A non-canonical role for pyruvate kinase M2 as a functional modulator of Ca$^{2+}$ signalling through IP$_3$ receptors

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ABSTRACT
Pyruvate kinase isoform M2 (PKM2) is a rate-limiting glycolytic enzyme that is widely expressed in embryonic tissues. The expression of PKM2 declines in some tissues following embryogenesis, whilst other pyruvate kinase isozymes are upregulated. However, PKM2 is highly expressed in cancer cells and is believed to play a role in supporting anabolic processes during tumour formation. In this study, PKM2 was identified as an inositol 1,4,5-trisphosphate receptor (IP$_3$R)-interacting protein by mass spectrometry. The PKM2:IP$_3$R interaction was further characterized by pull-down and co-immunoprecipitation assays, which showed that PKM2 interacted with all three IP$_3$R isoforms. Moreover, fluorescence microscopy indicated that both IP$_3$R and PKM2 localized at the endoplasmic reticulum. PKM2 binds to IP$_3$R at a highly conserved 21-amino acid site (corresponding to amino acids 2078-2098 in mouse type 1 IP$_3$R isoform). Synthetic peptides (denoted ‘TAT-D5SD’ and ‘D5SD’), based on the amino acid sequence at this site, disrupted the PKM2:IP$_3$R interaction and potentiated IP$_3$R-mediated Ca$^{2+}$ release both in intact cells (TAT-D5SD peptide) and in a unidirectional $^{45}$Ca$^{2+}$ flux-assay on permeabilized cells (D5SD peptide). The TAT-D5SD peptide did not affect the enzymatic activity of PKM2. Reducing PKM2 protein expression using siRNA increased IP$_3$R-mediated Ca$^{2+}$ signalling in intact cells without altering the ER Ca$^{2+}$ content. These data identify PKM2 as an IP$_3$R-interacting protein that inhibits intracellular Ca$^{2+}$ signalling. The elevated expression of PKM2 in cancer cells is therefore not solely connected to its canonical role in glycolytic metabolism, rather PKM2 also has a novel non-canonical role in regulating intracellular signalling.
HIGHLIGHTS

- PKM2 co-localizes with IP₃Rs at the endoplasmic reticulum
- PKM2 co-immunoprecipitates with the IP₃R in multiple cell types
- PKM2 interacts with amino acid sequence 2078-2098 of type 1 IP₃ receptor (denoted ‘Domain 5 subdomain’; D5SD)
- The D5SD peptide can be used as a decoy to acutely disrupt PKM2:IP₃R interaction in intact or permeabilized cells
- PKM2:IP₃R disruption or PKM2 knockdown increases IP₃-induced Ca²⁺ signalling by dis-inhibiting IP₃Rs

GRAPHIC ABSTRACT

ABBREVIATIONS

D5SD peptide: Domain 5 subdomain, peptide corresponding to amino acids 2078-2098 in type 1 IP₃R
ER: endoplasmic reticulum
HeLa 3KO: HeLa cells devoid of all IP₃R isoforms
IP$_3$: inositol 1,4,5-trisphosphate
IP$_3$R: inositol 1,4,5-trisphosphate receptor
PKM2: pyruvate kinase M2 isoform
TAT-D5SD peptide: TAT-conjugated D5SD peptide

KEYWORDS

PKM2; IP$_3$ receptor; endoplasmic reticulum; Ca$^{2+}$ signalling; T cells; B cells
1. Introduction

Intracellular Ca²⁺ signalling is initiated by activation of membrane receptors such as G protein-coupled receptors or tyrosine kinase receptors including T cell and B cell receptors. Activation of these receptors stimulates phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP₃). Following its production, IP₃ diffuses away from the cell membrane and binds to inositol 1,4,5-trisphosphate receptors (IP₃R); Ca²⁺-release channels that are primarily located on the endoplasmic reticulum (ER) [1, 2]. The binding of IP₃ to IP₃Rs triggers the release of Ca²⁺ ions from the ER, thereby producing Ca²⁺ signals that govern a wide variety of cellular processes, including cell metabolism, cell death and autophagy [3-5]. Such Ca²⁺ signals are not only directed towards the cytosol, but also towards organelles. For example, as the ER and mitochondria can be intimately linked, IP₃R-mediated Ca²⁺ signals are transmitted to the mitochondrial matrix [6, 7]. The sequestration of Ca²⁺ by mitochondria stimulates respiration and biosynthetic processes, but it can also promote cell death [8]. Many cancer cells possess mechanisms to reduce IP₃R-mediated cytosolic Ca²⁺ signals and subsequent mitochondrial Ca²⁺ uptake, thereby decreasing cell death susceptibility and thus acquiring a survival advantage [9-13].

Complementing their function as Ca²⁺-release channels, IP₃Rs act as scaffolds and signal integrators, bringing numerous proteins and protein complexes within proximity of the ER and mitochondria [14-16]. Several moieties, including kinases and phosphatases, bind to IP₃Rs, regulating channel opening and Ca²⁺ release [1, 17-21]. By regulating IP₃R activity, these allosteric protein interactions shape the amplitude and frequency of cellular Ca²⁺ signals, with consequential effects on cell function and viability [22-28]. Of particular importance to cell survival, IP₃Rs bind both pro-apoptotic and anti-apoptotic Bcl-2 family members. These interactions have been shown to profoundly impact cell death and survival [29].

An experimental approach that we have used to explore the functional significance of IP₃R-interacting proteins is to identify their binding site on IP₃R and use the corresponding sequence to synthesize a decoy peptide that can be delivered to intact cells. As such, a 20-amino acid synthetic peptide named BIRD-2, corresponding to the IP₃R-binding site for the anti-apoptotic protein Bcl-2, was developed. BIRD-2 functions as a competitive inhibitor of Bcl-2:IP₃R interaction [10]. The BIRD-2 peptide has been employed to assess the role of Bcl-2
in regulating IP₃R-mediated Ca²⁺ release from the ER. For example, using BIRD-2 to acutely antagonise Bcl-2:IP₃R interaction has demonstrated that Bcl-2 reduces IP₃R-mediated Ca²⁺ release [10], thereby abrogating the induction of cell death and providing a survival advantage for cancer cells that highly express Bcl-2 [30-35].

Another example where a decoy peptide has been used to elucidate the role of an IP₃R-interacting protein is the T cell-associated tyrosine kinase Lck [36]. In contrast to the Bcl-2:IP₃R interaction, the Lck:IP₃R interaction enhances IP₃R-mediated Ca²⁺ elevation following T-cell receptor activation [36]. A synthetic peptide corresponding to the Lck-binding site on IP₃R, referred to as D5SD (Domain 5 Subdomain), decreases Lck:IP₃R interaction and represses T cell receptor-mediated Ca²⁺ elevation [36]. These studies indicate that decoy peptides, such as BIRD-2 and D5SD, have great utility in determining the nature of allosteric regulation of IP₃R by accessory proteins, and can be used to provide insights into the physiological consequences of these interactions.

Here, we report a novel functional interaction between IP₃Rs and isoform M2 of pyruvate kinase (PKM2), which catalyses the final, rate-limiting reaction step of glycolysis [37]. This interaction was initially revealed using mass spectrometry and was subsequently confirmed using multiple biochemical assays with various cell types. Our results, including measurements of Ca²⁺ signals in intact cells and unidirectional ⁴⁵Ca²⁺-flux analysis performed with permeabilised cells, indicate that PKM2 is a functional modulator of IP₃Rs. In essence, PKM2 suppresses Ca²⁺ release through IP₃Rs. These data are consistent with a hypothesis in which PKM2 has a prominent non-canonical role in cancer cells that is additional to supplying metabolites: it binds directly to IP₃R and modulates intracellular signalling. The disruption of the PKM2:IP₃R interaction via a cell-permeant peptide therefore represents a useful approach to investigate the wider functions of PKM2 when tethered to IP₃R.
2. Results

2.1. PKM2 interacts with the D5SD sequence in domain 5 of the IP₃R

IP₃Rs are composed of six structurally stable domains located on the cytosolic side of the ER, together with a transmembrane channel pore between domains 5 and 6 (Supplementary Figure 1A). We previously found that the protein Lck binds to domain 5 of mouse IP₃R isoform 1 (IP₃R-1) at a 21-amino acid alpha helical region located between amino acids 2078-2098, called D5SD [36]. The D5SD sequence is conserved among species (Supplementary Figure 1B) and the three known IP₃R isoforms (Supplementary Figure 1C). The D5SD sequence appears unique to the IP₃R, as it is not detected in other proteins according to National Center for Biotechnology Information BLAST search [38].

To identify novel proteins that recognize and bind to the D5SD sequence, a D5SD peptide and a scrambled control peptide were both tagged with biotin and used for pull-down assays with WEHI7.2 lymphoma cell lysates. Coomassie Blue-stained SDS-PAGE revealed considerable differences in the pattern of proteins pulled down by the biotin-tagged D5SD (Biotin-D5SD) and control (Biotin-Control) peptides (Figure 1A). Proteins pulled down by Biotin-D5SD and Biotin-Control were identified by mass spectrometry, with any proteins pulled down by both biotin-tagged peptides being excluded from further consideration. Mascot software was used to validate the top twenty scoring proteins pulled down by Biotin-D5SD and not by Biotin-Control peptide, among which was PKM2 (Figures 1B-C). Western blot analysis verified that PKM2 was expressed in a variety of lymphoid and non-lymphoid cells, while PKM1 was highly prevalent in brain cells (Figure 1D), consistent with analysis of PKM2 expression in mouse tissues [39].
Figure 1. MS/MS prediction of PKM2:IP₃R interaction. (A) Coomassie Blue-stained SDS-PAGE resolving proteins in WEHI7.2 cell lysate (far right) and proteins pulled down from the cell lysate by biotin-tagged control (Biotin-Control) and biotin-tagged D5SD (Biotin-D5SD) peptide. (B) List of proteins, including PKM2, predicted by mass spectrometry to have been pulled down by Biotin-D5SD but not by Biotin-Control peptide. Proteins are ranked according to strength of prediction based on Mascot software analysis. (C) The PKM2 sequence is shown, with the sequences that served to predict PKM2 binding to the IP₃R in mass spectrometry marked in red. (D) Western blots indicating that PKM2 is the main pyruvate kinase isoform expressed in a wide variety of cells, including T-cells (WEHI7.2, Jurkat), B-cells (Raji), normal mouse spleen, and non-hematopoietic cells (COS-7, HEK-293T, HEK-293, HeLa), whereas PKM1 is the major isoform expressed in mouse brain. Actin was used as the loading control.
2.2. **PKM2 is a bona fide IP$_3$R interactor**

Validating the interaction of PKM2:IP$_3$R within cells was undertaken using a variety of approaches. The subcellular localisation of both proteins was characterised using HeLa and HEK-293 cells, which were fixed and immunostained with an antibody recognizing endogenous PKM2 (Figure 2). PKM2 immunostaining was observed to substantially overlap in HeLa cells with the localization of the ER marker DsRed2-ER (Figure 2A). Moreover, PKM2 had an overlapping expression pattern with YFP-tagged IP$_3$R-1 expressed in HEK-293 cells (Figure 2B). These data indicate that PKM2 and IP$_3$Rs are co-localized at the ER.

![Figure 2. PKM2 and IP$_3$R co-localize at the ER.](image)

(A) In HeLa cells, PKM2 (green) expression overlaps with DsRed2-ER, an ER marker (red). DsRed2-ER was transiently expressed in HeLa cells, which were fixed and immunostained with antibody recognizing endogenous PKM2. Co-localization of PKM2 with the ER marker is demonstrated by the yellow color in the merged image at the far right. (B) PKM2 (red) expression also overlaps with YFP-tagged IP$_3$R-1 (green). YFP-tagged IP$_3$R1 was transiently expressed in HEK-293 cells, which were fixed and immunostained with antibody recognizing endogenous PKM2. Nuclei are stained with
DRAQ5™ (blue). Co-localization of YFP-tagged IP3R-1 and PKM2 is shown by the yellow color in the merged image at the far right. Representative images were acquired by epifluorescence microscopy.

To further substantiate the PKM2:IP3R interaction and assess whether it is common to different cell types, pull-down assays and Western blot analyses were used (Figure 3). Biotin-D5SD pulled-down endogenous PKM2 from WEHI7.2 cells, while PKM2 was not pulled-down by Biotin-Control (Figure 3A). Purified recombinant PKM2 (His-tagged PKM2) was pulled-down by biotin-tagged D5SD, indicating a direct interaction (Figure 3B). In addition, GST-tagged IP3R domain 5, which includes the D5SD sequence, pulled-down endogenous PKM2, further supporting that PKM2 directly interacts with IP3R domain 5 (Figure 3C). Finally, PKM2:IP3R complexes were immunoprecipitated from cell lysates obtained from various cell lines, indicating that IP3Rs are an endogenous binding partner of PKM2 in WEHI7.2 and Jurkat T-cells (Figures 3D, E), Raji and OCI-LY-10 B-cell lymphomas (Figures 3F, G), and HEK-293 cells (Figure 3H).
Figure 3. Interaction of PKM2 with D5SD, domain 5 of IP$_3$R-1 and endogenous IP$_3$R-3 of various cell types. (A) Western blot showing that Biotin-D5SD pulls down endogenous PKM2 from WEHI7.2 cell lysates. N=3. (B) Western blot demonstrating that Biotin-D5SD pulls down purified 6xHis-PKM2. N=3. (C) Western blot demonstrating that GST-domain 5 (see Suppl. Fig. 1A for location of domain 5) pulls down endogenous PKM2 from WEHI7.2 cell lysates. N=3. (D-H) Western blot indicating that IP$_3$R-3 is pulled down by PKM2 immunoprecipitation from WEHI7.2 (D, N=3) and Jurkat T cells (E, N=3), Raji (F, N=3) and OCI-LY-10 (G, N=3) B-cell lymphoma cells and HEK-293 epithelial cells (H, N=4).

2.3. Uncoupling the PKM2:IP$_3$R interaction results in spontaneous Ca$^{2+}$ signalling

To acutely modulate the PKM2:IP$_3$R interaction, we employed a cell-permeable version of the D5SD peptide derived from the fusion of the HIV TAT sequence to the N-terminus of the D5SD peptide (TAT-D5SD). Immunoprecipitation experiments performed on cell lysates of WEHI7.2 (Figure 4A) and OCI-LY-10 (Figure 4B) cells after pre-treatment with either TAT-Control or TAT-D5SD peptides demonstrated that only the TAT-D5SD peptide could disrupt PKM2:IP3R interaction. The TAT-D5SD peptide evoked Ca$^{2+}$ oscillations in both WEHI7.2 and OCI-LY-10 cells (Figure 4C, D), while this was not the case for the TAT-Control peptide. Importantly, the TAT-D5SD peptide did not alter the catalytic activity of PKM2 (Supplementary Figure 2).

For further analysis of the potential effects of PKM2 on IP$_3$R-mediated Ca$^{2+}$ release we selected HeLa cells, as we verified PKM2 localization at the ER in these cells (Figure 2A). Moreover, HeLa cells have the additional advantages of expressing all three IP$_3$R isoforms [40], and being ideally suited for performing quantitative unidirectional $^{45}$Ca$^{2+}$-flux experiments after permeabilization [41, 42]. In addition, HeLa cells that are fully deficient in IP$_3$R expression (HeLa 3KO) are available [43] so that the role of the IP$_3$R can be clearly assessed.
Figure 4. PKM2:IP₃R interaction is disrupted by TAT-D5SD peptide, resulting in cytosolic Ca²⁺ elevation. (A, B) TAT-D5SD peptide disrupts the PKM2:IP₃R complex in (A) WEHI7.2 T-cells and (B) in the OCI-LY-10 B-cell lymphoma cell line. Cells were incubated with 200 μM TAT-D5SD or TAT-control peptide for 1 h followed by immunoprecipitation and Western blotting for IP₃R-3. N=3. (C, D) Cytosolic Ca²⁺ was measured by single-cell digital imaging using Fura-2 AM, as described in the Materials and Methods. (C) WEHI7.2 cells and (D) OCI-LY-10 cells were exposed to 20 μM TAT-control peptide (Ci, Di) or TAT-D5SD (Cii, Dii) for the indicated times (arrowheads indicate time of peptide addition). The traces represent the mean of three independent experiments; an average of 56 cells were analyzed in each experiment.

Co-immunoprecipitation experiments demonstrated that PKM2 interacted with all three IP₃R isoforms in HeLa cells (Figure 5A). Thereafter, using a unidirectional ⁴⁵Ca²⁺ efflux assay with permeabilized HeLa cells, we directly examined the impact of PKM2 on IP₃R-mediated Ca²⁺ release (Figure 5B, C). This assay allows the accurate quantification of the Ca²⁺ efflux.
from the ER lumen to the cytosol in the absence of any confounding sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase, plasma-membrane Ca\(^{2+}\)-ATPase or mitochondrial activity. The time course of the Ca\(^{2+}\) flux experiment is illustrated in Figure 5B, where the fractional release of Ca\(^{2+}\) from the ER (% efflux per 2-minute time window) is plotted as a function of time. Addition of IP\(_3\) (black bar) increased the rate of \(^{45}\text{Ca}^{2+}\) release. Pre-incubation with the D5SD peptide to disrupt binding of PKM2 to the IP\(_3\)Rs (TAT sequence was not needed, as cells were permeabilized), but not with the control peptide, enhanced IP\(_3\)-induced Ca\(^{2+}\) release compared to vehicle-treated conditions (Figure 5B, C). The D5SD peptide was added 4 min prior to IP\(_3\) addition (DSSD addition is shown by the grey rectangle in Figure 5B). The unaltered steady decline in \(^{45}\text{Ca}^{2+}\) efflux after addition of DSSD indicated that, in the absence of IP\(_3\), the DSSD peptide by itself did not enhance basal Ca\(^{2+}\) release from the ER. These data therefore indicate that the PKM2:IP\(_3\)R interaction can be acutely disrupted using a decoy peptide, thereby alleviating the inhibition of IP\(_3\)R-mediated Ca\(^{2+}\) release by PKM2. Finally, addition of the TAT-DSSD peptide to HeLa wild-type cells incubated in medium without Ca\(^{2+}\) evoked transient cytosolic Ca\(^{2+}\) signals, consistent with the mobilisation of an intracellular Ca\(^{2+}\) store (Figure 5D). The cytosolic Ca\(^{2+}\) signals caused by application of the TAT-DSSD peptide required expression of IP\(_3\)Rs, since HeLa 3KO cells in which all three IP\(_3\)R isoforms were deleted using the CRISPR/Cas9 method did not respond to application of the TAT-DSSD peptide (Figure 5E). These results indicate that disruption of the PKM2:IP\(_3\)R interaction leads to increased Ca\(^{2+}\) signalling that critically depends on the presence of IP\(_3\)Rs.
Figure 5. Interaction between PKM2 and IP$_3$R in HeLa cells and consequences for IP$_3$-induced Ca$^{2+}$ release. (A) Co-immunoprecipitation experiments using HeLa lysates demonstrating the interaction of PKM2 with IP$_3$R-1 (left panel), IP$_3$R-2 (middle panel) and IP$_3$R-3 (right panel). A representative experiment is shown. (B) Potentiation of IP$_3$-induced Ca$^{2+}$ release after pre-incubation with D5SD, as measured with a unidirectional $^{45}$Ca$^{2+}$ flux assay. The ER Ca$^{2+}$ stores of saponin-permeabilized HeLa cells were loaded until steady-state with $^{45}$Ca$^{2+}$ and release was induced by incubation in a Ca$^{2+}$-free efflux medium in the presence of thapsigargin (4 μM). Samples were taken every 2 min. The grey area indicates the addition of peptide (D5SD or control peptide; 10 μM each) or of vehicle (DMSO) and the black bar the addition of IP$_3$ (3 μM). IP$_3$-induced $^{45}$Ca$^{2+}$ release is expressed as fractional loss, i.e. the amount of Ca$^{2+}$ released during 2 min relative to the total amount of Ca$^{2+}$ present at that moment in the stores. Averaged traces are shown for 5 independent experiments, each performed in triplicate. The data points indicate mean ± standard deviation. (C) Bar graph showing quantitation of the experiments shown in panel D. The columns indicate mean ± SEM for 5 independent experiments, each performed in triplicate. (D, E) Time-course of the cytosolic Ca$^{2+}$ responses evoked in a cell population of (D) wild type HeLa cells (HeLa WT)
and (E) HeLa cells in which all three IP3R isoforms were knocked out (HeLa 3KO). The Ca\(^{2+}\) signals were measured in the absence of extracellular Ca\(^{2+}\) in cells loaded with Fura-2 AM, using a FlexStation 3 microplate reader. Vehicle (DMSO), and 10 μM of TAT-Control or TAT-D5SD peptide were added as indicated by the black arrows. The curves represent the mean (bold lines) ± SEM (dashed lines) of 3 independent experiments, each performed 5- or 6-fold.

2.4. **PKM2 inhibits IP3R-mediated Ca\(^{2+}\) signalling in lymphocytes**

HeLa cells provide a tractable and generic cell model that is used by many labs to study various aspects of cell and cancer biology. To further analyse the functional effects of the effect of PKM2 on IP3R-mediated Ca\(^{2+}\) release in additional cancer-relevant models, we returned to lymphocytic cells (T cells and B cells). Specifically, we focused on Jurkat (acute T cell leukaemia) and OCI-LY-10 (diffuse large B-cell lymphoma). Application of the TAT-D5SD peptide to Jurkat T cells evoked cytosolic Ca\(^{2+}\) signals (Figure 6A), which were not observed with the control peptide (Figure 6B). Single cell analyses revealed that the cytosolic Ca\(^{2+}\) signals caused by TAT-D5SD were heterogeneous and had variable latencies (Figure 6C), but all cells were found to respond. The TAT-D5SD-induced Ca\(^{2+}\) signals were significantly suppressed by the phospholipase C inhibitor U73122 but not by its inactive enantiomer U73343 (Figure 6D, F). Similar results were obtained with OCI-LY-10 B cells (Supplementary Figure 3).
Figure 6. The cytosolic Ca$^{2+}$ signal triggered in Jurkat T cells by TAT-D5SD depends on IP$_3$ synthesis. (A) Averaged trace of cytosolic Ca$^{2+}$ elevation in Jurkat cells induced by 10 µM TAT-D5SD. The averaged of a representative experiment is shown (N=6, 64 cells/experiment). (B) Averaged trace of cytosolic Ca$^{2+}$ in Jurkat cells treated with 10 µM TAT-ctrl peptide. The average of a representative experiment is shown (N=3, 82 cells/experiment). (C-E) Representative single-cell recordings of cytosolic Ca$^{2+}$ in Jurkat cells following addition of 10 µM TAT-D5SD (arrow) in untreated cells (C), cells pre-treated for 30 min with 0.5 µM U73343 (D), or cells pre-treated for 30 min with 0.5 µM U73122 (E). (F) The maximum amplitude (mean ± SEM) of the TAT-D5SD-induced Ca$^{2+}$ signal obtained in the three conditions (C-E) shown in a bar graph (N=3, 51-84 cells/experiment).
Knocking down PKM2 expression in Jurkat cells using siRNA (Figure 7A), increased the amplitude of Ca^{2+} signals evoked by application of an anti-CD3 antibody (Figure 7B, C). Similar results were obtained with OCI-LY-10 B cells when stimulated with IgG/IgM (Supplementary Figure 4). Importantly, PKM2 knockdown did not alter the ER Ca^{2+} content, as the magnitudes (area under the curve and maximal amplitude) of thapsigargin-evoked Ca^{2+} responses were unaffected (Supplementary Figure 5).

Taken together, these findings indicate that PKM2 functions in various cell types, including Hela and T and B cells, as a negative regulator of IP_{3}R-mediated Ca^{2+} release. This effect is due to its direct action on the IP_{3}Rs as no effect on the ER Ca^{2+} leak or on the ER Ca^{2+} content was observed. Moreover, downregulation of PKM2, or acute displacement of PKM2 from IP_{3}R by the TAT-DSSD decoy peptide, relieves this inhibition of IP_{3}R, thereby potentiating cytosolic Ca^{2+} signals.

**Figure 7. Enhancement of IP_{3}R-mediated Ca^{2+} signals after PKM2 knockdown in Jurkat T cells.** (A) Representative Western blot showing siRNA-mediated knockdown of PKM2 in Jurkat cells. (B) Representative single-cell Ca^{2+} traces showing that PKM2 knockdown enhances Ca^{2+} signals induced by 4 μg/mL of anti-CD3. (C) Quantification of the maximal amplitude of the Ca^{2+} signals induced by anti-CD3. Results are expressed as mean ± SEM of the Ca^{2+} amplitude observed in 410 (siPKM2-treated) or 423 (siCtrl-treated) cells.
3. Discussion

IP$_3$Rs are central players in intracellular Ca$^{2+}$ signalling. In addition to generating Ca$^{2+}$ signals essential for many important cellular processes, IP$_3$Rs also function as molecular scaffolds that bind a diverse array of accessory proteins and protein complexes [1, 19]. Many of the accessory proteins associated with IP$_3$Rs have been demonstrated to alter the magnitude and/or spatiotemporal properties of intracellular Ca$^{2+}$ signals in line with their physiological function. Some proteins alter Ca$^{2+}$ signalling by a direct allosteric modulation of IP$_3$R opening. For example, the anti-apoptotic protein Bcl-2 attenuates Ca$^{2+}$ signals to support cell survival (recently reviewed in [29, 44]), whereas Lck promotes IP$_3$R-mediated Ca$^{2+}$ signals to enhance activation of T cells [36]. Yet other accessory proteins, such as kinases and phosphatases, regulate IP$_3$R-mediated Ca$^{2+}$ signals via covalent modifications [21]. The activation of IP$_3$Rs is therefore not simply a function of IP$_3$ and Ca$^{2+}$ binding. Rather, the activity of IP$_3$Rs represents the summative effects of various modifiers including accessory proteins, and factors such as ions, nucleotides, and redox status [1].

The different modes of regulation of IP$_3$R by accessory proteins provides the means to alter Ca$^{2+}$ signals depending on cell type and physiological status. Significantly, there is a growing number of examples where the modulation of IP$_3$R-mediated Ca$^{2+}$ signals by accessory proteins is hijacked by cancer cells to provide a survival or metastatic advantage [13, 45-51]. The present work suggests that PKM2 may similarly provide an advantageous modulation of IP$_3$R-mediated Ca$^{2+}$ signals in cancer. PKM2 is highly expressed in cancer cells, where in most cases its expression correlates with poor prognosis [52]. Kaplan-Meier analyses of cancer patient-derived gene expression data sets emphasises the point that a high level of PKM2 expression correlates with significantly poorer patient prognosis for some cancer types. In the cases of lung, gastric, and liver cancers, the median survival times are approximately twice as long for low PKM2 expression versus high PKM2 expression (50 versus 99 months for lung cancer; 21 versus 8.5 months for gastric cancer; 71 versus 31 months for liver cancer) (Supplementary Figure 6). The interaction of PKM2 in cancer cells with several other proteins, including epidermal growth factor receptor, protein kinase B, death-associated protein kinase, the transcription factor STAT3, CD44, β-catenin, focal adhesion kinase and heat shock proteins, was previously described [53]. The data presented in this study demonstrate a novel, non-canonical role of PKM2 as an IP$_3$R accessory protein.
Although it is widely accepted that Ca\textsuperscript{2+} signalling can affect cellular metabolism [54-61], there is much less evidence for the reverse action, i.e., metabolic enzymes controlling Ca\textsuperscript{2+} signalling. The only example until now was glyceraldehyde 3-phosphate dehydrogenase that, via local NADH production, stimulates IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release [62]. The present study indicates that another glycolytic enzyme, PKM2, affects the function of the IP\textsubscript{3}R by direct interaction. These data point to complex interactions between metabolic enzymes and IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} signalling, which moreover, can be especially relevant for cancer cells.

The PKM2:IP\textsubscript{3}R interaction shown in this study was initially revealed by mass spectroscopy using a short peptide from domain 5 of IP\textsubscript{3}R-1 as bait (Supplementary Figure 1) and was further substantiated in pull-down assays performed in various cell types, including B and T cells (Figures 3, 4 and 5). Moreover, our data indicate that both PKM2 and IP\textsubscript{3}R are localised to the ER, with overlapping distributions (Figure 2). To acutely disrupt PKM2:IP\textsubscript{3}R interaction, the same 21-amino acid sequence of the IP\textsubscript{3}R that was initially used as bait to detect PKM2 interaction (Figure 1) was employed as a decoy peptide. This peptide, D5SD, could be used in permeabilized cells (Figure 5B, C), but to use it in intact cells a TAT sequence was added to make it cell permeant. The use of the (TAT-)D5SD peptide and its comparison with a (TAT-) Control peptide allowed the analysis of the effects of PKM2:IP\textsubscript{3}R interaction on Ca\textsuperscript{2+} signalling. We investigated this in various cell types and with several techniques (single-cell and cell population analysis of Ca\textsuperscript{2+} signals in intact cells, effect on IP\textsubscript{3}-induced Ca\textsuperscript{2+} release in permeabilized cells). All data point in the same direction; an increased Ca\textsuperscript{2+} signalling propensity was obtained after disruption of the PKM2:IP\textsubscript{3}R interaction while the control peptide was completely ineffective (Figures 4-6 and Supplementary Figure 3). The enhanced Ca\textsuperscript{2+} release was dependent on IP\textsubscript{3} (Figure 5B, 6D-F, Supplementary Figure 3) and on IP\textsubscript{3}R expression (Figure 5D, E). As the peptides did not affect the enzymatic activity of PKM2 (Supplementary Figure 2) these results indicate that PKM2, via interaction with IP\textsubscript{3}Rs, dampens agonist- and IP\textsubscript{3}-mediated Ca\textsuperscript{2+} signals, and that IP\textsubscript{3}R activity increases when the interaction is disrupted. siRNA-mediated knockdown of PKM2 had a similar effect on IP\textsubscript{3}R activity as disruption of the PKM2:IP\textsubscript{3}R interaction with the DSSD peptide (Fig. 7, Supplementary Figures 4-5). Our results complement a previous
study performed using breast cancer cell lines in which PKM2 was demonstrated to downregulate IP$_3$R expression, leading to decreased basal Ca$^{2+}$ levels in the mitochondria [63]. The decreased mitochondrial Ca$^{2+}$ levels consequently affected oxidative phosphorylation and shifted cellular metabolism towards aerobic glycolysis. Additionally, immunoprecipitation experiments also showed that PKM2 interacted with IP$_3$Rs, although the functional consequences of the interaction were not investigated [63].

We previously demonstrated that the T cell-associated tyrosine kinase Lck also bound to the D5SD sequence of the IP$_3$R [36]. However, it is important to note that we observe TAT-D5SD-induced Ca$^{2+}$ signals in cells that do not express Lck, such as HeLa cells (Figure 5D). Moreover, Lck has a stimulatory effect on IP$_3$R-mediated Ca$^{2+}$ signals, and its displacement would have the opposite effect to that seen in this study. Future work will examine whether PKM2 and Lck have discrete or overlapping binding sites within the D5SD domain, and whether there is any interaction between the proteins. Whereas Lck is expressed in all T-cell and only in some B-cell lymphocytes, PKM2 is broadly expressed (Figure 1D), though at especially high levels in embryonic tissues and cancer cells [52, 64]. We speculate that in many normally growing adult, non-leukocytic cells, neither PKM2 nor Lck occupy the DSD5 binding site. Within T lymphocytes, Lck may predominate at the DSD5 site (causing increased Ca$^{2+}$ signals), whereas within cancer cells, the highly expressed PKM2 may dominate (provoking reduced Ca$^{2+}$ signals).

Our mass spectrometry analysis identified several proteins that bound to the 21-amino acid D5SD bait sequence (Figure 1). The verification of the interaction of other proteins with IP$_3$Rs, and any physiological consequences of the interaction, will require further work. For the present study, we focused on PKM2 because of the enormous interest in PKM2’s role in cancer (reviewed in [52, 64-68]). Furthermore, the role of glycolysis in regulation of Ca$^{2+}$ signalling and cancer is increasingly recognized. For example, glycolysis fuels survival of pancreatic cancer cells by generating the ATP essential for the plasma-membrane Ca$^{2+}$-ATPase that maintains a pro-survival low cytosolic Ca$^{2+}$ concentration [69]. Inhibition of PKM2 in pancreatic cancer cells with the compound shikonin halted glycolysis, and lead to ATP depletion and subsequent toxic Ca$^{2+}$ overload [70]. The decision to pursue the role of
PKM2 as IP₃R accessory protein in this study was therefore based on established links between Ca²⁺ signalling, metabolism, and cancer cell survival.

In conclusion, we propose that, in addition to downregulation of IP₃R expression [63], PKM2, expressed in a variety of cancer cell types, suppresses IP₃R-mediated Ca²⁺ signals via direct allosteric regulation of the IP₃R. It is plausible that by reducing IP₃R-mediated Ca²⁺ signals, PKM2 enables cancer cells to rewire their cellular metabolism and generate proliferation and/or survival advantages leading to tumorigenesis. Our data add to burgeoning evidence of altered Ca²⁺ homeostasis and signalling, including IP₃R mutations and abnormal Ca²⁺ signalling checkpoints, in a variety of disease states including cancer [13, 16, 71-74].
4. Materials and Methods

4.1. Reagents and Antibodies

Fura-2 AM was purchased from Life Technologies. U73122 (Cat. #BML-ST391-0005) and U73343 (Cat. #BML-ST392-0005) were from Enzo Life Sciences. Purified human 6xHis-PKM2 was purchased from Biovision (Cat. #6372-100).

The following antibodies were used in this study: PKM2 (Cell Signaling, Cat. #4053); PKM1 (ProteinTech, Cat. #15821-1-AP); IP3R-1 (Rbt03, [75]); IP3R-2 (Novus, Cat. #NB100-246); IP3R-3 (BD Pharmingen, Cat. #610213); Actin (Sigma, Cat. #A-5441); anti-human IgG/IgM (Jackson ImmunoResearch, Cat. #109-006-127); anti-human CD3 (Thermo Scientific, Cat. #14-0037-82), anti-rabbit Alexa 488 (ThermoFisher, Cat # A32731) and Alexa 568-conjugated antibody (ThermoFisher, Cat #A20184), heavy and light chain anti-mouse (Invitrogen Cat. #31430) and light chain-specific anti-rabbit (Jackson ImmunoResearch, Cat. #211-032-171) HRP-conjugated secondary antibody.

The D5SD peptide and control peptide were synthesized by Genscript of Lifetein, purified by liquid chromatography/mass spectrometry to more than 95% purity, and quantified by amino acid analysis. The D5SD sequence is KKRMDLVLELKNNASKLLLAI. The control peptide sequence is NLNHSQFAENLSHICGGHG. The TAT cell-penetrating peptide sequence (RKKRRQRRRGG) or biotin were added to the N-terminus of each peptide, where indicated under Results. TAT-D5SD and TAT-Control peptide were dissolved in water, D5SD and control peptide in DMSO and Biotin-D5SD and Biotin-Control peptide were dissolved in 20% DMSO.

4.2. Cell culture

WEHI7.2, HEK-293, HEK-293T, and HeLa cells were cultured in DMEM containing 10% FCS, 100 μM non-essential amino acids, and 2 mM L-glutamine. HeLa cells deficient in all three IP3R isoforms (HeLa 3KO) were cultured as previously stated [43]. Jurkat and Raji cells were cultured in RPMI-1640 medium with 10% FBS, 100 μM non-essential amino acids, and 2 mM L-glutamine. OCI-LY-10 cells were cultured in IMDM with 20% FBS, 2 mM glutamine and 50 μM 2-mercaptoethanol. All cell lines were incubated in a humidified incubator at 37°C in 5% CO2 except for WEHI7.2 cells, which were incubated in 7% CO2. All cell lines except for WEHI7.2 (Univ. California San Francisco) were purchased from the American Type Culture
Collection. Cell lines were routinely tested for mycoplasma. All procedures followed the guidelines and regulations in accordance with internal review board protocol ICC2902/11-02-28 of Case Western Reserve University Cancer Center/University Hospitals of Cleveland Ireland Cancer Center. Spleen cells and brain tissue were isolated from normal mice with approval of the Case Western Reserve University Institutional Animal Care and Use Committee (number 2014-0074).

4.3. **Sample Preparation Prior to Mass Spectrometry**
A total of 20 μL of either Biotin-D5SD pull down or Biotin-Control pull down WEHI7.2 eluate were loaded onto a one-dimensional SDS-PAGE gel (4-20% Tris-HCl). The gel was stained using Bio-Rad Biosafe Coomassie (Bio-Rad Hercules, CA), thus comparing proteins pulled down by biotin-D5SD with proteins pulled down by Biotin-Control peptide. Protein mixtures pulled down by each biotin-tagged peptide were transferred to a 1.5 mL Eppendorf tube for proteolytic digestion. Briefly, 30 μL of 50 mM ammonium bicarbonate was added to each sample and incubated at room temperature. Following incubation, the supernatant was removed, and a series of additional washes was performed using a solution of 50% acetonitrile in 25 mM ammonium bicarbonate. Samples were then taken to complete dryness using a Savant Speedvac Concentrator (Thermo Scientific) and reduced with 30 μL of 10 mM dithiothreitol and alkylated with 30 μL of 55 mM iodoacetamide. The supernatant was removed, and the gel was taken to complete dryness. Proteolytic digestion was performed with 100 ng of bovine trypsin (Promega, Madison, WI) overnight at 37°C.

4.4. **Liquid Chromatography and Mass Spectrometry**
Samples prepared as described above were analyzed using a Waters NanoAcquity Ultra-high-pressure liquid chromatography system (Waters, Milford, MA) and an LTQ-OrbitrapVelos mass spectrometer (Thermo, Waltham, MA). The instrument was mass calibrated immediately before the analysis using the instrument protocol. Raw data were processed and subsequently searched by Mascot version 2.2.0 (Matrix Science London, UK). The mouse International Protein Index (56,957 sequences) database was used. Search settings were as follows: trypsin enzyme specificity; mass accuracy window for precursor ion, 10 ppm; mass accuracy window for fragment ions, 0.8 Daltons; modifications included a fixed modification of carbamidomethylation of cysteines and variable modification oxidation of methionine. The criteria for peptide identification were a mass accuracy of ≤10 ppm and
an expectation value of \( p \leq 0.05 \). Proteins that had 2 or more peptides matching the above criteria were considered confirmed assignments while proteins identified with one peptide regardless of the Mascot score were considered tentative assignments.

### 4.5. **SDS-PAGE and Western blot analysis**

Cell lysates containing 20–40 \( \mu \)g of protein were separated on NuPAGE 4–12% Bis/Tris SDS-polyacrylamide gels using MES/SDS-running buffer (Invitrogen) and transferred onto Immobilon-P PVDF membranes (Millipore). The membranes were blocked with TBS containing 0.1% Tween and 5% non-fat dry milk powder, and incubated overnight with the primary antibody. Next, membranes were incubated for 1 h with a secondary horseradish peroxidase (HRP)-conjugated antibody (dilution 1:2000 in 0.1% Tween/TBS). Protein detection was performed with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Bands quantification was performed using Image lab 5.2 software.

### 4.6. **Cell transfection**

Five million WEHI7.2 or OCI-LY-10 cells were transfected utilizing the Amaxa® Cell Line Nucleofector® Kit L (Lonza, Basel, Switzerland), program C-05 with 500 nM siCTRL (ON-TARGET plus, Non-targeting Control pool, from Dharmacon), and 500 nM siPKM2 (hs.Ri.PKM.13.1, from IDT). At 48 h post-transfection, the cells were used for experiments and collected for Western blot analysis to confirm knockdown. For \( \text{Ca}^{2+} \) measurements, the cells were co-transfected with 1 \( \mu \)g of GCaMP5G plasmid [76]. pCMV-GCaMP5G was a gift from Douglas Kim & Loren Looger & GENIE Project (Addgene plasmid #31788; http://n2t.net/addgene:31788; RRID:Addgene_31788).

For immunofluorescence experiments, cells were transfected with either DsRed2-ER (Clontech; 2 \( \mu \)g for HeLa cells) or YFP-IP\(_3\)-R-1 (pcDNA3.1-EYFP-IP\(_3\)-R-1, provided by Llewellyn Roderick, Univ. Cambridge, UK; 4 \( \mu \)g for HEK-293 cells) using Lipofectamine-2000 (Life Technologies) according to the manufacturer’s recommendations.

### 4.7. **Immunofluorescence**

Plasmid expression was confirmed by epifluorescence microscopy 24 hr after transfection. Transfected cells with DsRed2-ER or YFP-IP\(_3\)-R1 constructs were fixed for 30 min in a fresh solution of 3.7% formaldehyde in PBS, pH 7.4. Samples were washed with PBS and blocked
for 30 min at room temperature in blocking solution (0.5% saponin and 5% goat serum in PBS). Samples were subsequently incubated for 1 hr at room temperature with either the PKM2 antibody or rabbit IgG isotype control. Samples were washed three times, then incubated with Alexa-conjugated secondary antibody for 30 min in blocking solution at room temperature. DRAQ5™ (5 μM) was added for staining of the nuclei. After three wash steps with PBS, images were acquired using an UltraView VoX spinning-disk confocal system (PerkinElmer) mounted on a DMI6000B microscope (Leica Microsystems) equipped with an HCX PL APO 100x/1.4 oil immersion objective. With Volocity software, confocal images of YFP-IP₃R-1, DsRed2-ER and DRAQ5™ were collected using solid-state diode 488-nm, 561-nm and 640-nm lasers, respectively, and with the appropriate emission filters. In addition, proper imaging controls were used to ensure that fluorescence of YFP-IP₃R-1 and fluorescence of DsRed2-ER were spectrally distinct.

**4.8. Biotin-Streptavidin Pull Down**

Biotin-streptavidin pull downs were performed as described previously using the Pierce Pull-Down Biotinylated Protein:Protein Interaction Kit (ThermoFisher) [35]. Briefly, Biotin-D5SD peptide or Biotin-Control peptide (200 μM in 400 μL of solution containing 0.8% DMSO) were immobilized on streptavidin-coated beads by incubation with 50 μL suspension of beads for 4 hours at 4°C with gentle rotation. The beads were incubated with a biotin-containing buffer provided by the pull-down kit to block streptavidin molecules not bound to peptides, and beads were washed three times with Tris-Buffered Saline (TBS; 25 mM Tris-HCl, 0.15 M NaCl, pH 7). WEHI7.2 cells were washed twice with ice-cold PBS and incubated for 30 minutes on ice in CHAPS lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% CHAPS, 50 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, protease inhibitor cocktail (Roche), and phosSTOP (Roche). Cell lysates were centrifuged for 15 minutes at 13,000 rpm at 4°C. Lysates (2.8 mg total protein) were incubated for 18 hours at 4°C with gentle rotation with Biotin-D5SD or Biotin-Control peptides immobilized to streptavidin-coated beads or with streptavidin-coated beads alone. The beads were washed four times with TBS and incubated for 5 minutes with 50 μL elution buffer provided by the kit prior to centrifugation at 1,250 rpm for 60 seconds. Eluate samples were analyzed by mass spectrometry and western blotting. The biotin-streptavidin pull down with purified 6xHis-PKM2 was performed as above, using purified human 6xHis-PKM2 (10 μg) instead of cell lysate.
4.9. **GST Pull-Down Experiments**

GST pull-down experiments were performed as described previously [10]. The pGEX6p2 expression vectors containing the IP$_3$R domain fragment 5 fused with GST at its N terminus and the GST control were expressed and purified as previously described [77]. GST-IP$_3$R domain 5 (10 µg) in 300 µL PBS was incubated with 50 µL of a 50% slurry of Glutathione Sepharose 4B beads (GE Healthcare) in PBS for 2.5 hours at 4°C with gentle rotation to allow for formation of domain fragment-bead complexes. WEHI7.2 cells were washed two times with ice-cold PBS, incubated for 30 minutes on ice in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na$_3$PO$_4$, and protease inhibitor cocktail (Roche)), and centrifuged for 15 minutes at 14,000 rpm at 4°C. Lysate samples (250 µL containing 300 µg total protein) were pre-cleared with 50 µL of a 50% slurry of Glutathione Sepharose 4B beads in PBS for 1 hours at 4°C with gentle rotation. The lysate was separated from the pre-clearing beads by centrifugation, and NaCl and BSA were added to the pre-cleared lysate samples to final concentrations of 300 mM NaCl and 1% BSA to reduce non-specific binding during the binding step. The supernatant was removed from the GST-IP$_3$R domain fragment-bead complexes, and the pre-cleared lysate samples were incubated with the domain fragment-bead complexes for 1 hour at 4°C with gentle rotation. The beads were washed seven times with 500 µL lysis buffer, boiled for 5 minutes in 2X SDS sample buffer, and prepared for western blotting.

4.10. **Co-immunoprecipitation experiments**

Co-immunoprecipitation experiments were as described previously [9, 10]. Briefly, cells were washed twice with ice-cold PBS, incubated for 30 minutes in ice-cold CHAPS lysis buffer (composition described above), and centrifuged for 15 minutes at 14,000 rpm at 4°C. Lysate (1.1 mg in 1 mL volume per sample) was pre-cleared with 100 µL of a slurry of 50% Protein G agarose beads (Millipore) in PBS for 3 hours at 4 °C with gentle rotation. The beads were pelleted and removed, and the pre-cleared lysate was incubated for 18 hours with the indicated antibodies (2.16 µg antibody per sample) at 4°C with gentle rotation. 50 µL of a slurry of 50% Protein G agarose beads in PBS was added to samples and incubated for 2 hours at 4°C with gentle rotation. The beads were washed two times with ice-cold PBS, then five times with ice-cold CHAPS lysis buffer. The beads were boiled for 5 minutes in 2X SDS sample buffer and prepared for western blotting. Co-immunoprecipitation experiments
in the presence of peptides were performed as above with the following modifications: TAT-D5SD or TAT-Control peptide was added to total cell lysate to a final concentration of 200 μM in 1 mL total volume for 1 hour at 4°C with gentle rotation prior to addition of antibodies as described above.

4.11. Cytosolic single-cell Ca²⁺ measurements

Methods of single-cell digital imaging with Fura-2 AM were described previously in detail and used here with only minor modifications [9, 10]. Briefly, cells were plated on poly-L-lysine coated 35 mm glass-bottom dishes (MatTek Corporation) to yield a final concentration of 8 x 10⁵ cells/mL in culture medium. Cells were incubated with 1 μM Fura-2 AM for 45 minutes at room temperature. The medium was then replaced with extracellular buffer (ECB) consisting of Hanks’ Balanced Salt Solution supplied by Life Technologies (138 mM NaCl, 0.34 mM Na₂HPO₄, 5.33 mM KCl, 0.44 mM KH₂PO₄, 1.26 mM CaCl₂, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 5.56 mM D-glucose) supplemented with 20 mM Hepes, pH 7.4-7.45. Incubation was continued for 30 minutes at room temperature to allow for Fura-2 AM de-esterification. Ca²⁺ imaging was performed and analyzed using an Ion-Lite Dual Emission Fluorescence Imaging System (Intracellular Imaging), using a 20× fluor objective (UPlanAPO; Olympus) [9, 10]. Reagents, including peptides and thapsigargin, were diluted into ECB and gently added to MatTek plates so as not to disturb cells while continuously measuring cytosolic Ca²⁺. Ca²⁺ concentrations were calculated as previously described, based on Fura-2 emission at 510 nm with alternating excitation at 340 and 380 nm and using a Kᵩ of 220 nM for Fura-2 [9, 10].

For single-cell Ca²⁺ measurements in cells transfected with GCaMP5G, cells were washed with modified Krebs solution (150 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM HEPES (pH 7.3), 11.5 mM glucose and 1.5 mM CaCl₂), and seeded in a 35 mm glass bottom dish with 4 chambers (Cellvis) at 500,000 cell/well in modified Krebs solution without Ca²⁺ (150 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM HEPES (pH 7.3), 11.5 mM glucose and 3 mM EGTA). A Zeiss Axiovert 100M LSM 510 confocal microscope equipped with a 20×/0.75 air objective (Nikon) were used for these measurements. Changes in fluorescence were monitored with an Argon 488 nm laser equipped with a LP 505 filter. The baseline was measured before adding the compound and the data were plotted as F/F₀. The area under
the curve and the amplitude were calculated using GraphPad Prism 9.1.0 software. At least three independent experiments were performed.

4.12. **Cytosolic Ca\(^{2+}\) measurements in cell populations**

HeLa WT, HeLa 3KO or OCI-LY-10 cells were seeded in poly-L-lysine-coated 96-well plates (Greiner) at 250,000 cell/well. Cells were incubated in modified Krebs solution (150 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 11.6 mM HEPES pH 7.3, 11.5 mM glucose and 1.5 mM CaCl\(_2\)) with 1.25 µM Fura-2 AM for 30 min, followed by a 30 min de-esterification step in the absence of Fura-2 AM. The buffer was exchanged by the modified Krebs solution without Ca\(^{2+}\) (150 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 11.6 mM HEPES pH 7.3, 11.5 mM glucose and 3 mM EGTA) previously to fluorescence monitoring in a FlexStation 3 microplate reader (Molecular Devices). The Ca\(^{2+}\) signal was quantified by alternately exciting the Ca\(^{2+}\) indicator at 340 and 380 nm and collecting emitted fluorescence at 510 nm. The data were plotted as F\(_{340}\)/F\(_{380}\) and the baseline was measured before adding the compound. The area under the curve was calculated using GraphPad Prism 9.1.0 software. At least three independent experiments were performed.

4.13. **Unidirectional \(^{45}\)Ca\(^{2+}\)-Flux Assay**

\(^{45}\)Ca\(^{2+}\) fluxes were performed on confluent monolayers of HeLa cells, obtained 7 days after plating the cells at 60,000 cells/well in 12-well clusters [42, 78]. In short, after 10 minutes permeabilization of the cells with saponin (20 µg/mL), the non-mitochondrial Ca\(^{2+}\) stores were loaded for 45 minutes at 30 °C with 150 nM free \(^{45}\)Ca\(^{2+}\) with a final specific activity of 0.3 MBq/mL in the presence of 10 mM NaN\(_3\). After washing the cells with efflux medium (120 mM KCl, 30 mM imidazole-HCl pH 6.8, 1 mM EGTA) containing 4 µM thapsigargin, \(^{45}\)Ca\(^{2+}\) efflux was followed for 18 minutes by adding and replacing the efflux medium every 2 minutes. After 10 minutes, 3 µM IP\(_3\) (Cayman Chemical company, Cat. #60960) was added. DSSD and control peptides were added from 4 min before the addition of IP\(_3\) to 2 minutes after the addition of IP\(_3\). At the end of the experiment, the \(^{45}\)Ca\(^{2+}\) remaining in the stores was released by adding 2% SDS for 30 minutes. Ca\(^{2+}\) release is plotted as fractional loss, which is obtained by measuring the amount of Ca\(^{2+}\) released in 2 minutes divided by the total Ca\(^{2+}\) store content at that time. The IP\(_3\)-sensitive Ca\(^{2+}\) release was quantified as the difference in fractional loss after and before the addition of IP\(_3\) and normalized to the IP\(_3\)-induced Ca\(^{2+}\) release in cells treated with vehicle.
4.14. **Pyruvate Kinase Activity**

Pyruvate kinase enzyme activity was measured by a standard lactate dehydrogenase (LDH)-coupled assay [79, 80] whereby the absorbance change due to oxidation of NADH to NAD+ was measured at room temperature at 340 nm at 12 second intervals with a UV/Vis spectrophotometer (DU 730; Beckman Coulter). For the experiments with purified 6xHis-PKM2, the reaction buffer contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 1.5 mM ADP (Sigma), 5 mM phosphoenolpyruvate (PEP; Roche), 200 μM NADH (Roche), 10 μM fructose 1,6-bisphosphate (Sigma), and 8 U LDH (Roche). The reaction was initiated by addition of 125 ng 6xHis-PKM2 to the reaction buffer. Absorbance was measured for 5 minutes prior to treatment addition to determine the basal rate of enzyme activity. Then, peptide (or vehicle) was added to the indicated final concentration and absorbance was measured for an additional 20 minutes. For measurement of enzyme activity with cell lysate as PKM2 source, 10⁶ WEHI7.2 or Raji cells were washed three times in ice-cold PBS and incubated for 10 minutes in lysis buffer: Tris-HCl, (pH 7.5), 1.5 mM MgCl₂, 20 mM NaCl, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Roche). Cells were lysed on ice by Dounce homogenization (B pestle). Each lysate was centrifuged twice for 30 minutes at 14,000 rpm at 4°C. Basal PKM2 activity was measured by adding 2 μg lysate to 50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1.5 mM ADP, 0.5 mM PEP and 200 μM NADH. Treatment with peptides for 45 minutes to 24 hours is as indicated in the figure legends.

4.15. **Experimental Reproducibility, Statistical Analysis, and Kaplan-Meier Plots**

Data are presented as mean ± SD or SEM as appropriate. For quantitative data, a minimum of three repeats was performed in all experiments. The number of cells analyzed per individual experiment in Ca²⁺ measurements using Fura-2 AM is indicated in the legends. A Student’s t-test or an Analysis of variance (ANOVA) followed by Tukey’s post-test were used to determine significant differences between groups. A two-tailed p value of 0.05 was the minimal threshold for significance. For the Kaplan—Meier plots, we analyzed data from the world’s largest collection of patients’ transcriptomic data for gastric, lung and liver cancer (Kaplan-Meier Plotter, accessible on: http://kmplot.com/analysis/) (Menyhart O, Nagy A, Gyorffy B. Determining consistent prognostic biomarkers of overall survival and vascular invasion in hepatocellular carcinoma. R Soc Open Sci., 2018 Dec 5;5(122):181006. doi: 10.1098/rsos.181006). We queried the database for the effect of
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Supplementary Figure 1. Domain 5 Subdomain (D5SD) peptide and IP$_3$R structure. (A) Linear representation of IP$_3$R-1, showing functional regions, the stable domains numbered 1-6, and the 21-amino acid sequence referred to as D5SD. (B) D5SD sequence is highly conserved among the IP$_3$R-1 from various species. Species-specific amino acid sequence numbering is indicated. (C) The three known IP$_3$R isoforms share a high degree of sequence similarity at the level of D5SD.
Supplementary Figure 2. TAT-D5SD peptide does not directly inhibit PKM2's glycolytic activity. (A) PKM2 kinase activity using purified human 6xHis-tagged PKM2. PKM2 activity was measured for 5 min, whereupon TAT-D5SD or TAT-control peptide (20 μM each) was added to the reaction buffer. PKM2 enzyme activity was subsequently measured for an additional 20 min. Enzyme activity is expressed in percent, relative to baseline value. Results are mean ± SEM of three experiments. Pyruvate kinase activity measured in cell lysates from WEHI7.2 T cells (B) or Raji B cells (C). After determining baseline enzyme activity, the lysates were incubated for up to 90 min either in the absence of peptide or in the presence of 20 μM or 200 μM TAT-D5SD or TAT-control peptide. Enzyme activity is expressed in percent, relative to baseline value. (D) Pyruvate kinase activity in WEHI7.2 or Raji cell lysates after 24 h incubation in the absence of peptides or in the presence of TAT-D5SD or TAT-control peptide (20 μM each). Enzyme activity is expressed in percent, relative to the activity in the untreated samples. Results are presented as mean ± SEM of 2 experiments.
Supplementary Figure 3. The cytosolic Ca^{2+} signal triggered in OCI-LY-10 B cells by TAT-D5SD depends on IP_{3} synthesis. (A, B) Cell population analysis of the cytosolic Ca^{2+} concentration in OCI-LY-10 cells following addition of 10 µM TAT-Control (blue curves) or TAT-D5SD (red curves) in cells pre-treated for 30 min with 2.5 µM of the inactive enantiomer U73343 (A) or of the phospholipase C inhibitor U73122 (B). The Ca^{2+} signals were measured in the absence of extracellular Ca^{2+} using a FlexStation 3 microplate reader in cells loaded with Fura-2 AM. TAT-Control or TAT-D5SD were added as indicated by the arrow. Cytosolic Ca^{2+} was normalized to baseline fluorescence. The curves are presented as the mean (bold line) ± SEM (dashed line) of 3 independent experiments, each performed in 3- or 4-fold. (B) Quantification of the area under the curve is presented as mean ± SEM. Significance was analyzed by the ANOVA, Tukey’s post-test (*p<0.05; ****p<0.0001).
Supplementary Figure 4. Enhancement of the IP₃R-mediated Ca²⁺ signals after PKM2 knockdown in OCI-LY-10 cells. (A) Western blot showing siRNA-mediated knockdown of PKM2 in OCI-LY-10 cells (4 independent samples). Actin was used as the loading control. (B) Cytosolic Ca²⁺ traces in OCI-LY-10 cells co-transfected with pCMV-GCaMP5G plasmid and siCtrl or siPKM2. After 48 h, single-cells Ca²⁺ signals were measured by fluorescence microscopy in the absence of extracellular Ca²⁺. As indicated by the arrow, 12 μg/mL anti-human IgG/IgM was added after 60 s of baseline measurement. Cytosolic Ca²⁺ was normalized to baseline fluorescence. The curves are presented as the mean (bold line) ± SEM (dashed line) of 3 independent experiments, each performed on 15-20 cells. (C)
Quantification of the area under the curve of the Ca\textsuperscript{2+} signals induced by IgG/IgM, shown as mean ± SEM. Significance was analyzed by Student T-test (**p< 0.01).

Supplementary Figure 5. PKM2 knockdown does not affect ER Ca\textsuperscript{2+} content. (A) Cytosolic Ca\textsuperscript{2+} traces in OCI-LY-10 cells co-transfected with pCMV-GCaMP5G plasmid and siCtrl or siPKM2. After 48 h, single-cells Ca\textsuperscript{2+} signals were measured by fluorescence microscopy in the absence of extracellular Ca\textsuperscript{2+}. As indicated by the arrow, 10 μM thapsigargin was added after 60 s of baseline measurement in order to induce full release of the ER Ca\textsuperscript{2+}. Cytosolic Ca\textsuperscript{2+} was normalized to baseline fluorescence. The curves are presented as the mean (bold
line) ± SEM (dashed line) of 3 independent experiments, each performed on 15-20 cells. Quantification of (B) the area under the curve and (C) the maximum amplitude of the Ca^{2+} signals are presented as means ± SEM.
Supplementary Figure 6. Association of PKM2 with overall survival. Plots (A), (B), and (C) illustrate Kaplan-Meier analysis for overall survival of (A) lung, (B) gastric, and (C) liver.
cancer patients respective to PKM2 expression levels. The P values derived from a log-rank test and hazard ratios indicate significantly altered survival outcomes among the high PKM2-expressing subjects.
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