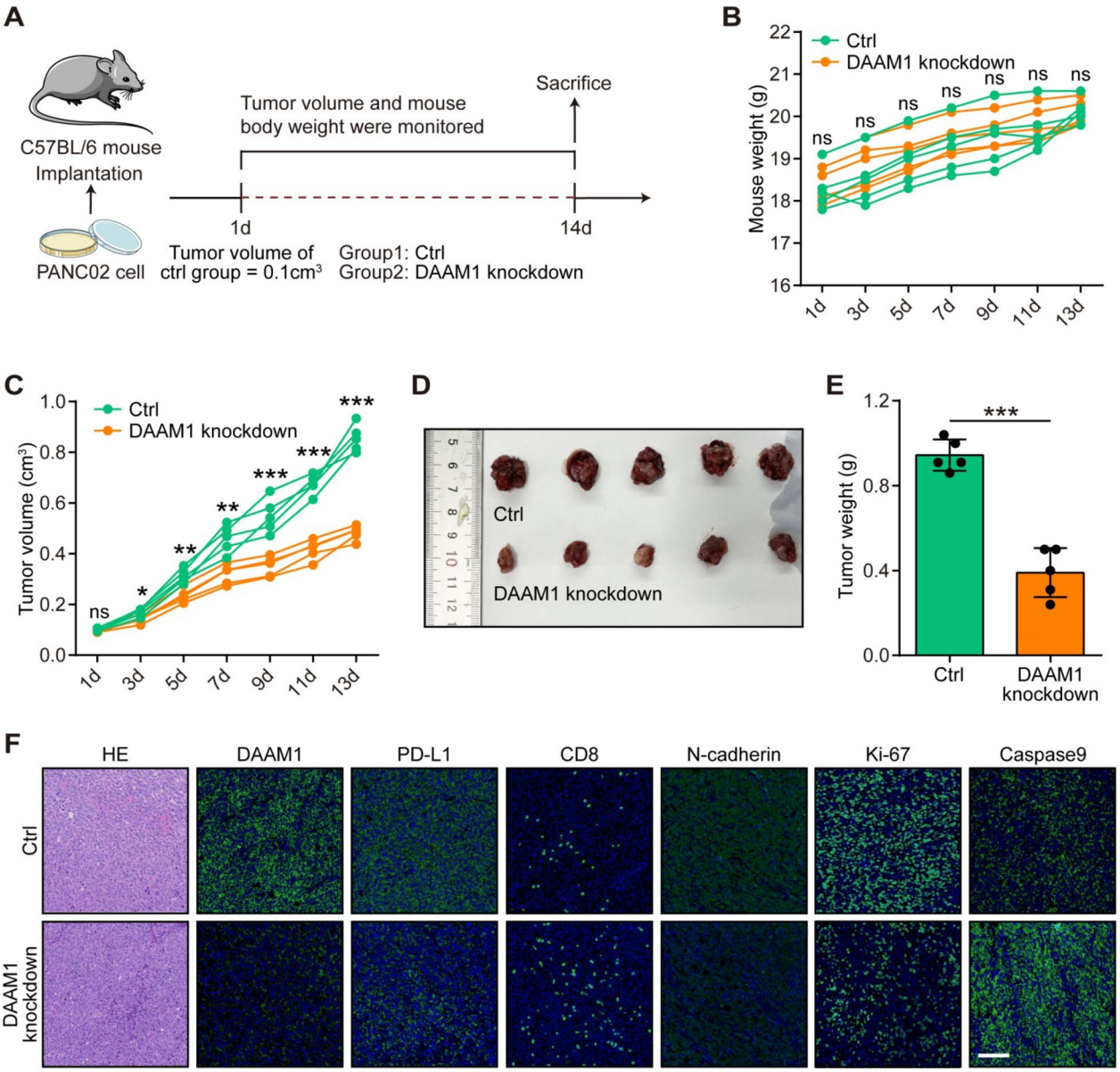

Fig. 5 Inhibition of DAAM1 inhibited pancreatic cancer progression in vivo. **A** Schematic protocol of the effects of DAAM1 knockdown on immunocompetent C57BL/6 mice. **B**, **C** Mouse weight and tumor growth curves of mice bearing PANC02 cells infected with the control and DAAM1 shRNA lentivirus. Difference was checked by Student's t test. ns, non-significance, *P < 0.05, **P < 0.01, ***P < 0.001. **D**, **E** Representative images showing the tumors harvested from mice bearing PANC02 cells infected with the control and DAAM1 shRNA lentivirus, and weight of the harvested tumors. Difference was checked by Student's t test. ***P < 0.001. **F** Representative images showing the levels of DAAM1, PD-L1, CD8, N-cadherin, Ki67, and Caspase9 in tumor tissues from mice in different groups

STAT can pass through intermolecular interactions. These interactions form either homologous or heterodimers, which are then translocated to the cell nucleus, where they bind DNA regulatory elements and induce gene transcription [35, 36].

PD-L1, encoded by the *CD274* gene, is a membrane protein involved in immune regulation that plays an critical role in regulating T cell activity and immune tolerance. When PD-L1 binds to PD-1, it inhibits T cell activation and proliferation, thereby reducing the recognition and attack of antigens [37, 38]. A key factor influencing the efficacy of immunotherapy is the regulation of PD-L1 expression [39]. It has been demonstrated by earlier research that both transcriptional and post-translational modifications can control the expression of PD-L1 [26]. Transcription factors such as

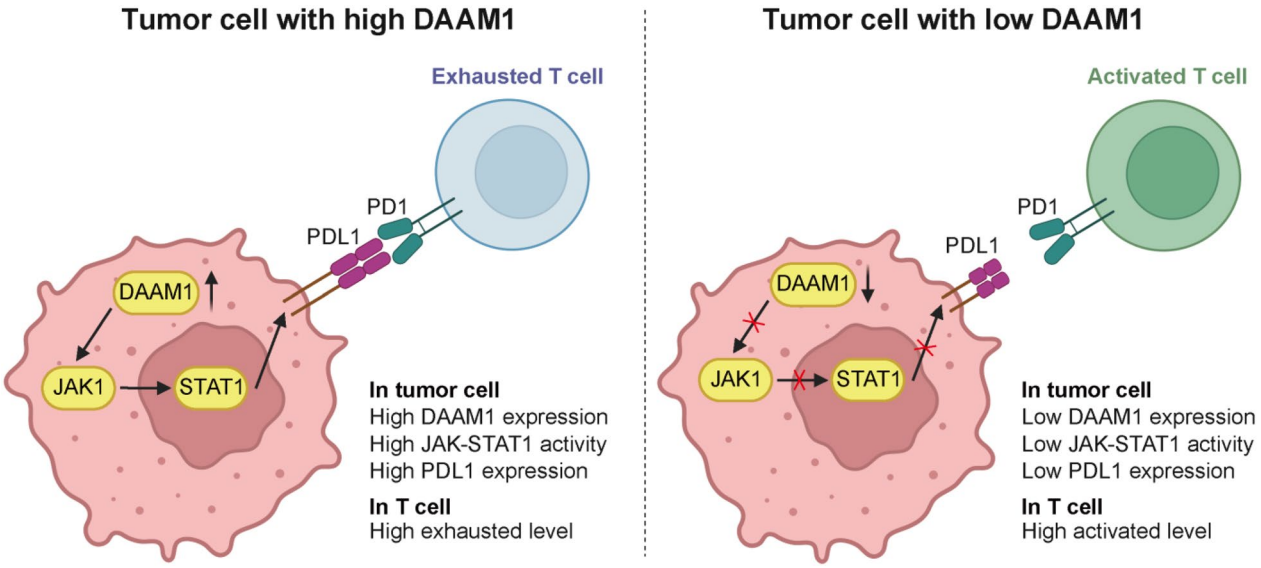

Fig. 6 Schematic overview in this study. In pancreatic cancer cells, DAAM1 positively regulated the JAK1-STAT1 pathway, and then upregulated PD-L1, which induced T cell exhaustion. Inhibition of DAAM1 expression suppressed the JAK1/STAT1/PD-L1 axis and recovered anti-tumor activities of T cells

Myc, hypoxia-inducible factors HIF1α/2α, IRF1, and NF-κB directly bind to the PD-L1 promoter, upregulating its transcriptional expression [40–43]. Additionally, the JAK1/STAT1 axis has been shown to be directly upstream of PD-L1 [25, 26]. The results of our study demonstrates that DAAM1 could regulate PD-L1 expression dependent on modulating the JAK1/STAT1 signaling pathway in pancreatic cancer.

It has been reported that Formin proteins, including DIAPH1, DAAM2, and FMNL1, are associated with increased with intratumoral immune cell infiltration [9, 16, 44]. In this research, we also investigated the relationship between DAAM1 and the abundance of intratumoral immune cells. Using the the TIMER algorithm, we showed that DAAM1 is strongly correlated with abundance of CD8+ T cells. Given that DAAM1 was positively correlated with PD-L1 in pancreatic cancer as well, we supposed that high level of DAAM1 promoted the exhaustion of CD8+ T cells. Given that DAAM1 is also positively correlated with PD-L1 in pancreatic cancer, we hypothesized that high DAAM1 levels promote CD8+ T cell exhaustion. Overall, our study reveals a novel role of DAAM1 in negatively regulating T cell activity by positively regulating PD-L1 expression in pancreatic cancer cells.

Limitations of the study

We acknowledge certain limitations of our study. The first relates to the detailed molecular mechanisms of DAAM1-mediated JAK1/STAT1 pathway activation. Given that DAAM1 was a scaffold protein [4], we guessed that DAAM1 might phosphorylation-related regulators. In addition, we also did not know whether JAK1/STAT1-indenpdent pathway involved in DAAM1-mediated PD-L1 expression, which warrants further investigation.

Conclusion

In conclusion, we report that DAAM1 functions as a critical oncogene in pancreatic cancer and regulates STAT1-induced PD-L1 transcription (Fig. 6). This study is the first to link the formin protein DAAM1 with PD-L1, providing new insights into DAAM1-mediated tumorigenesis and immune evasion.

Abbreviations

DAAM1	Dishevelled-associated activator of morphogenesis1
ICB	Immune checkpoint blockade
TMA	Tissue microarray
IHC	Immunohistochemistry
CPS	Combined positive score
qRT-PCR	Quantitative real-time PCR
PBMC	Peripheral blood mononuclear cells
UCSC	University of California Santa Cruz
TCGA	The Cancer Genome Atlas

Supplementary Information

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Supplementary Material 1.

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Not applicable.

Author contributions
Jing Sun, Yichao Zhu, and Jiayue Yang designed the study and participated in coordination and project control. Rui Xu, Jiadong Pan, Jie Mei, and Yan Shen collected the public data and conducted the bioinformatics analysis. Jie Mei collected the clinical samples and evaluated the relative clinical data. Mengyu Wan, Jie Mei, and Ji Zhou performed in vitro assays. Rui Xu, Mengyun Wan, and Jiadong Pan wrote the manuscript. Jing Sun, Yichao Zhu, and Jiayue Yang revised the manuscript. Jing Sun, Yichao Zhu, and Jiayue Yang got financial support. All authors reviewed and approved the final edition.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Ethical approval for the study of TMAs was granted by the Outdo Biotech Clinical Research Ethics Committee, and ethical approval for the collection of peripheral blood mononuclear cells (PBMC) was granted by the Clinical Research Ethics Committee of The Affiliated Wuxi People's Hospital of Nanjing Medical University. All animal experiments were approved by the Research Ethics Committee in The Affiliated Wuxi People's Hospital of Nanjing Medical University.

Consent for publication
All the listed authors have participated in the study, and have approved the manuscript.

Competing interests
The authors declare no competing interests.

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