Calcium Channel \(\alpha_{2\delta1}\) is Essential for Pancreatic Tumor-Initiating Cells through Sequential Phosphorylation of PKM2

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SUMMARY
Voltage-gated calcium channel \(\alpha_{2\delta1}\) was identified as a functional marker and therapeutic target of pancreatic tumor-initiating cells. It mediated calcium influx to activate CaMKII\(d\), which phosphorylated PKM2 at Thr454 that led to subsequent PKM2-Tyr105 phosphorylation to induce stem-like properties.

BACKGROUND & AIMS: Tumor-initiating cells (TICs) drive pancreatic cancer tumorigenesis, therapeutic resistance, and metastasis. However, TICs are highly plastic and heterogenous, which impede the robust identification and targeted therapy of such a population. The aim of this study is to identify the surface marker and therapeutic target for pancreatic TICs.

METHODS: We isolated voltage-gated calcium channel \(\alpha_{2\delta1}\) subunit (isoform 5)-positive subpopulation from pancreatic cancer cell lines and freshly resected primary tissues by fluorescence-activated cell sorting and evaluated their TIC properties by spheroid formation and tumorigenic assays. Coimmunoprecipitation was used to identify the direct substrate of CaMKII\(d\).

RESULTS: We demonstrate that the voltage-gated calcium channel \(\alpha_{2\delta1}\) subunit (isoform 5) marks a subpopulation of pancreatic TICs with the highest TIC frequency among the known pancreatic TIC markers tested. Furthermore, \(\alpha_{2\delta1}\) is functionally sufficient and indispensable to promote TIC properties by mediating Ca\(2^+\) influx, which activates CaMKII\(d\) to directly phosphorylate PKM2 at T454 that results in subsequent phosphorylation at Y105 to translocate into nucleus, enhancing the stem-like properties. Interestingly, blocking \(\alpha_{2\delta1}\) with its specific antibody has remarkably therapeutic effects on pancreatic cancer xenografts by reducing TICs.

CONCLUSIONS: \(\alpha_{2\delta1}\) promotes pancreatic TIC properties through sequential phosphorylation of PKM2 mediated by CaMKII\(d\), and targeting \(\alpha_{2\delta1}\) provides a therapeutic strategy against TICs for pancreatic cancer. (Cell Mol Gastroenterol Hepatol 2023;15:373–392; https://doi.org/10.1016/j.jcmgh.2022.10.006)

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the disease. Although early detection is undoubtedly critical for the survival of PDAC patients, a better understanding of the biology of PDAC associated with its progression is also imperative for the development of more efficient therapeutic strategies.3

As a subpopulation of cancer cells with stem cell–like properties such as self-renewal, differentiation, and tumorigenic capacity, tumor-initiating cells (TICs), or so-called cancer stem cells, have the ability to recapitulate the whole heterogeneity of a tumor and therefore are postulated as a sustaining force to drive and maintain fully malignancy of a tumor.5,6 Targeting these cells thus is expected to bring a “cure” for the patients. Although several surface markers such as CD133, CD44, EpCAM, DCLK1, and CD96–8 have been used to define TICs for PDAC in literature, the reliable identification of such a population in PDAC is hampered by the need for specific markers that can be used for isolation and clinical targeting.

The auxiliary subunit of voltage-gated calcium channels (VGCC) α2δ1 is a product of single gene that is post-transationally cleaved into α2 and δ1 subunits, which subsequently form a dimer via disulfide bonds. It can promote membrane expression of VGCC and modulate its current properties by interacting with the core forming α1 subunit.7 A change in voltage moves the switch that in turn pulls the “gate” of the channel open, which subsequently leads to influx of calcium ions (Ca2+) into cells.8 By serving as a major second messenger, elevated Ca2+ in the cells relays signals in the form of spikes or oscillations, which are decoded by several downstream sensor and adaptor proteins including calmodulin (CaM), to control a variety of cellular processes including gene transcription, cell metabolism, proliferation, motility, cell death, and survival.9,10 Ca2+/CaM complex subsequently binds and activates a number of downstream enzymes such as the Ca2+/CaM-dependent protein kinase II (CaMKII), a multifunctional serine/threonine kinase family of 4 closely related isoforms (CaMKIIα, β, γ, and δ) that show differential but overlapping expression patterns among tissues and during development.12,13 As one of the most widely studied Ca2+-regulated kinases, CaMKII comprises 12 identical subunits that form a dodecameric holoenzyme. Activation of individual subunits of the holoenzyme by Ca2+/CaM leads to the phosphorylation of adjacent enzyme subunits at Thr286/287 (the α isoform at Thr286, the β, γ, and δ isoforms at Thr287). Once autophosphorylated, CaMKII acquires enhanced enzymatic activity that remains even after Ca2+/CaM dissociation.14,15

Breakdown of Ca2+ homeostasis and/or aberrant expression of calcium signaling pathway molecules have been linked to many pathologic processes including cancer.10,12,16 Extensive remodeling in the expression of proteins and/or the activity directly involved in calcium signaling have been observed as consequences of oncogenic pathways. On the other hand, many oncogenic machineries are sensitive to the regulation by specific calcium signal(s) that can initiate the formation of some cancers, promote tumor cell growth, angiogenesis, migration, invasion, and metastasis, prevent tumor cell death, as well as regulate the response to therapeutic agents and the acquisition of therapeutic resistance.16–19 Recently, a number of studies indicated that changes in intracellular calcium ([Ca2+]i) also play essential roles in the self-renewal capacity and differentiation of stem cells and cancer stem cells, as results of the expression of a wide variety of calcium channels such as VGCC and/or store release channels (IP3R and RYR).18–20 We have previously identified that the isoform 5 of the VGCC α2δ1 subunit, which is specifically recognized by the monoclonal antibody (mAb) B150-1, is sufficient and indispensable to promote TIC properties by mediating Ca2+ influx into cells, hence serving as a functional marker and therapeutic target of TICs of liver,21,22 lung,23 and gastric24 origins. However, the calcium signaling pathways mediated by α2δ1 in the determination of TIC properties remain elusive.

PKM2 is the M2 isoform of pyruvate kinase (PK) that regulates the final rate-limiting step of glycolysis by transferring the phosphate from phosphoenolpyruvate to adenine diphosphate to generate pyruvate and adenosine triphosphate.25 In addition to its role in glycolysis, PKM2 can function as a protein kinase or a transcriptional co-activator to activate the transcription of those genes that are essential for tumorigenesis.18,20–24 Moreover, the roles of PKM2 in oncogenesis are dependent on its posttranslational modification. For example, the phosphorylation of PKM2 at Tyr105 (Y105) by multiple tyrosine kinases results in the formation of PKM2 dimers that induce cancer stem cell–like properties in human breast cancer by enhancing Yes-associated protein (YAP) nuclear translocation.26

Here, we show that α2δ1 is a functional marker and therapeutic target for pancreatic cancer TICs. The expression of α2δ1 is sufficient to induce stem-like properties via Ca2+-mediated activation of CaMKIīδ, which directly phosphorylates PKM2 at T454 that subsequently resulted in the phosphorylation of PKM2 at Y105 in a sequential mode. Importantly, blocking calcium influx with mAb B150-1 against α2δ1 can selectively reduce TICs, providing a promising approach of targeted therapy for pancreatic cancer.

Results
α2δ1 Defines a TIC Subpopulation of PDAC
To test whether α2δ1 marks a subpopulation of TICs in PDAC, we first detected the expression of α2δ1 in the PDAC

Abbreviations used in this paper: CaM, calmodulin; FACS, fluorescence-activated cell sorting; GEM, gemcitabine; i.p., intraperitoneally; IRS, immunoreactive score; mAb, monoclonal antibody; NOD/SCID, nonobese diabetic/severe combined immunodeficient; OE, over-expression; PDAC, pancreatic ductal adenocarcinoma; PDX, patient-derived xenograft; PK, pyruvate kinase; s.c., subcutaneously; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; TIC, tumor-initiating cell; VGCC, voltage-gated calcium channels; YAP, Yes-associated protein.

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cell lines PANC-1 and BxPC-3, as well as the normal human pancreatic duct cell line HPDE6-C7 by immunofluorescent cytochemistry with mAb1B50-1. As shown in Figure 1A, α2δ1 localized in the cell membrane of a minor population in the PDAC cell lines, whereas it is undetectable in the immortalized normal pancreatic duct cell line HPDE6-C7. The percentage of α2δ1-positive (α2δ1+) cells varied from 1.33% to 4.66% across a panel of PDAC cell lines including AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1 as detected by flow cytometry (Figure 1B). Furthermore, the α2δ1− cells stained by 1B50-1 were confirmed to be positive for a commercial α2δ1 antibody by both immunofluorescent cytochemistry and flow cytometry (Figure 1C and D). We then performed fluorescence-activated cell sorting (FACS) to purify α2δ1+ and α2δ1− cells from these PDAC cell lines to assay their cancer stem cell capacities both in vitro and in vivo. Sorted α2δ1+ cells from these PDAC cell lines could form spheres at much higher rates than their respective negative subsets in serum-free medium (Figure 1E and F). Single cells dissociated from the spheres formed by these α2δ1+ cells could be passaged and clonally expanded with enhanced sphere formation efficiencies, suggesting that the α2δ1+ cells possess the in vitro self-renewal capacity (Figure 1E and F). The FACS-purified α2δ1+ and α2δ1− cells were then serially transplanted in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice subcutaneously (s.c.) at serial dilutions of 1000 and 100 cells to assay their tumorigenicity. The α2δ1+ subpopulations from these cell lines generated tumors in almost all the transplanted mice, whereas the respective α2δ1− cells were either completely non-tumorigenic or formed tiny nodules only occasionally. Furthermore, α2δ1+ subpopulations re-sorted from the tumors formed by the α2δ1+ cells were able to generate tumors in the secondary recipient mice (Figure 1G, Table 1). The histopathologic features of the tumors formed by the α2δ1+ cells resembled those of the tumors formed by which the α2δ1+ cells originated as demonstrated by H&E staining (Figure 1H), suggesting that α2δ1+ cells were able to initiate the formation of the heterogeneous tumors they derived.

In addition, the α2δ1+ cells expressed much higher levels of a panel of stem-associated molecules, eg, ABCG2, BM11, NANOG, SOX2, than their negative ones as demonstrated by Western blotting (Figure 1F). It was hypothesized that TICs could undergo asymmetric cell division that resulted in the production of a stem cell and a progenitor cell, the latter of which could differentiate into non-TICs.19,20 Hence, we detected whether sorted α2δ1+ cells were able to generate α2δ1− cells both in vitro and in vivo. The percentage of α2δ1+ fractions purified from both the PAN-1 and BxPC-3 cell lines decreased from about 90% to 2%–5%, which were similar to those in parent cell lines, after the purified α2δ1+ cells were cultivated in medium containing 10% fetal bovine serum for 2 weeks or transplanted into NOD/SCID mice (Figure 1I), suggesting the differentiation ability of α2δ1+ into negative ones. On the contrary, the α2δ1− cells purified from both cell lines failed to differentiate into α2δ1+ cells after being cultured in serum-containing medium for 2 weeks (Figure 1K).

All of these data confirmed that the VGCC α2δ1 marked a TIC subpopulation with stem-like properties of pancreatic cancer.

**TICs Are Enriched the Most in α2δ1+ Cells among CD9+, CD44+, EpCAM+, and DCLK1+ Ones**

To further characterize the α2δ1+ PDAC TICs, we determined the correlation between α2δ1 and CD9+, CD44+, EpCAM+, or DCLK1, the surface markers that have been used to isolate PDAC TICs, in the PDAC cell lines PAN-1 and BxPC-3 by dual-color flow cytometry. More than 90% of α2δ1+ cells were also positive for CD9+, CD44+, EpCAM+, and DCLK1, whereas less than one third (3.5%–31.1%) of CD9+, CD44+, EpCAM+, or DCLK1+ subpopulations were positive for α2δ1 in both PAN-1 and BxPC-3 cell lines (Figure 2A and B), indicating that α2δ1+ TICs are a shared subpopulation of the pancreatic cancer TICs defined by these known markers.

We then sought to address which marker was the best to identify TICs of PDAC by carrying out a side-by-side comparison of the tumorigenic potential among α2δ1+, CD9+, CD44+, EpCAM+, and DCLK1+ PAN-1 cells in NOD-SCID mice with limited dilution. Compared with those of CD44+, EpCAM+, and DCLK1+ fractions, the TIC frequencies of α2δ1+ cells were 11.09, 11.09, and 37.9 times those of CD44+, EpCAM+, and DCLK1+ cells, respectively (Figure 2C, Table 2). In another experiment set, the TIC frequency of α2δ1+ cells was about 1000 times that of CD9+ fraction (Figure 2D, Table 2). These data indicate that α2δ1 is the most robust surface marker for defining PDAC TICs among these molecules tested.

**Clinical Significance of 2δ1+ Cells in Pancreatic Cancer and Surgical Margin Tissues**

To address the clinical relevance of α2δ1 expression in pancreatic cancer, we performed immunofluorescent staining in 74 paired frozen pancreatic cancer and paracancerous tissues that were obtained from the pancreatectomy margins using mAb1B50-1. The cells positive for α2δ1 staining, which were also positive for CK19 staining, showed a scattered distribution in 59.5% (44/74) of paracancerous tissues (Figure 3A) and 89.2% (66/74) of all the pancreatic cancer tissues tested, whereas they were detected only as isolated ones in 35.5% (26/74) of all the paracancerous tissues tested. The α2δ1+ fraction of 9/41 (22%) of the tumors in the pancreatectomy margins was positive for α2δ1 in both the same tissues in all the transplanted mice (Figure 3B). H&E staining demonstrated that the histologic features of tumors formed by the α2δ1+ fractions also resembled those from which they derived, retaining the phenotypic heterogeneity (Figure 3C).

Kaplan-Meier curves showed that the α2δ1 staining status in the tumor tissues did not correlate with overall survival of these patients, but the median overall survival in the patients...
with $\alpha\delta 1$ negative staining in the paracancerous tissues was 7.5 months longer than those with positive staining (Figure 3D and E). Multi-variant Cox regression analysis showed the presence of $\alpha\delta 1^+$ cells in paracancerous tissues was an independent risk factor of poor prognosis for PDAC patients (Figure 3F).

### Roles of $\alpha\delta 1$ in the Acquisition and Subsequent Maintenance of TIC Properties

To test whether $\alpha\delta 1$ plays any roles in the determination of the properties of PDAC TICs, we performed both gain-of-function and loss-of-function studies. Ectopic expression of $\alpha\delta 1$ in the pancreatic cancer cell lines PANC-1 and BxPC-3 led to significant up-regulation of a panel of stem-related genes and remarkably increased the ability of initiating the formation of spheroids that could be expanded in subsequent propagation (Figure 4A-C). On the contrary, knockdown of $\alpha\delta 1$ with short hairpin RNAs (shRNAs) in $\alpha\delta 1^+$ cells resulted in remarkable down-regulation of the stem-related genes detected (Figure 4D), the suppression of spheroid formation abilities (Figure 4E and F), as well as the retardation of tumorigenicity (Figure 4G and H). The role of $\alpha\delta 1$ in the acquisition and subsequent maintenance of in vitro self-renewal ability of PDAC TICs was further validated in the AsPC-1 and MIA PaCa-2 cell lines by spheroid formation assay (Figure 4B, C, E, and F).

All these data confirmed that $\alpha\delta 1$ is sufficient to enable pancreatic cancer cells to acquire the stem-like properties and is necessary for the subsequent maintenance of pancreatic cancer TIC properties.

### Role of $\alpha\delta 1$ in Promoting TIC Properties Is CaMKII$\delta$-Dependent

Many of the previous studies have linked $\alpha\delta 1$ to its role in mediating calcium influx into cells. Here, we confirmed that forced expression of $\alpha\delta 1$ led to significantly elevated levels of [$Ca^{2+}$], in the pancreatic PANC-1 and BxPC-3 cell lines, and that the [$Ca^{2+}$] levels were much higher in the $\alpha\delta 1^+$ fractions than their negative ones (Figure 5A and B). Further treatment of $\alpha\delta 1^+$ PANC-1 and BxPC-3 cells with 10 $\mu$mol/L EGTA-AM could completely inhibit the sphere formation ability of these cells (Figure 5C and D), indicating that the in vitro self-renewal ability of $\alpha\delta 1^+$ cells was dependent on intracellular calcium. To delineate the signaling pathway(s) mediated by calcium in the determination of pancreatic TICs, we focused on the members of Ca$^{2+}$/CaMKII$\delta$.
dependent protein kinase II family, one of the critical calcium decoders. Of the 4 members of CaMKII, CaMKII\(\text{d}\) was found to be the most dominantly and consistently up-regulated isoform after forced expression of \(\alpha_2d_1\) in both the PANC-1 and BxPC-3 cell lines, as demonstrated by Western blotting (Figure 5\(E\)). Moreover, the level of phosphorylated CaMKII at Thr286/287 was also up-regulated following \(\alpha_2d_1\) over-expression, suggesting the activation of CaMKII (Figure 5\(E\)). In addition, the expression of CaMKII\(\text{d}\) was much higher in the \(\alpha_2d_1^+\) fractions than their negative ones (Figure 5\(F\)). Knockdown of the expression of CaMKII\(\text{d}\) with its specific shRNAs in the \(\alpha_2d_1^+\) overexpressing (OE) cells resulted in down-regulation of stem-related genes detected, significant retardation of spheroid formation efficiencies and tumorigenicity (Figure 5\(G–J\), Table 3). Further knockdown of the expression of CaMKII\(\text{d}\) in the purified \(\alpha_2d_1^+\) TICs led to similar effects with the down-regulation of the same panel of stem-related genes and suppression of the abilities of sphere formation and tumorigenicity (Figure 5\(K–N\), Table 3). These results indicate that CaMKII\(\text{d}\) is required for \(\alpha_2d_1^+\)-mediated acquisition and subsequent maintenance of the properties of pancreatic TICs.

CalMKII\(\text{d}\) Directly Phosphorylates PKM2 at T454

We then performed immunoprecipitation with anti-FLAG M2 beads in the cell lysates of 293FT cells transiently transfected with CaMKII\(\text{d}\)-Flag construct to identify potential substrate(s) of CaMKII\(\text{d}\) that were involved in the TIC properties promoted by \(\alpha_2d_1\). Compared with the anti-FLAG M2 beads incubated with the cell lysates of 293FT cells transfected with empty control vector, the anti-FLAG M2 beads incubated with the CaMKII\(\text{d}\)-Flag cell lysates precipitated CaMKII\(\text{d}\) itself as expected, and several other top candidates including PKM2, whose phosphorylation at Y105 has been demonstrated as inducing cancer stem cell–like phenotypes in human breast cancer,\(^{28}\) as revealed by mass spectrometry analyses of the bands indicated in Figure 6\(A\), and thus was selected for further characterization. The physiological binding between CaMKII\(\text{d}\) and PKM2 was verified by carrying out co-immunoprecipitation of extracts from PANC-1 cells overexpressing \(\alpha_2d_1\) with antibodies against CaMKII\(\text{d}\) and PKM2. As expected, PKM2 was co-immunoprecipitated by antibody against CaMKII\(\text{d}\) and vice versa (Figure 6\(B\)), demonstrating that the 2 proteins bind each other specifically.
We next performed mass spectrometry analyses for the phosphorylation site(s) of PKM2 immunoprecipitated from α2δ1-OE PANC-1 cells. The phosphorylation of PKM2 at T454 was identified in the immunoprecipitated products from the α2δ1-OE cells (Figure 6C and D). Further Western blot using a site-specific antibody against phosphorylated PKM2 at T454 (Phospho-PKM2-T454, p-PKM2-T454) confirmed that the phosphorylation levels of PKM-T454 were much higher in the α2δ1-OE and α2δ1+ PANC-1 and BxPC-3 cells than the respective vector alone control cells and sorted α2δ1+ subpopulations, respectively, whereas the total PKM2 remained the same (Figure 6E). Ectopic expression of CaMKIIδ in PANC-1 cells also dramatically led to this phosphorylation, whereas the construct CaMKIIδ with T287A mutation that led to the disruption of the kinase activity failed to induce such phosphorylation (Figure 6F). Treatment of α2δ1-OE PANC-1 cells with KN-93, a specific inhibitor of CaMKII, could remarkably decrease the levels of phosphorylated PKM2-T454 in a dose-dependent manner (Figure 6G). Furthermore, knockdown of CaMKIIδ with specific shRNAs could also decrease the levels of phosphorylated PKM2-T454 (Figure 6H). These data suggest that PKM2 is a substrate for CaMKIIδ.

To further verify that CaMKIIδ could directly phosphorylate PKM2, we carried out in vitro phosphorylation assay. Incubation of GST-PKM2 expressed in Escherichia coli with CaMKIIδ could significantly lead to the phosphorylation of PKM2-T454, whereas the kinase-dead CaMKIIδ-T287A only resulted in trace level of phosphorylated PKM2-T454 (Figure 6I).

Taken together, these data demonstrated that PKM2 is a bona fide substrate for CaMKIIδ, which phosphorylated it directly at T454.

**Phosphorylated PKM2-T454 Is Essential for the Phosphorylation of PKM2-Y105 and Its Subsequent Role in Pancreatic TIC Properties**

The fact that phosphorylated PKM2-Y105 could induce stem-like properties in breast cancer cells28 prompted us to address whether phosphorylated PKM2-Y105 was also related to α2δ1+ pancreatic TICs, and whether there was any relationship between phosphorylated PKM2-T454 and phosphorylated PKM2-Y105. Notably, the levels of phosphorylated PKM2-Y105 were positively correlated with those of phosphorylated PKM2-T454, showing also much higher levels in α2δ1+ pancreatic TICs and α2δ1-OE PANC-1 and BxPC-3 cells (Figure 6E). Ectopic expression of CaMKIIδ in PANC-1 cells also dramatically led to the phosphorylation of PKM2-Y105, whereas the kinase-dead construct CaMKIIδ-T287A failed to induced such a phosphorylation (Figure 6F). Treatment of α2δ1-OE PANC-1 cells with CaMKII inhibitor KN-93, or knockdown of CaMKIIδ with specific shRNAs, could also decrease the levels of phosphorylated PKM2-Y105, which is a similar change to phosphorylated PKM2-T454 did (Figure 6G and H). We then tested whether the phosphorylation of PKM2 at T454 was required for the phosphorylation of PKM2-Y105 by reconstituting the expression of RNAi-resistant wild-type, a phosphodefective mutant PKMT-454A, and phosphomimetic mutant PKM2-T454D in α2δ1-OE PANC-1 cells with endogenous PKM2 knocked down (PKM2-KD) by specific shRNA. Interestingly, the phosphomimetic mutant PKM2-T454D was enough to induce the phosphorylation of PKM2 at Y105, whereas the mutant PKM2-T454A failed to induce such a phosphorylation (Figure 6F), suggesting that the phosphorylation of PKM2-T454 is critical for the phosphorylation of PKM2-Y105.

It was reported that the phosphorylation of PKM2 at Y105 could result in PKM2 homotetramer dissociation into dimers by releasing fructose 1,6-bisphosphate from tetramers, which decreased the PK activity of PKM2, and was linked to translocation of it into nucleus and increased tumorigenicity.28 Interestingly, we found that most of the phosphomimetic mutant PKM2-T454D proteins formed dimers and translocated into nucleus, which was co-stained by phospho-PKM2-Y105 antibody, whereas the majority of phosphodefective mutant PKM2-T454A failed to form dimers and was mainly localized in cytoplasm (Figure 7A and B).

Next, we detected the effects of the phosphorylation of PKM2 on the sphere formation and tumorigenicity abilities
of these phospho-defective and phospho-mimetic mutants. Expression of PKM2-T454D or Y105D remarkably led to increased spheroid formation efficiencies and enhanced tumorigenicity, compared with the cells expressing PKM2-WT, whereas expression of PKM2-T454A and PKM2-Y105F did not (Figure 7C–E, Table 4). However, when ectopic expression of PKM2-T454D with an additional Y105F mutation (PKM2-Y105F-T454D), the prompting effects of PKM2-T454D on the sphere-forming and tumorigenic abilities of Panc-1 cells diminished (Figure 7C–E, Table 4). These data suggested that the phosphorylation of PKM2 at T454 was sufficient to induce stem-like properties of pancreatic TICs, which was dependent on the phosphorylation of PKM2 at Y105 synergistically.

As additional evidence to support the role of α2δ1 in promoting PDAC TIC properties through CaMKII δ-mediated

**Figure 3.** Clinical significance of α2δ1 expression in PDAC and paracancerous tissues. (A) Representative images showing results of immunofluorescent staining for α2δ1 with mAb1B50-1 and cytokeratin 19 in cryostat sections of PDAC and paracancerous tissues. Nuclei were stained with DAPI. Scale bars: 50 μm. (B) Tumorigenicity of α2δ1+ and α2δ1− fractions purified directly from freshly resected PDAC tissues. Data are expressed as number of tumors formed/number of sites injected, and the numbers in parentheses represent the numbers of cells injected. (C) Representative images of H&E staining showing histology of tumors formed by α2δ1+ cells from PDAC tissues, as well as that of original tumor tissues from respective patients. Scale bars: 50 μm. (D and E) Kaplan-Meier curves showing overall survival for PDAC patients divided by α2δ1 staining status in the cancer (D) and paracancerous tissues (E). (F) Multivariate analysis predicts risk factors of poor survival for PDAC patients. Ca, cancer tissue; PCa, paracancerous tissue; RR, relative risk.
subsequent phosphorylation of PKM2 at T454 and Y105, the expression of α2δ1 was positively correlated with CaMKII, p-PKM2-Y105, and p-PKM2-T454, and there was a positive correlation between p-PKM2-T454 and p-PKM2-Y105 as demonstrated by immunohistochemical staining in 30 cases of PDAC specimen (Figure 7). Blocking α2δ1 Reduces TICs and Inhibits the Growth of PDAC in Vivo

The abovementioned results led us to test whether blocking the function of α2δ1 with mAb1B50-1 could have any therapeutic effects on PDAC by reducing TICs. NOD/SCID mice bearing established xenografts of the cell lines PANC-1 and BxPC-3 were administered intraperitoneally (i.p.) with mAb1B50-1 at 800 μg per mouse alone, gemcitabine (GEM) (60 mg/kg), or combination of both. The treatment with mAb1B50-1 alone could suppress significantly the growth of both the xenografts in NOD/SCID mice, whereas the combinational treatment of mAb1B50-1 with GEM led to superior inhibition of the growth of the tumors to any single regimen by ratios of as many as 78.8% and 75% on PANC-1 and BxPC-3 xenografts, respectively.
lasting for additional periods after termination of the treatments (Figure 8A–F). Notably, there were no significant side effects observed for these treatments because the body weights of the mice remained stable during the treatment period (Figure 8G). The therapeutic effects of these treatments were further validated in 2 patient-derived xenografts (PDX) (Figure 8H and I). Interestingly, the overall survival of the mice was also improved significantly after combinational therapy in these PDX models (Figure 8J and K).
To address whether the treatments with mAb1B50-1 could reduce TICs, we first analyzed the TIC proportions in the residues of treated PANC-1 engraftments by flow cytometry. The population of $\alpha$2$\delta^{+}$ TICs decreased upon mAb1B50-1 treatments, especially with the combinational regimen, whereas the proportion of such population enriched by a rate of about 5.5-fold after GEM alone treatment (Figure 8L). Moreover, re-transplantation of 10,000 cells from the residual tumor that received mAb1B50-1 treatment into secondary mice could only initiate the formation of very tiny nodules in 2 of 5 mice, whereas the cells from the control and GEM-treated tumors subsequently generated tumors in almost all the transplanted mice, with GEM-treated cells developing tumors faster and bigger. Notably, the residual tumor cells from the combinational therapy group only formed a negligible nodule in 1 of 5 mice injected (Figure 8M), suggesting that TICs were reduced after mAb1B50-1 treatments.

### Discussion

Here, we found that the $\alpha$2$\delta^{+}$ cells isolated from PDAC cell lines and fresh tissues could generate heterogenous tumors that histologically recapitulated the primary tumors they derived. These $\alpha$2$\delta^{+}$ cells were commonly shared by CD44$^{+}$, EpCAM$^{+}$, DCLK1$^{+}$, and CD9$^{+}$ PDAC cells, previously reported TIC subpopulations of PDAC. Moreover, $\alpha$2$\delta$ mediated calcium influx into cells, which subsequently activated CaMKII$\delta$ to enable the acquisition of stem cell–like properties by phosphorylating PKM2. Therefore, we identified $\alpha$2$\delta$ as a robust and functionally significant marker for PDAC TICs.

Aberrant expression and/or activation of a particular member of CaMKIIs have been linked to cancer cell proliferation, epithelial-to-mesenchymal transition, invasion and metastasis, and their roles in TICs or cancer stem cells are emerging. In the current study, we identified CaMKII$\delta$ was up-regulated by $\alpha$2$\delta$ and served as the major CaMKII that was responsible for $\alpha$2$\delta$-mediated acquisition

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**Figure 5.** (See previous page). CaMKII$\delta$ is required for stem cell–like properties promoted by $\alpha$2$\delta$. (A and B) [Ca$^{2+}$]i levels were measured using flow cytometry with Ca$^{2+}$ probe Fluo-4/AM in indicated cells overexpressing $\alpha$2$\delta^{+}$ (A) and FACS-sorted $\alpha$2$\delta^{+}$ and $\alpha$2$\delta^{-}$ fractions from indicated cell lines (B). (C and D) Representative phase contrast micrographs (C) and histograms (D) showing spheroid formation abilities of $\alpha$2$\delta^{+}$ cells from indicated sources after treatment with EGTA-AM at 10 $\mu$mol/L. Scale bars: 100 $\mu$m. (E and F) Western blotting results showing expression of indicated molecules in indicated cell lines overexpressing $\alpha$2$\delta^{+}$ (E) and sorted $\alpha$2$\delta^{+}$ and $\alpha$2$\delta^{-}$ fractions from indicated sources (F). (G) Western blot analysis of indicated molecules in $\alpha$2$\delta^{+}$-OE cells of indicated sources after knockdown of CaMKII$\delta$ by specific shRNAs. (H and I) Representative phase contrast images (H) and histograms (I) demonstrating spheroid formation abilities of $\alpha$2$\delta^{+}$-OE cells from indicated sources after knockdown of CaMKII$\delta$. Scale bars: 100 $\mu$m. (J) Tumorigenicity of indicated cell lines overexpressing $\alpha$2$\delta^{+}$ infected with lentiviruses harboring shRNA expression cassettes for scramble and CaMKII$\delta$. Bars = 1 cm. (K) Expression of indicated molecules was analyzed in sorted $\alpha$2$\delta^{+}$ fractions from indicated cell lines after knockdown of CaMKII$\delta$ by specific shRNAs. (L and M) Phase contrast micrographs (L) and histograms (M) showing change of spheroid formation efficiencies in FACS-purified $\alpha$2$\delta^{+}$ fractions upon knockdown of CaMKII$\delta$ by specific shRNAs. Scale bars: 100 $\mu$m. (N) Tumorigenicity of $\alpha$2$\delta^{+}$ fractions purified from indicated sources after knockdown of CaMKII$\delta$ by specific shRNAs. Bars = 1 cm. Data in A, B, D, I, and M represent mean ± standard deviation of 3 independent experiments. *Two-tailed Student t test.
of TIC properties of PDAC. Although it requires more work to address how CaMKII was up-regulated by α2δ1, it is possible that calcium influx mediated by α2δ1 will activate the basal level of one or more member(s) of the CaMKII family through Ca2+/CaM binding, resulting in activated calcium signaling cascade(s) to up-regulated CaMKIIδ. This hypothesis was supported by the fact that the phosphorylated CaMKII at Thr286/287 was enhanced after forced expression of α2δ1. Moreover, PKM2 was demonstrated as a novel substrate for CaMKIIδ involved in such a process. The phosphorylation of PKM2 at T454 mediated by CaMKIIδ led to subsequent phosphorylation of PKM2 at Y105. Because

Figure 6. CaMKIIδ directly phosphorylates PKM2 at T454. (A) SDS-PAGE analysis of immunoprecipitated products with Flag-resin in PANC-1 cells forced expression of CaMKIIδ-Flag construct. Precipitated products in the cells transfected with vector alone serve as a control. Bands that contained PKM2 and CaMKIIδ as identified by mass spectrum are shown. (B) Western blotting analysis of immunoprecipitated products with indicated antibodies in α2δ1-OE PANC-1 cells. (C) PKM2 was immunoprecipitated from cell lysates of PANC-1 cells infected with vector alone or α2δ1-OE lentivirus and was separated by SDS-PAGE for phosphorylation analysis by mass spectrum. (D) Analysis of phosphorylated sites of PKM2 by mass spectrometry in PANC-1 cells overexpressing vector alone or α2δ1. (E) Western blot analysis with indicated antibodies in sorted α2δ1+ and α2δ1− fractions, as well as the cells overexpressing α2δ1 from indicated sources. (F) PANC-1 cells were transiently transfected with vector, CaMKIIδ, and CaMKIIδ-T287A and were analyzed by Western blotting with indicated antibodies. (G) Western blotting analysis with indicated antibodies in cell lysates of α2δ1-OE PANC-1 cells treated with KN-93 at indicated concentration for 48 hours. (H) Western blotting analysis with indicated antibodies in cell lysates of α2δ1-OE PANC-1 cells after knockdown of CaMKIIδ with shRNAs. (I) Western blotting analysis of in vitro phosphorylation assay products with indicated antibodies. Purified GST-PKM2 on glutathione agarose beads were incubated with purified CaMKIIδ-Flag or CaMKIIδ-T287A-Flag in the presence of CaM and ATP. (J) Cell lysates of PKM2-KD PANC-1 cells overexpressing the indicated RNAi-resistant constructs were analyzed by Western blotting with indicated antibodies. IB, immunoblot; t-PKM2, total PKM2.
the phosphorylated PKM2-Y105 was previously reported to have decreased PK activity after the dissociation of PKM2 tetramers into dimers, which was associated with the acquirement of stem-like properties by inducing the translocation of YAP to activate downstream YAP signaling pathway.\textsuperscript{28} we proposed that CaMKII\textsubscript{d} relayed elevated calcium signaling to the transition of the role of PKM2 in PK activity to non-metabolic function in the determination of
cancer stem-like properties of αδ1⁺ PDAC TICs through the phosphorylation of PKM2 at T454 to activate YAP signaling pathway.

The phosphorylation of PKM2 could occur at multiple sites, such as Ser37, Tyr105, and Thr454 as reported here. Each of the phosphorylated PKM2 at Ser37, Tyr105, or Thr454 was able to result in the translocation of PKM2 into nucleus. Our data supported a sequential phosphorylation mode for PKM2. The phosphorylation occurred first at Thr454 of PKM2 triggered by CaMKII, which possibly increased the accessibility of other kinases to Tyr105, and subsequently led to the phosphorylation at Y105. The phosphorylation of PKM2-Y105 was both necessary and sufficient for its phosphorylation at Y105. Hence, the role of phosphorylated PKM2-Y105 in inducing stem-like properties of cancer cells as reported in literature was indeed dependent on the phosphorylation of T454. It would be interesting to determine whether the modifications of PKM2 at other sites such as Ser37 were also affected by T454 phosphorylation.

Here, we demonstrated that the αδ1⁺ cells were also presented in some of the paracancerous tissues of PDAC, and the existence of such a population in the paracancerous tissues was predictive of poor prognosis of PDAC patients. These findings are consistent with our previous work on hepatocellular carcinoma, supporting the hypothesis that αδ1⁺ TICs in paracancerous tissues represent a putative cell-of-origin for the recurrence of respective cancers including PDAC. Further prospective studies using clinical cohorts are warranted to address the prognostic value of the presence of αδ1⁺ TICs in the paracancerous tissues for PDAC patients.

Calcium influx mediated by αδ1 triggers a plethora of intracellular signaling cascades such as MAPK signaling and NOTCH pathway, which are essential for the self-renewal, survival, drug-resistance, and tumorigenic properties of the TICs of a variety of cancer types; hence targeting αδ1 with mAb1850-1 to prevent calcium influx can block these signaling pathways, providing a novel strategy for targeted therapy against TICs of liver and lung origins. Our study here revealed that αδ1 was also a therapeutic target for PDAC, further indicating that this strategy was also possibly applied to other cancer types.

In conclusion, the results presented here reveal the role of CaMKIIδ, which senses elevated calcium mediated by αδ1 to transit PKM2 from PK activity to nonmetabolic function through a sequential phosphorylation mode, in the acquisition and subsequent maintenance of the properties of PDAC TICs. Future study is warranted to address whether this calcium signaling pathway is a general mechanism applied to all the other αδ1⁺ TICs from different cancer types. The identification of αδ1 as a more robust TIC surface marker and therapeutic target for PDAC not only lays the foundation for a better understanding of the nature of TICs, but also provides novel strategies for the prediction of prognosis and targeted therapy against PDAC TICs.

Materials and Methods

Cell Lines and Clinical Samples

The pancreatic carcinoma cell lines AsPC-1, Mia PaCa-2, PANC-1, and BxPC-3 were purchased from American Type

![Table 4. Tumorigenicity of the PKM2-KD PANC-1 Cells that Ectopically Expressed the Indicated RNAi-Resistant PKM2 Mutants](image)

| Group                      | Tumor formation | Tumorigenic cell frequency (95% CI) | P value |
|----------------------------|-----------------|-------------------------------------|---------|
| Set 1 experiment           |                 |                                     |         |
| PKM2-Wild-type             | 3/5             | 1/711 (1/1928–1/262)                |         |
| PKM2-Y105A                 | 1/5             | 1/2458 (1/10394–1/581)              | 0.122†  |
| PKM2-Y105D                 | 5/5             | 1 (1/125–1)                        | 3.3E-05*|
| Set 2 experiment           |                 |                                     |         |
| PKM2-Wild-type             | 2/5             | 1/1445 (1/4728–1/442)               |         |
| PKM2-T454A                 | 1/5             | 1/2458 (1/10394–1/581)              | 0.559†  |
| PKM2-T454D                 | 5/5             | 1 (1/125–1)                        | 1.5E-06*|
| PKM2-Y105F-T454D           | 2/5             | 1/1445 (1/4728–1/442)               | 1.08†   |

*Compared with the respective PKM2-Wild type group.
Figure 8. Therapeutic effects of mAb1B50-1 on PDAC xenografts. (A–C) Growth curves (A), photograph of dissected tumors (B), and tumor weights (C) of the PANC-1 engraftments treated with mAb1B50-1 (800 µg/mice), gemcitabine (GEM) (50 mg/kg), or combination of both after the tumors were visible. (D–F) Growth curves (D), photograph of dissected tumors (E), and tumor weights (F) of BxPC-3 xenografts received the indicated treatments. (G) Body weights of the mice during the treatment period. (H and I) Growth curves showing therapeutic effects of indicated regimens on 2 pancreatic PDX models. (J and K) Kaplan-Meier curves showing the overall survival of PDX-1 (J) and PDX-2 (K) models after the treatments. (L) Flow cytometry analysis of percentage of α2δ1+ cells in the residual tumors of PANC-1 xenografts after the indicated treatments. (M) Dissected tumors showing the tumor-initiating ability of the PANC-1 engraftment residues after the indicated treatments, which was assayed by re-transplanting 10^4 cells per site with Matrigel into NOD/SCID mice. *Two-tailed Student t test. Arrows in A, D, H, and I show the time points that the indicated regimens were administered. Bars = 1 cm.
were maintained in an atmosphere of 5% CO2 at 37
were cleared off mycoplasma contamination. All cell lines
authenticated using short tandem repeat DNA pro
factor (Thermo Fisher Scientific
supplemented with 50
Keratinocyte Serum Free Medium (Thermo Fisher Scientific
obtained from Kerafast Inc (Boston, MA) and cultured in
human pancreatic duct epithelial cell line HPDE6-C7 was
Tomycin (Thermo Fisher Scientific
were polymerase chain reaction amplified from
cDNAs that were reverse-transcribed from total RNAs
extracted from the hepatocellular carcinoma cell line Hep-
were subcloned into pLentiv6 vector (Thermo Fisher Scientific) using standard DNA recombinant tech-
ique. For all the mutant constructs, respective point mu-
tations were introduced using overlapped polymerase chain
reaction with primers harboring the mutations and were
cloned into plenti6 vector. The shRNA-resistant wild-type
and mutant PKM2 constructs were further made by
replacing the shRNA targeting sequence with synonymous
codons. For the CaMK2D and PKM2 shRNA constructs,
synthetic target oligos were cloned into PSIH-H1-Puro vec-
tors. All the primers used are listed in Table 5, and the
constructs were validated by sequencing. Lentiviral particles
were produced in 293FT cells as described previously.

Vector Construction and Lentivirus Packaging
The construction of α2δ1 overexpression and shRNA
knockdown lentivirus vectors was described in our previous
article. The open reading frames of wild-type CaMK2D and
PKM2 were polymerase chain reaction amplified from

| Name                  | Primer          | Sequences                                                                 |
|-----------------------|-----------------|---------------------------------------------------------------------------|
| PKM2 Wild-type        | Forward         | 5'-CGGGATCCATGTCGAGCAGAGCCATAGTGAAGC-3                                    |
|                       | Reverse         | 5'-CGGCTGAGCGCCACAGAACACAGCATG-3                                           |
| PKM2 shRNA            | Forward         | 5'-gatccCTGGAGCTCTAGACACTAAAttcctgtcagaTTTAGTGTCTAGAGCCACAGttttG-3         |
|                       | Reverse         | 5'-aattcAAAAACTGTGGCTCTAGACACTAAAtctgcagaggaTTTATGTCTAGAGCCACAGG-3         |
| PKM2-Y105F            | Forward         | 5'-CCTCTCGGCGCGTTGTCTTGTG-3                                               |
|                       | Reverse         | 5'-GCCGGAAGAGGATGGGTCAAGAHC-3                                              |
| PKM2-Y105D            | Forward         | 5'-CCGGATCATGTCGAGCAGCCCTAGTGAAGG-3                                       |
|                       | Reverse         | 5'-CGGCTGAGGTGAGCTCATCAGAAGG-3                                            |
| PKM2-T454A            | Forward         | 5'-GAGGCTGATGCTGAGACTGTTGAGAGAAGG-3                                       |
|                       | Reverse         | 5'-CCGCTGAGCATGTCGAGCAGAGACACAG-3                                         |
| PKM2-T454D            | Forward         | 5'-ATCACTGATGCTGAGACTGTTGAGAGAAGG-3                                       |
|                       | Reverse         | 5'-CGGCTGAGCTGAGACTGTTGAGAGAAGG-3                                         |
| CaMKII Wild-type      | Forward         | 5'-CGGGATCCATGTCGAGCAGAGCCATAGTGAAGC-3                                    |
|                       | Reverse         | 5'-CGGCTGAGCGCCACAGAACACAGCATG-3                                           |
| CaMKII shRNA1         | Forward         | 5'-gatccGATCAAGGCGTTGAGCTCATCAGAAGG-3                                     |
|                       | Reverse         | 5'-aattcaaaaaGATCAAGGCGTTGAGCTCATCAGAAGG-3                                  |
| CaMKII shRNA2         | Forward         | 5'-gatccGGTGAGAAGATGTATGAAAGTGCAGAGGACAGTGAAGG-3                           |
|                       | Reverse         | 5'-aattcaaaaaGATCAAGGCGTTGAGCTCATCAGAAGG-3                                  |
| CaMKII T287A          | Forward         | 5'-CATCAACAGGCGTTGAGACTGTTGAGAGAAGG-3                                     |
|                       | Reverse         | 5'-AATTTTTCAGGTCAAGAGCAGCATGTCGAGCTTGTG-3                                   |
| Scrambled shRNA       | Forward         | 5'-gatccGAGGACAGGCGTCAAGAGATGATGAGTGAAGG-3                                  |
|                       | Reverse         | 5'-aattcaaaaaACAGGAGAGGCGTCAAGAGATGATGAGTGAAGG-3                           |

Culture Collection (Manassas, VA) and were cultured in
RPMI 1640 medium or Dulbecco modified Eagle medium as
suggested by the vendor, supplemented with 10% fetal
bovine serum, 100 U/mL penicillin, and 100 μg/mL strep-
tomycin (Thermo Fisher Scientific, Waltham, MA). Normal human pancreatic duct epithelial cell line HPDE6-C7 was
obtained from Kerafast Inc (Boston, MA) and cultured in
Keratinocyte Serum Free Medium (Thermo Fisher Scientific)
supplemented with 50 μg/mL bovine pituitary extract
(Thermo Fisher Scientific) and 5 ng/mL epidermal growth
factor (Thermo Fisher Scientific). All cell lines were
authenticated using short tandem repeat DNA profiling and
were cleared off mycoplasma contamination. All cell lines
were maintained in an atmosphere of 5% CO2 at 37°C.

Primary PDAC specimens and paracancerous tissues
were obtained from patients who underwent duodenopan-
createctomy or pancreatosplenectomy in Peking University
Third Hospital with written informed consent. The acquisition
and use of these tissues were approved by the Ethics
Committee of Peking University Third Hospital (no. G-
2014005), and the study was compliant with all relevant
ethical regulations regarding research involving human
participants. The PDX were established by transplanting
mechanically minced PDAC specimens immediately after
surgery into NOD/SCID mice (NOD.CB17-Prkdc<sup>scid</sup>; Vital
River Laboratories, Beijing, China).

Lentivirus Infection
Adherent cells were incubated with lentivirus for over-
night, followed by adding selection antibiotics 48 hours
later. Surviving cells were expanded for subsequent exper-
iments. For shRNA knockdown assay, FACS-sorted α2δ1<sup>+</sup>
TICs were incubated with lentivirus for 4 hours at 37°C by
spinning slowly in an incubator and were cultured in serum-
free medium for additional 72 hours for Western blot assay
or were proceeded directly for spheroid formation assay or
tumorigenicity assay.

Living Cell Immunofluorescent Staining and Flow
Cytometry
The preparation of single cell suspension, immunofluo-
rescent staining, and flow cytometry was done essentially
the same as previously described. In brief, single- cell
suspensions from PDAC cell lines, tumor tissues were incubated with the antibodies including mAb1B50-1 against α2δ1 and DCLK1 (Abcam, Cambridge, MA), which were conjugated with fluorescein isothiocyanate or phycoerythrin-cyanin 5 using BD Lightning conjugation kits (Expedeon Ltd, Cambridge, UK), as well as CD44-PE (Miltenyi Biotec, Auburn, CA), EpCAM-FITC (R&D Systems, Minneapolis, MN), and CD9-APC (Thermo Fisher Scientific), followed by washing with phosphate-buffered saline 3 times. After being filtered through a 40-μm nylon mesh, the single-cell suspensions were gated, analyzed, or sorted using a FACSAria II flow cytometer (Becton Dickinson, San Jose, CA). The respective isotype controls were used as references, and data were processed using FlowJo VX software.

Table 6. Patient Information for the Pancreatic Carcinoma PDX Models

| Variable                  | PDX1                   | PDX2                   |
|---------------------------|------------------------|------------------------|
| Gender                    | Male                   | Male                   |
| Age/year                  | 43                     | 61                     |
| Pathologic type           | Pancreatic ductal adenocarcinoma | Pancreatic ductal adenocarcinoma |
| Tumor size                | 5.5 cm                 | 1.2 cm                 |
| Pathologic grades         | 2                      | 3                      |
| Lymphatic metastasis      | Yes                    | No                     |
| Neural invasion           | Yes                    | Yes                    |
| Intravascular carcinoma emboli | Yes             | Yes                    |
| Choledochus infiltration  | Yes                    | Yes                    |

Intracellular Calcium Measurement

Intracellular calcium levels were measured using the Fluo-4/AM probe (Thermo Fisher Scientific) following the published protocol. Labeled cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

Immunofluorescent and Immunohistochemical Staining

For cultured cell immunofluorescent staining, cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes, permeabilized with 0.5% Triton X-100, and were incubated with primary antibody and secondary antibody as previously described. The immunofluorescent staining of frozen tissues was performed following the protocol described in literature. For immunohistochemical staining, formalin-fixed, paraffin-embedded PDAC specimen sections were deparaffinized and rehydrated, followed by heating for antigen retrieval for 15 minutes in 10 mmol/L citrate (pH 6.0) according to standard protocol. Sections were then incubated with primary antibody at 4°C overnight. Diaminobenzidine staining was performed using DAB detection Kit (polymer) (Beijing Zhongshan Goldenbridge Biotechnology Co, Ltd, Beijing, China) according to the vendor’s instructions, followed by hematoxylin counterstaining. The information of the primary antibodies was listed in Table 6. The immunohistochemistry staining was quantified using the immunoreactive score (IRS) (system in a double-blind manner, giving a score range of 0–12 by multiplication of the positive cell proportion score (0 = 0%, 1 = 1%-10%, 2 = 11%-50%, 3 = 51%-80%, and 4 = 81%-100% stained cells) and the staining intensity score (0 = negative, 1 = weak, 2 = moderate, and 3 = strong).

Table 7. Information for Antibodies

| Name            | Vendor     | Cat. no. | Species                     | Dilution  |
|-----------------|------------|----------|----------------------------|-----------|
| ABCG2           | Epitomics  | 3765-1   | Rabbit monoclonal IgG      | WB: 1:2000 |
| Nanog           | Abcam      | ab109250 | Rabbit monoclonal IgG      | WB: 1:2000 |
| SOX2            | Abcam      | ab97959  | Rabbit monoclonal IgG      | WB: 1:2000 |
| BMI1            | Abcam      | Ab126783 | Rabbit monoclonal IgG      | WB: 1:5000 |
| FLAG            | Origene    | TA50011  | Mouse monoclonal IgG       | WB: 1:5000 |
| α2δ1            | Abcam      | ab2864   | Mouse monoclonal IgG       | WB: 1:2000 |
| α2δ1            | Novus      | Nb120-2864 | Mouse monoclonal IgG      | WB: 1:2000 |
| CaMKIIα         | Cell Signaling | 3357S   | Rabbit polyclonal IgG      | WB: 1:2000 |
| CaMKIIβ         | Invitrogen | 13-9800  | Mouse monoclonal IgG       | WB: 1:2000 |
| CaMKIIβ         | Santa Cruz | SC-100362 | Mouse monoclonal IgG      | WB: 1:200; IP: 1:20 |
| CaMKIIγ         | Sigma-Aldrich | SAB1400039 | Rabbit polyclonal IgG      | WB: 1:2000 |
| p-PKM2(Y105)    | Cell Signaling | 3827S   | Rabbit monoclonal IgG      | WB: 1:2000 |
| PKM2            | Cell Signaling | 4053S   | Rabbit monoclonal IgG      | WB: 1:2000 |
| GAPDH           | Bioworld   | BS606030 | Rabbit polyclonal IgG      | WB: 1:20000 |
| PKM2            | Abcam      | AB131021 | Rabbit monoclonal IgG      | IP: 1:20   |
| CD44-PE         | Miltenyi   | 130-113-335 | Mouse monoclonal IgG   | IF: 1:50  |
| EpCAM-FITC      | R&D Systems | FAB9601F | Mouse monoclonal IgG       | IF: 1:50  |
| DCLK1           | Abcam      | Ab37994  | Rabbit polyclonal IgG      | IF: 1:30  |
Sphere Formation Assay

Sphere formation assay was performed in ultralow attachment 96-well plates (Corning Incorporated Life Sciences, Acton, MA) by plating 100 cells per well in DMEM/F12 medium supplemented with B27 (Invitrogen, Waltham, MA), 20 ng/mL EGF, 10 ng/mL HGF (Peprotech, Rocky Hill, NJ), 20 ng/mL bFGF (Invitrogen), and 1% methylcellulose (Sigma-Aldrich, St Louis, MO). After cultivation in 5% CO₂ incubator for 2–3 weeks, the spheres with diameter ≥100 μm were counted under an Axio Observer A1 inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Tumorigenicity Assay

Female 4- to 6-week-old NOD/SCID mice (Beijing Vital River Laboratory Animal Technology Co, Ltd, Beijing, China) were used in this study following the National Institutes of Health Guide for the Care and Use of Laboratory Animals with protocols approved by the Peking University Cancer Hospital Animal Care and Use Committee. For the tumorigenic potential assay, cells suspended in 100 μL of 1:1 mixture of plain RPMI 1640 medium and Matrigel (BD Biosciences, Bedford, MA) were transplanted s.c. into the backs of mice. Tumor formation was monitored weekly, and the tumorigenic cell frequency was determined on the basis of extreme limiting dilution analysis using a web tool at http://bioinf.wehi.edu.au/software/elda/. For the therapeutic assay, both PDAC cell line-derived xenografts and PDX (Table 7) were established by transplanting tumor cells/tissues s.c. into the backs of mice. When all the tumors reached palpable size, the mice were randomly separated into 4 groups and were administered i.p. with control vehicle, anti-α2δ1 mAb1B50-1 (800 μg/mice), GEM (50 mg/kg), and the combination of mAb1B50-1 with GEM for each respective group. The tumors were measured every other day with calipers, and individual tumor volumes (V) were determined using the formula: $V = \text{length} \times \text{width}^2 \times 0.5$.

Western Blot

Cells were homogenized in radioimmunoprecipitation assay buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, phosphatase inhibitor cocktail, and Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany). Equal amounts of proteins were electrophoresed on 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to Immobilon-P PVDF membrane (0.45 μm pore size; Millipore, Billerica, MA). The membranes were blocked with 5% non-fat milk and incubated with primary and secondary antibodies following the protocol described previously.22 The polyclonal rabbit antibody specifically recognizing phosphorylated PKM2 at Thr454 (p-PKM2-T454) was produced by immunizing rabbits with phosphorylated peptides C-RAPIIAVT(p)RNPQTRAR (Huiou Biotech Inc, Shanghai, China). The information on other antibodies used is provided in Table 6. The immuno-complexes were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore), and the signals were captured with MiniChemi 610 Chemiluminescent Imaging and Analysis System (Beijing Sage Creation Science Co, Ltd., Beijing, China).

Immunoprecipitation Assay

Cells were extracted with ice-cold CellLytic M lysis buffer (Sigma-Aldrich) plus phosphatase inhibitor cocktail and protease inhibitor cocktail on ice, followed by sonication. Cell extracts were then clarified by centrifugation at 12,000 rpm for 5 minutes, and the supernatants were subjected to immunoprecipitation with mouse anti-CaMKIIδ monoclonal and rabbit anti-PKM2 polyclonal antibodies at 4°C overnight, followed by additional incubation with Protein A/G PLUS-Agarose beads (GE Healthcare, Uppsala, Sweden) for an additional 3 hours. After washing with ice-cold lysis buffer 3 times, proteins binding to the beads were eluted with 1× sodium dodecyl sulfate loading buffer. For the Flag-tagged proteins, cell extracts were incubated with anti-Flag M2 affinity gel (A2220; Sigma-Aldrich) overnight at 4°C, and the precipitated proteins were eluted following the anti-Flag M2 gel vendor’s recommendation.

Mass Spectrometry Analysis

The immunoprecipitation products were detected on SDS-PAGE using Pierce Silver Stain for MS kit (Thermo Fisher Scientific). The bands of target proteins were sliced and digested with sequencing grade trypsin in 50 mmol/L NH₄HCO₃ overnight at 37°C, followed by nano-liquid chromatography with tandem mass spectrometry analysis on an LTQ-velos mass spectrometer interfaced with an EASY nano-LC system (Thermo Fisher Scientific). The liquid chromatography with tandem mass spectrometry data were searched against the human sequence library in the Uniprot protein sequence database using SEQUEST HT algorithm in the Proteome Discoverer 1.4 software package (Thermo Fisher Scientific). The probability of phosphosite localization was calculated using the phosphoRS 3.0 software implemented into the Proteome Discoverer.

In Vitro Phosphorylation Assay

Flag-tagged CaMKIIδ and CaMKIIδ-T287A mutant were purified from FreeStyle 293 cells (Invitrogen) transiently transfected with respective expression constructs using anti-Flag M2 affinity gel. The fusion protein glutathione S-transferase-PKM2 was expressed in E.coli Rosetta (DE3) and was purified using glutathione-agarose 4B (GE Healthcare, Uppsala, Sweden) following standard protocol. For the in vitro PKM2 phosphorylation assay, purified wild-type CaMKIIδ or mutant CaMKIIδ-T287A protein was pre-incubated in a reaction mixture containing 35 mmol/L HEPES, pH 8.0, 10 mmol/L MgCl₂, 0.5 μmol/L CaM (Prospec, Rehovot, Israel), 5 μmol/L adenosine triphosphate, and 1 mmol/L CaCl₂ at 30°C for 10 minutes, followed by the addition of glutathione Sepharose 4B beads that bound glutathione S-transferase-PKM2 and further incubation for 2 hours. Glutathione S-transferase-PKM2 were eluted using 2× SDS-PAGE loading buffer. The phosphorylation of PKM2...
was detected by Western blotting using p-PKM2-T454 antibody.

Detection of PKM2 Oligomerization
Cultured cells were collected, washed with phosphate-buffered saline, and resuspended in CHAPS buffer (20 mmol/L HEPES-KOH, pH 7.5, 5 mmol/L MgCl₂, 0.5 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride) containing 4 mmol/L disuccinimidyl suberate (Thermo Fisher Scientific) at room temperature for 30 minutes to crosslink proteins. After the samples were centrifuged at 5000g for 8 minutes at 4°C, cell pellets were lysed with Tris-free radioimmunoprecipitation assay buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 0.1% SDS) supplemented with proteinase inhibitor cocktail and phosphatase inhibitors on ice for 30 minutes, followed by sonication. The supernatants were subjected to SDS-PAGE and Western blot analysis.28,42

Statistical Analysis
The data were analyzed using GraphPad Prism software (San Diego, CA). The significance of differences was determined with a double-sided Student t test unless otherwise specified. A P value ≤ 0.05 was considered statistically significant.

References
1. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. N Engl J Med 2014;371:1039–1049.
2. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. CA Cancer J Clin 2021;71:7–33.
3. Crawford HC, Pasca di Magliano M, Banerjee S. Signaling networks that control cellular plasticity in pancreatic tumorigenesis, progression, and metastasis. Gastroenterology 2019;156:2073–2084.
4. Clara JA, Monge C, Yang Y, Takebe N. Targeting signalling pathways and the immune microenvironment of cancer stem cells: a clinical update. Nat Rev Clin Oncol 2020;17:204–232.
5. Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. Cell Stem Cell 2012;10:717–728.
6. Wang VM, Ferreira RMM, Almagro J, Evan T, Legrave N, Zaw Thin M, Frith D, Carvalho J, Barry DJ, Snijders AP, Herbert E, Nye EL, MacRae J, Behrens A. CD9 identifies pancreatic cancer stem cells and modulates glutamine metabolism to fuel tumour growth. Nat Cell Biol 2019;21:1425–1435.
7. Herrmann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschhen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 2007;1:313–323.
8. Gzil A, Zarebska I, Bursiewicz W, Antosik P, Grzanka D, Szyberg L. Markers of pancreatic cancer stem cells and their clinical and therapeutic implications. Mol Biol Rep 2019;46:6629–6645.
9. Campiglio M, Flucher BE. The role of auxiliary subunits for the functional diversity of voltage-gated calcium channels. J Cell Physiol 2015;230:2019–2031.
10. Clapham DE. Calcium signaling. Cell 2007;131:1047–1058.
11. Smedler E, Uhlen P. Frequency decoding of calcium oscillations. Biochim Biophys Acta 2014;1840:964–969.
12. Brzozowski JS, Skelding KA. The multi-functional calcium/calmodulin stimulated protein kinase (CaMK) family: emerging targets for anti-cancer therapeutic intervention. Pharmaceuticals (Basel) 2019;12:8.
13. Bayer KU, Schulman H. CaM kinase: still inspiring at 40. Neuron 2019;103:380–394.
14. Yang E, Schulman H. Structural examination of autoregulation of multifunctional calcium/calmodulin-dependent protein kinase II. J Biol Chem 1999;274:26199–26208.
15. Rosenberg OS, Deindl S, Sung R-J, Nairn AC, Kuriyan J. Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. Cell 2005;123:849–860.
16. Roderick HL, Cook SJ. Ca²⁺ signalling checkpoints in cancer: remodelling Ca²⁺ for cancer cell proliferation and survival. Nat Rev Cancer 2008;8:361–375.
17. Monteith GR, Prevarskaya N, Roberts-Thomson SJ. The calcium–cancer signalling nexus. Nat Rev Cancer 2017;17:373–380.
18. O’Reilly D, Buchanan P. Calcium channels and cancer stem cells. Cell Calcium 2019;81:21–28.
19. Forostyak O, Forostyak S, Cortus S, Sykova E, Verkhratsky A, Dayanithi G. Physiology of Ca²⁺ signalling in stem cells of different origins and differentiation stages. Cell Calcium 2016;59:57–66.
20. Sun C, Shui B, Zhao W, Liu H, Li W, Lee JC, Doran R, Lee FK, Sun T, Shen QS, Wang X, Reining S, Kotlikoff MI, Zhang Z, Cheng H. Central role of IP3R2-mediated Ca²⁺ oscillation in self-renewal of liver cancer stem cells elucidated by high-signal ER sensor. Cell Death Dis 2019;10:396.
21. Han H, Du Y, Zhao W, Li S, Chen D, Zhang J, Liu J, Suo Z, Bian X, Xing B, Zhang Z. PXB3 is targeted by multiple miRNAs and is essential for liver tumour-initiating cells. Nat Commun 2015;6:8271.
22. Zhao W, Wang L, Han H, Jin K, Lin N, Guo T, Chen Y, Cheng H, Lu F, Fang W, Wang Y, Xing B, Zhang Z. A 556-1, a mAb raised against recurrent tumor cells, targets liver tumor-initiating cells by binding to the calcium channel α2β1 subunit. Cancer Cell 2013;23:541–556.
23. Ma Y, Yang X, Zhao W, Yang Y, Zhang Z. Calcium channel α2β1 subunit is a functional marker and therapeutic target for tumor-initiating cells in non-small cell lung cancer. Cell Death Dis 2021;12:257.
24. Zhang Z, Zhao W, Lin X, Gao J, Zhang Z, Shen L. Voltage-dependent calcium channel alpha2delta1 subunit is a specific candidate marker for identifying gastric cancer stem cells. Cancer Manag Res 2019;11:4707–4718.
25. Mazurek S. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. Int J Biochem Cell Biol 2011;43:969–980.
26. Yang W, Xia Y, Hawke D, Li X, Liang J, Xing D, Aldape K, Hunter T, Alfred Yung WK, Lu Z. PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. Cell 2012;150:685–696.

27. Yang W, Xia Y, Ji H, Zheng Y, Liang J, Huang W, Gao X, Aldape K, Lu Z. Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. Nature 2011; 480:118–122.

28. Zhou Z, Li M, Zhang L, Zhao H, Sahin Ö, Chen J, Zhao JJ, Songyang Z, Yu D. Oncogenic kinase–induced PKM2 tyrosine 105 phosphorylation converts non-oncogenic PKM2 to a tumor promoter and induces cancer stem–like cells. Cancer Res 2018;78:2248–2261.

29. Luo W, Hu H, Chang R, Zhong J, Knabel M, O’Meally R, Cole RN, Pandey A, Semenza GL. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 2011;145:1–12.

30. Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. Annu Rev Cell Dev Biol 2007;23:675–699.

31. Si J, Collins SJ. Activated Ca2+/calmodulin-dependent protein kinase II is a critical regulator of myeloed leukemia cell proliferation. Cancer Res 2008;68:3733–3742.

32. Li N, Jiang P, Du W, Wu Z, Li C, Qiao M, Yang X, Wu M. Si6a1 suppresses epithelial-mesenchymal transition and metastasis of tumor cells by inhibiting stathmin and stabilizing microtubules. Proc Natl Acad Sci USA 2011;108:12851–12856.

33. Daft PG, Yuan K, Warram JM, Klein MJ, Siegel GP, Zayzafoon M. Alpha-CaMKII plays a critical role in determining the aggressive behavior of human osteosarcoma. Mol Cancer Res 2013;11:349–359.

34. Cuddapah VA, Sontheimer H. Molecular interaction and functional regulation of CDC-3 by Ca2+/calmodulin-dependent protein kinase II (CaMKII) in human malignant glioma. J Biol Chem 2010;285:11188–11196.

35. Terrie E, Coronas V, Constantin B. Role of the calcium toolkit in cancer stem cells. Cell Calcium 2019;80:141–151.

36. Yang W, Zheng Y, Xia Y, Ji H, Chen X, Guo F, Lysiosiotis CA, Aldape K, Cantley LC, Lu Z. ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. Nat Cell Biol 2012;14:1295–1304.

37. Yu Z, Zhao X, Huang L, Zhang T, Yang F, Xie L, Song S, Miao P, Zhao L, Sun X, Liu J, Huang G. Proviral insertion in murine lymphomas 2 (PIM2) oncogene phosphorylates pyruvate kinase M2 (PKM2) and promotes glycolysis in cancer cells. J Biol Chem 2013;288:35406–35416.

38. Sainz B Jr, Heeschen C. Standing out from the crowd: cancer stem cells in hepatocellular carcinoma. Cancer Cell 2013;23:431–433.

39. Xu XL, Xing BC, Han HB, Zhao W, Hu MH, Xu ZL, Li JY, Xie Y, Gu J, Wang Y, Zhang ZQ. The properties of tumor-initiating cells from a hepatocellular carcinoma patient’s primary and recurrent tumor. Carcinogenesis 2010; 31:167–174.

40. Zhang ZQ, Bish LT, Holtzer H, Sweeney HL. Sarcomeric-alpha-actinin defective in vinculin-binding causes Z-line expansion and nemaline-like body formation in cultured chick myotubes. Exp Cell Res 2009;315:748–759.

41. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods 2009; 347:70–78.

42. Fernández-Duran I, Quintanilla A, Tarrats N, Birch J, Hali P, Millar FR, Lagnado AB, Smer-Barreto V, Muir M, Brunton VG, Passos JF, Acosta JC. Cytoplasmic innate immune sensing by the caspase-4 non-canonical inflammasome promotes cellular senescence. Cell Death Differ 2022;29:1267–1282.