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Inhibition of glycolysis and Src/Akt signaling reduces Caveolin-1-enhanced metastasis

Layla Simón [a,b,c,*,1], Keila Torres [d,e], Pamela Contreras [b,c], Natalia Díaz-Valdivia [b,c], Lisette Leyton [b,c], Andrew F.G. Quest [b,c,**]

[a] Nutrition and Dietetic School, Universidad Finis Terrae, Santiago, Chile
[b] Cellular Communication Laboratory, Center for Studies on Exercise, Metabolism and Cancer (CEMC), Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Santiago, Chile
[c] Advanced Center for Chronic Diseases (ACCDiS), Faculty of Medicine, University of Chile, Santiago, Chile
[d] Advanced Center for Chronic Diseases (ACCDiS), Pontificia Universidad Católica de Chile, Santiago, Chile
[e] Department of Hematology-Oncology, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

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ABSTRACT

Metastasis is the leading cause of cancer-related deaths, making the development of novel, more effective therapies imperative to alleviate patient suffering. Metabolic switching is a hallmark of cancer cells that facilitates metastasis. Cancer cells obtain most of their energy and intermediate metabolites, which are required to proliferate and metastasize, through aerobic glycolysis. Previous work from our laboratory has shown that Caveolin-1 (CAV1) expression in cancer cells promotes glycolysis and metastasis. Here, we sought to determine if limiting glycolysis reduced CAV1-enhanced metastasis and to identify the mechanism(s) involved. We evaluated the effects of the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) in metastatic melanoma and breast cancer cell lines expressing or not CAV1. Non-cytotoxic concentrations of 2-DG (1 mM) inhibited the migration of B16-F10 melanoma and MDA-MB-231 breast cancer cells. CAV1-mediated activation of Src/Akt signaling was required for CAV1-enhanced migration and was blocked in the presence of 2-DG. Moreover, inhibition of Akt reduced CAV1-enhanced lung metastasis of B16-F10 cells. Collectively, these findings highlight the importance of CAV1-induced metabolic reprogramming for metastasis and point towards possible therapeutic approaches to prevent metastatic disease by inhibiting glycolysis and Src/Akt signaling.

1. Introduction

Metastasis is responsible for about 90 % of cancer deaths, and as such is the leading cause of deaths due to this disease [1,2]. Melanoma is an example of a highly metastatic cancer for which the 5-year overall survival decreases from 98 % to 15 % of the patients depending on whether melanoma is detected in early (within the primary site) or later (metastatic melanoma) stages, respectively [3]. Also, metastatic breast cancer has a poor prognosis and only 26 % of women with metastasis survive more than 5 years [4]. Cancer cells acquire several traits that facilitate the development of primary and metastatic tumors [5,6]. Particularly the metabolic switch in cancer cells is a hallmark that facilitates metastasis [6–9]. For that reason, Choi and collaborators suggest viewing cancer as a metabolic disorder and propose targeting metabolism as an alternative, more efficient cancer treatment [10].

Caveolin-1 (CAV1) is a 22-kDa membrane protein that adopts a hairpin-like structure with an intramembrane domain, and both N and C termini facing the cellular cytoplasm. The protein contains a tyrosine 14 (Y14) residue, which can be phosphorylated [11]. CAV1 interactions with many signaling molecules are regulated by Src-family

Abbreviations: 2-DG, 2-Deoxy-D-Glucose; Akti, Akt inhibitor; PT308Akt, Akt phosphorylation on Threonine 308; CAV1, Caveolin-1; CSD, Caveolin Scaffolding Domain; DST, Dasatinib; LY, LY294002; NR, Not reached; CAV1/Y14E, phosphomimetic CAV1; PCAV1, phosphorylated CAV1; Y14, tyrosine 14; CAV1, Wild-Type CAV1; Wort, Wortmannin.

* Correspondence to: Av. Pedro de Valdivia 1509, 7501015, Providencia, Santiago, Chile.
** Correspondence to: Av. Independencia 1027, 8380453; Independencia, Santiago, Chile.
E-mail addresses: lsimon@uft.cl (L. Simón), aquest@u.uchile.cl (A.F.G. Quest).
[1] Twitter handle @lsimonujam

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kinase-mediated Y14 phosphorylation in response to several stimuli, and phosphorylated CAV1 (pCAV1) is known to interact with numerous downstream targets [12,13]. Previous work from our laboratory has shown that CAV1 promotes migration, invasion and metastasis of cancer cells through its Y14 phosphorylated residue [14–16] and does so by activation of a Rab5-Rac1 signaling pathway [17].

Given the relevance of metabolism in promoting metastasis, mechanisms controlling glycolysis are of considerable interest. Indeed, growth factors regulate the metabolism of cancer cells via activation of the PI3K/Akt/mTOR pathway which stabilizes HIF-1α and increases the expression of HIF-1-target genes, such as glucose transporters, glycolytic enzymes (hexokinase, phosphofructokinase, and lactate dehydrogenase A) [18]. On the other hand, inhibition of PI3K signaling reduces glucose uptake and tumor progression [8]. Recent results from our group demonstrated that CAV1 overexpression, and specifically its phosphorylation on tyrosine 14, induces glycolysis and metastasis [19]. CAV1 increases glucose consumption and lactate production by binding to glycolytic enzymes (phosphofructokinase and aldolase) or by modulating the expression of hexokinase (reviewed in [20]) and the glucose transporter 3 (GLUT3 [21,22]).

Furthermore, the expression of CAV1 and Akt are positively correlated in tumors [23] and CAV1 increases migration through the formation of lamellipodia in an Akt-dependent manner [24–26]. In addition, CAV1 activates Rac1 promoting migration [17], and there is evidence for the existence of a PI3K/PDK1/Akt/Rac1 signaling pathway [19,27,28].

Interestingly, Src kinase is maintained in an active state by pCAV1 [29], which promotes Akt activation. For instance, Src-mediated PDK1 (phosphoinositide-dependent protein kinase-1) and Akt tyrosine phosphorylation increase Akt activation [30–32]. On the other hand, treatment of CAV1 overexpressing cells with Src inhibitors, such as PP2, reduces their metastatic potential [15]. Based on the available evidence, we hypothesized that this could potentially relate not only to CAV1 dephosphorylation but also to Akt inactivation.

Therefore, we sought here to determine whether glycolytic restriction reduces CAV1-enhanced migration by inhibiting Akt signaling. To that end, we used 2-DG as a glycolysis inhibitor at non-cytotoxic concentrations and analyzed its effects on Akt activation and migration of cells overexpressing CAV1. We provide evidence that indeed inhibition of glycolysis by 2-DG suffices to reduce CAV1-enhanced migration and metastasis and show for the first time that signaling downstream of CAV1 via an Akt-dependent pathway is essential for metastasis.

2. Material and methods

2.1. Cell culture

Metastatic murine melanoma cells B16-F10 (ATCC, #CRL6475, provided by Laurence Zitvogel, Institut Gustav Roussy, Villejuif, France), and metastatic human breast cancer MDA-MB-231 (ATCC HTB-26) cells were cultured in RPMI 1640 medium. All media were supplemented with 10 % FBS and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Cells were maintained at 37°C and 5 % CO_2. B16-F10 cells were transfected with the plasmid pLacIOP alone (Mock) or with inserts permitting overexpression of either wild type CAV1 (CAV1) or phosphomimetic CAV1 (CAV1/Y14E) [16] following induction with 1 mM IPTG for 48 h. On the other hand, CAV1 was silenced in MDA-MB-231 cells by transduction with a short hairpin construct specific for CAV1 (shCAV1). Cell transduced with a short hairpin specific for luciferase (shC) were used in this case as controls [14,33,34].

2.2. Drug treatments

Glycolytic restriction: The effects were evaluated in B16-F10 melanoma and MDA-MB-231 breast cancer cells after treatment for 24 h with 1 mM 2-deoxy-D-glucose (2-DG, #D8375, Sigma-Aldrich, St. Louis, MO, USA).

PI3K/PDK1/Akt inhibition: The effects on Akt phosphorylation (activation) were evaluated in cells following treatments with either 100 nM wortmannin (Wort, #W1628, Sigma-Aldrich, St. Louis, MO, USA) or 10 μM LY294002 (LY, #440202, Sigma-Aldrich, St. Louis, MO, USA) for 1 h, 1 μM Akt inhibitor VIII (Akti, Calbiochem #124018) or 4 μM BX-912 (PDK1 inhibitor, Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Src inhibition: 100 nM Dasatinib (DST, #S1021, Selleckchem, UK) was used to treat B16-F10 cells for 2 h [15].

2.3. Analysis of cell viability

The effects of 2-DG on viability were determined using the MTS assay. Cells (3×10^3 cells/well) were plated in 96 well plates. B16-F10 cells were induced with IPTG for 48 h. After an initial 24 h period in culture, cells were treated with increasing concentrations of 2-DG (0–8 mM) for 24 h. Viability was measured according to the manufacturer's instructions (Promega, Madison, WI). The IC50 values were obtained using the GraphPad Prism software (San Diego, CA). Additionally, the number of dead cells was also determined by Trypan blue staining.

2.4. L-Lactate measurement

L-Lactate in the medium was determined with the Eton Bioscience kit (#1200011002, San Diego, CA). Cell culture supernatants were collected and stored at -80°C. L-Lactate was measured using the supernatants diluted 4 times and comparing with the standard curve according to the manufacturer's recommendations.

2.5. Transwell migration assay

Migration was assayed in Boyden Chambers (Transwell Costar, 6.5 mm diameter, 8 μm pore size, Corning, NY, USA) according to the manufacturer's instructions. Briefly, the lower surfaces of the inserts were coated with fibronectin (2 μg/ml). B16-F10 cells (2×10^5) induced with IPTG for 48 h, or MDA-MB-231 cells (1×10^5) were treated for 24 h with 2-DG (1 mM), BX-912 (4 μM) or the Akti (1 μM), and 1 h with Wort (100 nM) or LY (10 μM). Then, cells were re-suspended in serum-free medium, plated in the upper chambers of the inserts and allowed to migrate towards the lower chamber containing complete media for 2 h. Finally, inserts were removed, fixed/stained with 0.1 % crystal violet in 20 % methanol overnight and trans-migrated cells were counted using an inverted microscope.

2.6. Metastasis assays

C57BL/6 mice between 8 and 12 weeks of age were sub-divided into different groups, according to the experiments performed. These groups received an intravenous injection into the tail vein of 2×10^5 B16-F10 cells, pretreated with 1 mM IPTG for 48 h, and Akti for the last 24 h. On day 21 post injection, mice were euthanized, the lungs were removed and fixed in Feketes solution (70 % ethanol, 10 % formalin and 5 % glacial acetic acid) [33,35]. Once fixed, the lungs were weighed. Then, the tumor nodules were separated from the lung parenchyma and weighed. Metastasis is expressed as black tissue mass/total lung mass (%), as previously described [33]. The sample size was determined using the following equation: Sample Size = $2 * [(Za+Zb) * SD/(Xa-Xb)]^2$ [36]. Here, the standard deviation (SD) is 11 % and the difference between means (Xa-Xb) is 25 % (based on previous results from our group) [34,37,38]. Considering a statistical power of 95 % (Zb=1,65) and a level of significance of α=0,05 (Za=1,64), the minimal number of animals per group is N=4. However, there is an approximately 20 % loss of animals in this assay, which increases the N required to 5 per group. This study was performed according to the rules and standards established by

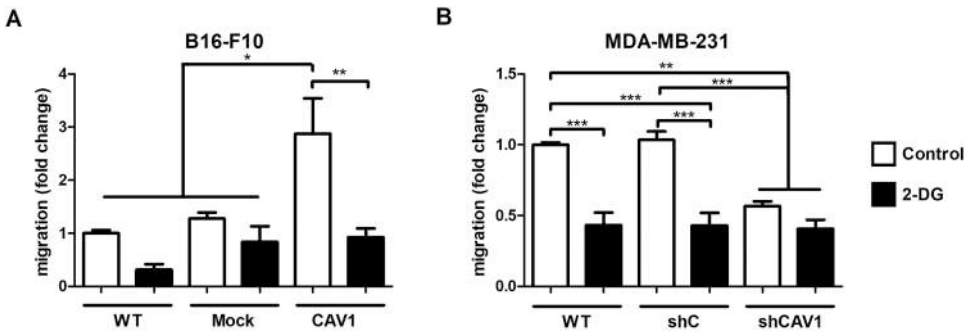

Fig. 1. Inhibition of glycolysis with 2-DG reduced CAV1-enhanced migration of melanoma and breast cancer cells. Values obtained for cells without (white bars) or with treatments (black bars) are shown. A: B16-F10 melanoma cells were induced with IPTG for 48 h, treated with 1 mM 2-DG for 24 h, seeded (2×10^5 cells) in serum-free medium in the upper chamber and allowed to migrate towards the bottom chamber filled with complete medium for 2 h. WT: wild type cells. Mock: cells transfected with empty plasmid. CAV1: cells overexpressing wild type CAV1. Statistically significant differences between groups are indicated (* $p < 0.05$; ** $p < 0.01$; n = 3). B: MDA-MB-231 breast cancer cells were treated with 1 mM 2-DG for 24 h and seeded (1×10^5 cells) in transwell plates for migration assays (2 h). WT: wild type cells. shC: short hairpin control cells. shCAV1: short hairpin CAV1 knock-down cells. Statistically significant differences between groups are indicated (** $p < 0.01$, *** $p < 0.001$; n = 3).

the Bioethics Committee for Animal Research in the Faculty of Medicine, University of Chile (CBA 1169 FMUCH, year of approval: 2021).

2.7. Western blot analysis

Cells were washed and harvested in ice-cold PBS containing 1 mM orthovanadate, 10 μg/ml benzamidine, 2 μg/ml antipain, 1 μg/ml leupeptin, and 1 mM phenylmethyl-sulphonylfluoride (OVA-BAL-PMSF). Cells were centrifuged at 3000 x *g* for 5 min at 4°C and the respective cell pellets were lysed by sonication in extraction buffer (20 mM HEPES pH 7.4, 0.1 % NP-40, and 0.1 % SDS plus OVA-BAL-PMSF). Extracts were centrifuged at 12000 x *g* for 2 min at 4°C and protein concentrations in supernatants were determined using the BCA protein assay kit. Protein samples were separated by SDS-PAGE (50 μg/lane), transferred to nitrocellulose, blocked for 1 h in blocking buffer (1 % Tween-20 containing 5 % bovine serum albumin) and probed overnight at 4°C with anti-CAV1 (1:5000, #610060, BD, San Jose, CA, USA), anti-pAkt (1:500, #4060 and #4056, CST, Danvers, USA), anti-Akt (1:500, #9272, CST, Danvers, USA), anti-pSrc (1:1000, #2101, CST, Danvers, USA) antibodies diluted in blocking buffer. Anti-β-actin (1:3000, A5060, Sigma-Aldrich, St. Louis, MO, USA) or anti-HSP90 (1:3000, sc-515081, Santa Cruz Biotechnology, Dallas, Texas, USA) antibodies were used to confirm equal protein loading in the different wells. Bound antibodies were detected using secondary anti-rabbit or anti-mouse antibodies coupled to HRP and the ECL system. Protein bands were quantified by densitometric analysis using the ImageJ software.

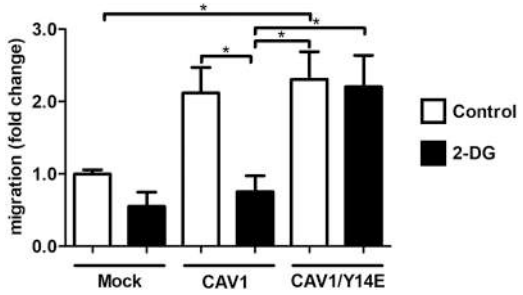

Fig. 2. The glycolysis inhibitor 2-DG failed to prevent phosphomimetic CAV1/Y14E-enhanced migration. Migration after treatment with 1 mM 2-DG for 24 h. B16-F10(Mock), (CAV1) or (CAV1/Y14E) cells were induced with IPTG for 48 h, treated with 2-DG (1 mM) for 24 h, seeded (2×10^5 cells) in serum-free medium in the upper chamber and allowed to migrate towards the lower chamber with complete medium for 2 h. Values shown were averaged from three different experiments (mean ± standard deviation). Statistically significant differences between groups are indicated (* $p < 0.05$; n = 3). White bars: control cells without inhibitor; Black bars: cells with inhibitor.

2.8. Rac1-GTP pull-down assay

Rac1 activity was measured using the p21 binding domain (GST-PBD) in pull-down assays as previously described [14,17]. Briefly, cells were lysed in a buffer containing 25 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 % NP40, 10 % glycerol, 1 mM dithiothreitol and protease inhibitors. Extracts were incubated for 5 min on ice and clarified by centrifugation for 2 min at 10000 × *g*, 4°C. Supernatants were used for pull-down assay with 30 μg of GST-PBD pre-coated GSH beads per condition. Beads were incubated with the supernatants for 15 min at 4°C on a rotating shaker. Thereafter, beads were collected, washed with lysis buffer containing 0.01 % NP-40 and samples were solubilized in sample buffer, boiled and separated by SDS PAGE for analysis by Western blotting.

2.9. Statistical analysis

Results were analyzed in GraphPad Prism (San Diego, CA) using ANOVA followed by a Tukey post-test. All results were averaged from three or more independent experiments and are shown as the mean ± SEM. A value for $p < 0.05$ was considered statistically significant. Data analysis using different post-tests is indicated in the respective figure legends.

3. Results

In our previous studies, we provided evidence showing that glycolysis inhibition with 1 mM 2-DG prevented CAV1-enhanced migration and metastasis of B16-F10 mouse melanoma cells without affecting cell viability. In order to extend the relevance of such findings to breast cancer cells and to shed light on a new signaling pathway connecting CAV1 phosphorylation on tyrosine-14 to the activation of Akt in both melanoma and breast cancer cells (MDA-MB-231), it was essential first to confirm that 2-DG (1 mM) did not affect viability of either MDA-MB-231 or B16-F10 cells (see Supplementary Figures 1, 2 and text), as well as to confirm that 2-DG at that concentration also inhibited glycolysis (lactate release) in both cell lines (see Supplementary 3 and text).

3.1. Inhibition of glycolysis decreases CAV1-induced cell migration

Previous results obtained by our group showed that 2-DG treatment reduces CAV1-enhanced metastasis of B16-F10 cells [19]. Bearing this in mind, we compared here the effect of 2-DG on the migration of B16-F10

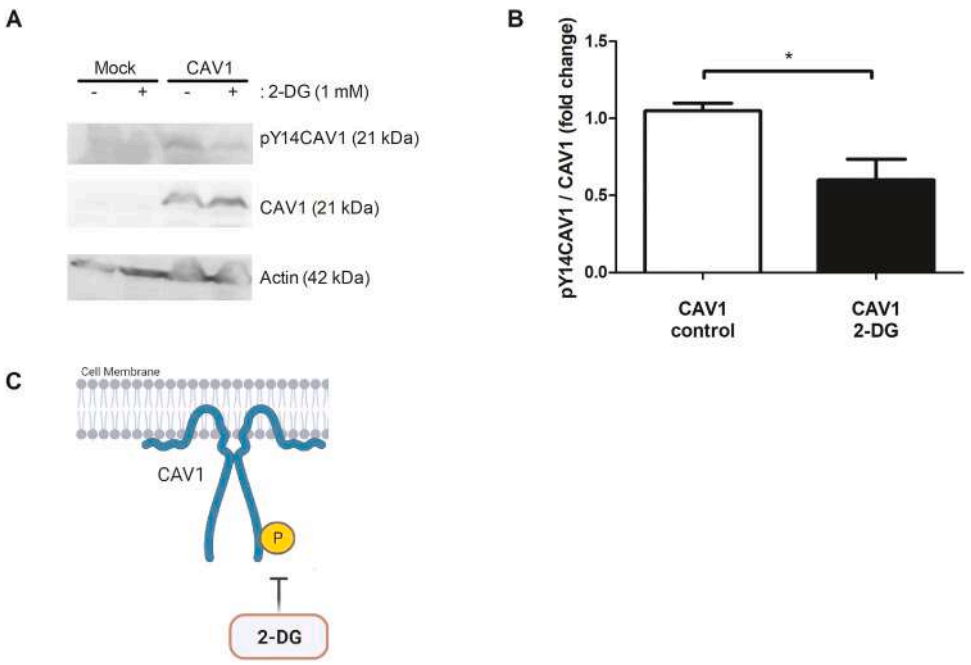

Fig. 3. Glycolysis inhibition with 2-DG reduced the phosphorylation of CAV1 on tyrosine-14. A: Western blot analysis of pY14CAV1 and CAV1 in extracts from B16-F10(Mock) and (CAV1) cells treated with 1 mM 2-DG for 24 h. Actin was used as a loading control. B: CAV1 phosphorylation on Y14 normalized to total CAV1 (* $p < 0.05$; n = 3; t-test). C: Section from summary model in Fig. 9 showing that 2-DG prevents CAV1 phosphorylation.

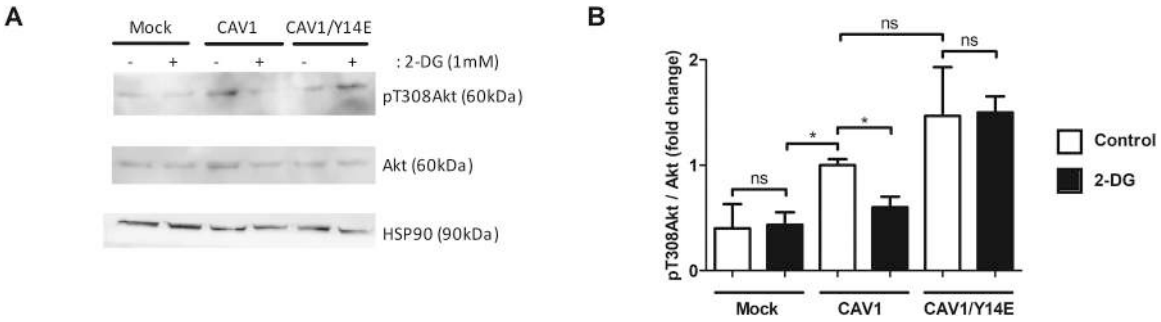

Fig. 4. Inhibition of glycolysis reduced CAV1-induced Akt phosphorylation. A: Western blot analysis of pT308Akt levels in extracts from B16-F10(Mock), (CAV1) and (CAV1/Y14E) cells treated (+) or not (-) with 2-DG (1 mM). HSP90 was included as a loading control. B: Akt phosphorylation on T308 normalized to total Akt (* $p < 0.05$; ns: non-statistically significant differences; n = 3; t-test).

and MDA-MB-231 cells. As anticipated, the expression of CAV1 increased migration, while the treatment with 2-DG reduced the migration of B16-F10(CAV1) cells to levels similar to those observed for B16F10(WT) or (Mock) cells (Fig. 1A). Moreover, 2-DG decreased migration of MDA-MB-231(WT) and (shC) cells to levels like those observed for (shCAV1) cells (Fig. 1B). These results demonstrate that the glycolysis inhibitor 2-DG effectively quenches the ability of CAV1 to promote migration of melanoma and breast cancer cells.

3.2. Inhibition of glycolysis failed to prevent CAV1/Y14E-enhanced migration

Previous studies by our group have shown that phosphorylation of CAV1 on tyrosine-14 induces metabolic reprogramming and thereby promotes migration of cancer cells [19]. To determine whether 2-DG-inhibited events upstream or downstream of CAV1 phosphorylation that are required for migration, B16-F10(Mock), (CAV1) and (CAV1/Y14E) cells were treated with 2-DG (1 mM). As anticipated, 2-DG blocked CAV1-enhanced migration but was unable to reduce the migration of cells overexpressing phosphomimetic CAV1/Y14E (Fig. 2). In addition, treatment with 2-DG reduced CAV1 phosphorylation in B16-F10(CAV1) cells (Fig. 3). The observations that 2-DG blocked CAV1 phosphorylation and that phosphomimetic CAV1/Y14E bypassed the effects of 2-DG on CAV1-enhanced migration, suggested that this inhibitor blocks events both upstream and downstream of CAV1 phosphorylation.

3.3. Inhibition of glycolysis impaired the Src/Akt signaling axis

CAV1-enhanced metastasis of cancer cells requires phosphorylation on tyrosine-14 by Src-family kinases [15] and can be bypassed by expressing CAV1/Y14E. Interestingly, Src is known to phosphorylate and activate PDK1 thereby promoting Akt phosphorylation on Threonine 308 (pT308Akt) [30]. Thus, we analyzed the effect of 2-DG on pT308Akt in B16-F10 cells expressing CAV1 or phosphomimetic CAV1/Y14E. In both cases, we detected an increment in pT308Akt (Fig. 4). Importantly, however, treatment with 2-DG (1 mM) reduced pT308Akt/Akt levels in CAV1, but not CAV1/Y14E cells (Fig. 4). These observations suggest that Akt phosphorylation on T308 is enhanced by CAV1 presence and specifically phosphorylation on tyrosine-14. This explains why CAV1/Y14E-expressing cells were insensitive to 2-DG treatment and migrated at similar rates regardless of the presence or absence of 2-DG (see Fig. 2). Thus, 2-DG inhibits an event that occurs

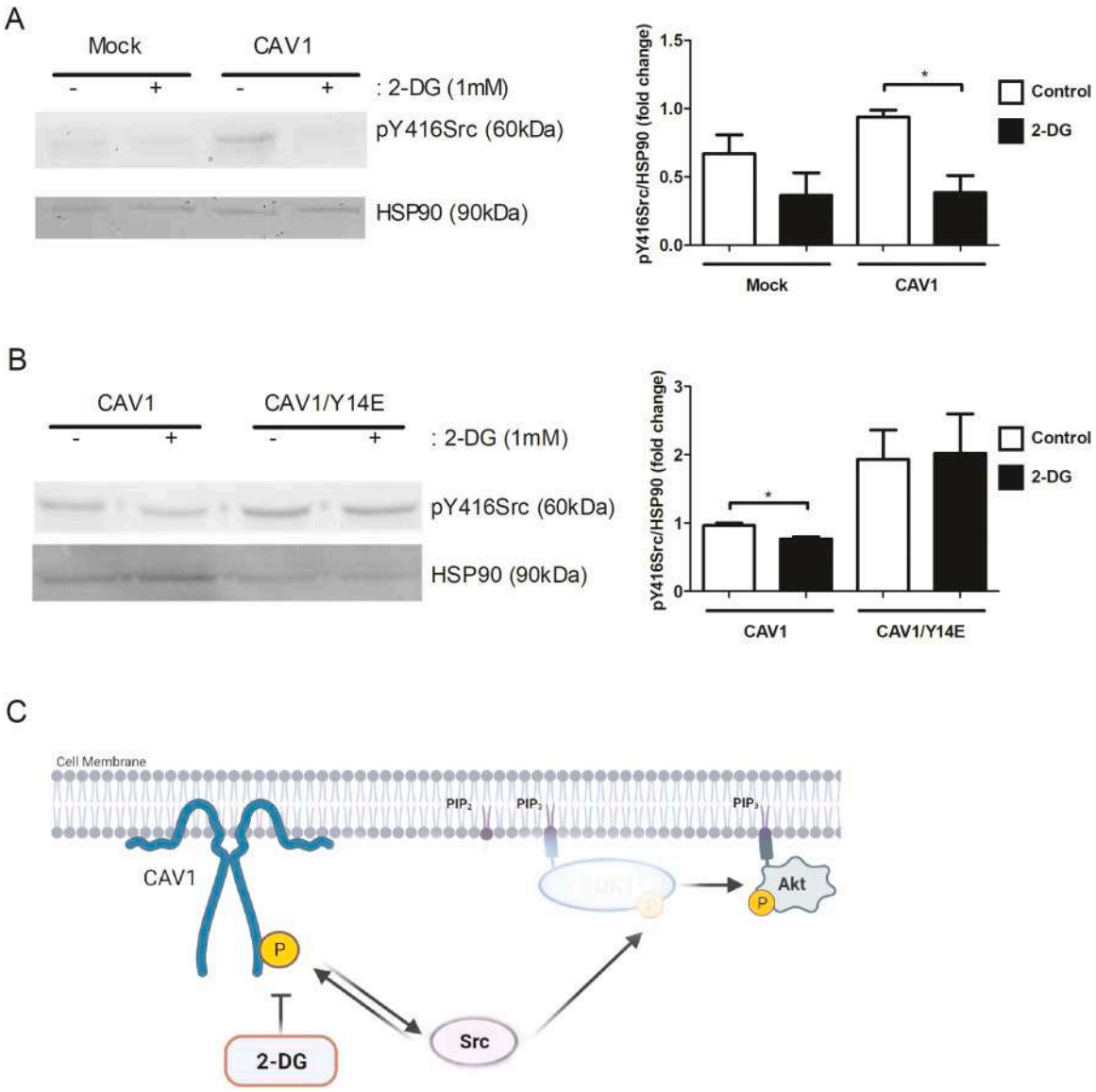

Fig. 5. Inhibition of glycolysis prevented CAV1-induced Src activation. A: Western blot analysis of pY416Src levels in extracts from B16-F10 Mock and CAV1 cells treated or not with 2-DG (1 mM). Values shown were obtained by scanning densitometry and averaged from three independent experiments. Statistically significant differences following treatment with 2-DG are indicated (* $p < 0.05$; n=4). B: Western blot analysis of pY416Src levels in B16-F10 (CAV1) and (CAV1/Y14E) cells treated or not with 2-DG (1 mM). Statistically significant differences following treatment with 2-DG are indicated (* $p < 0.05$; n=3; t-test). C: Section from summary model in Fig. 9 showing that 2-DG prevents CAV1/Src/Akt activation.

upstream of Akt phosphorylation.

We then analyzed the effect of 2-DG on Src activation loop phosphorylation on amino acid Y416. In cells overexpressing CAV1, levels of pY416Src (activated Src) increased and treatment with 2-DG reduced Src phosphorylation at this site (Fig. 5A). On the other hand, the presence of phosphomimetic CAV1/Y14E increased Src phosphorylation to a slightly greater extent. The treatment with 2-DG maintained similar pY416Src levels to those detected with wild type CAV1 in the absence of the inhibitor (Fig. 5B), which explains why CAV1/Y14E-expressing cells were insensitive to 2-DG treatment and migrated in the presence or absence of 2-DG (see Fig. 2). Taken together the data suggest that CAV1 phosphorylation is required for Src and Akt activation loop phosphorylation. Importantly, glycolytic restriction using 2-DG impaired this sequence of events (Fig. 5C).

3.4. Src/PDK1 inhibition reduced CAV1-enhanced migration

Previous studies from the laboratory have demonstrated that Dasatinib (DST), a Src kinase inhibitor, reduces CAV1-enhanced migration and metastasis [15]. Thus, we evaluated the effect of DST on the Src/Akt pathway. We observed reduced pT308Akt levels following DST treatment in cells expressing CAV1. In the case of phosphomimetic CAV1/Y14E cells, DST treatment slightly reduced pT308Akt levels but differences were not significant (Fig. 6A).

PDK1-dependent phosphorylation of Akt on the activation loop residue T308 is essential for Akt activation. Alternatively, PDK1 is reportedly activated by Src-dependent phosphorylation [31]. Since CAV1 expression increased Src activation (Fig. 5A), we suspected that PDK1 may also be activated, and that inhibiting PDK1 could reduce CAV1-enhanced migration. Indeed, treatment of B16-F10 and MDA-MB-231 cells with the PDK1 inhibitor BX-912 reduced CAV1-enhanced migration (Fig. 6B and C). Viability after BX-912 treatment was maintained as evidenced by Trypan blue staining (Supplementary table 1). This result suggests that CAV1 increases migration by promoting the activation of a Src/PDK1/Akt signaling pathway, which can be inhibited either by glycolytic restriction or by blocking any

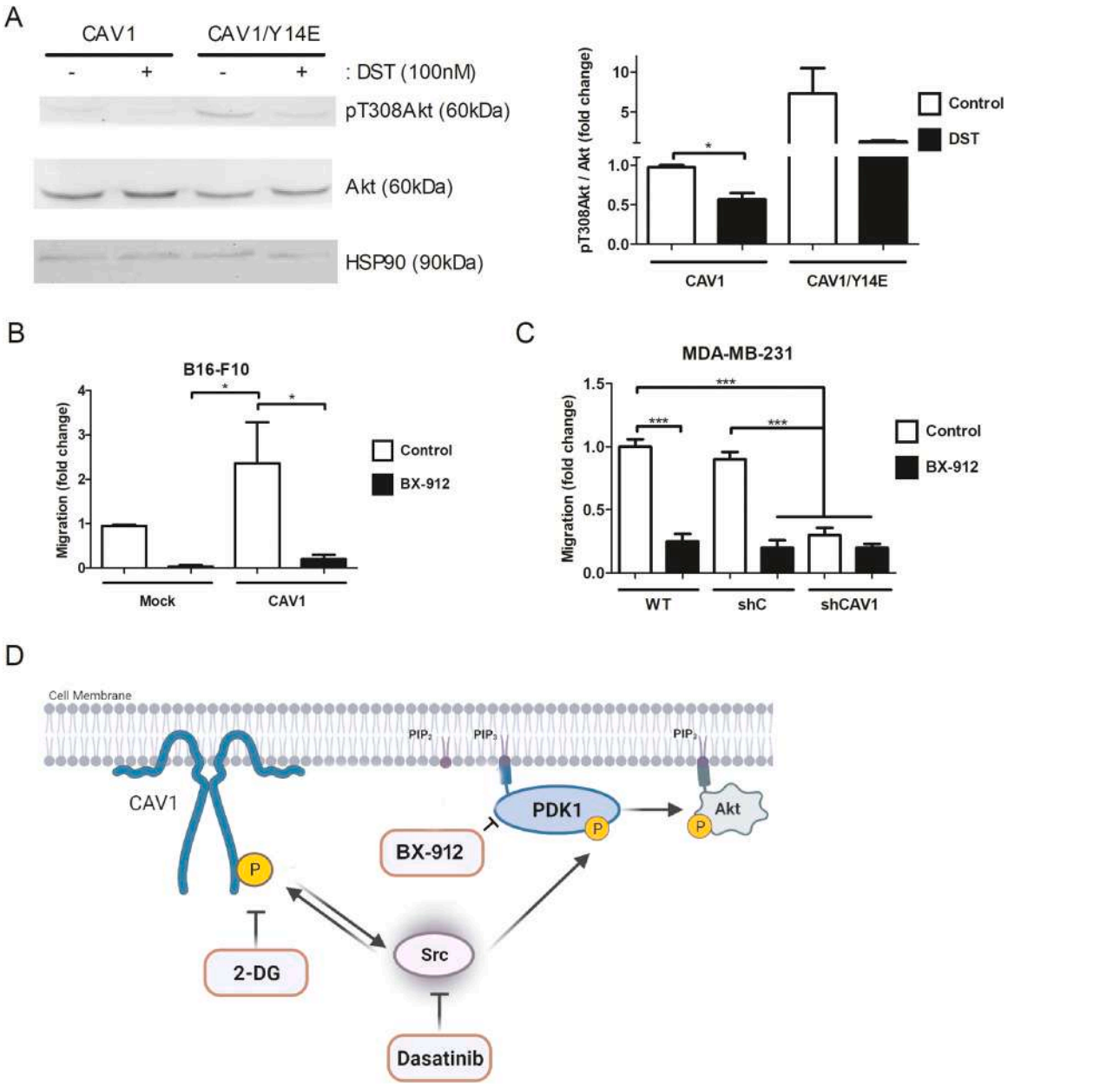

Fig. 6. CAV1-enhanced migration was prevented by Src and PDK1 inhibition. A: Effects of the Src-family kinase inhibitor Dasatinib on Akt T308 phosphorylation. Western blot analysis of pT308Akt levels in extracts from B16-F10(CAV1) and (CAV1/Y14E) cells treated or not with Dasatinib (DST, 100 nM) for 1 h. Statistically significant differences following DST treatment are indicated (* $p < 0.05$; n=4; t-test). B: Effects of the PDK1 inhibitor BX-912 (4 μM) on B16-F10 migration enhanced by CAV1 expression. Migration after treatment with BX-912 for 24 h. B16-F10(Mock) or (CAV1) cells were induced with IPTG for 48 h, treated with BX-912 for 24 h, seeded (2 x 10^5 cells) in serum-free medium in the upper chamber and allowed to migrate towards the lower chamber with complete medium for 2 h. C: Effects of BX-912 on MDA-MB-231 migration. MDA-MB-231(WT), (shC) and (shCAV1) cells were treated with BX-912 for 24 h, seeded (1 ×10^5 cells) in serum-free medium in the upper chamber and allowed to migrate towards the lower chamber with complete medium for 2 h. White bars: cells without inhibitor; Black bars: cells with inhibitor. Statistically significant differences between groups are indicated (* $p < 0.05$; *** $p < 0.001$; n = 3). D: Section from summary model in Fig. 9 showing how Src and PDK1 inhibition prevents CAV1 from connecting to PDK1/Akt precluding thereby migration.

one of the kinases involved (Fig. 6D).

3.5. Inhibition of PI3K reduced CAV1-induced migration

PI3K/Akt signaling is deregulated in cancer and associated with several processes that favor migration and invasion [39,40]. Therefore, to confirm the relevance of PI3K/Akt signaling in these processes, we analyzed the effects of the PI3K inhibitors wortmannin and LY294002. Wortmannin (100 nM) and LY294002 (10 μM) reduced CAV1-enhanced migration in B16-F10 and MDA-MB-231 cells to basal levels (Fig. 7A and 7B, respectively), mimicking thereby the effects previously described for 2-DG. These results show that PI3K/Akt signaling is required for CAV1-enhanced migration (Fig. 7C). Furthermore, they suggest that 2-DG might suppress CAV1-induced migration/invasion by inhibiting PI3K/Akt signaling. Of note, viability, as determined by Trypan blue staining (Supplementary Table 1), was not reduced by PI3K inhibitors at the concentrations indicated.

3.6. Akt inhibition reduced CAV1-enhanced migration in vitro and metastasis in vivo.

Previous studies by our group showed that either glycolysis or Src

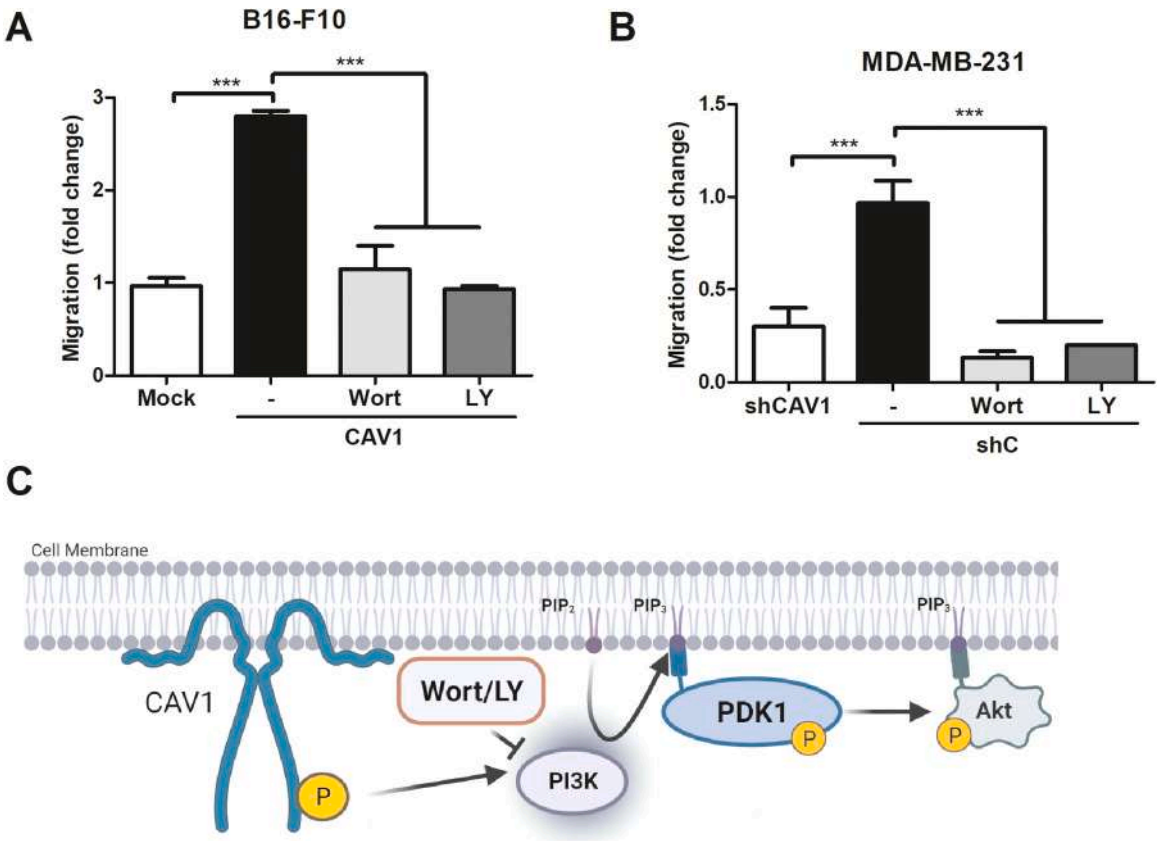

Fig. 7. Inhibition of PI3K reduced CAV1-enhanced migration. A: Migration of B16-F10 melanoma cells. Cells were induced with IPTG for 48 h, treated with Wort (100 nM), LY (10 μM), or DMSO, seeded (2×10^5 cells) in serum-free medium in the upper chamber and allowed to migrate towards the lower chamber with complete medium for 2 h. Mock (white bar), CAV1 (DMSO, black bar), CAV1 (Wort, light grey bar), CAV1 (LY, dark grey bar). B: Migration of MDA-MB-231(shCAV1) cells (white bar) or MDA-MB-231(shC) cells treated with DMSO (black bar), Wort (light grey bar), LY (dark grey bar), seeded (1×10^5 cells) in serum-free medium in the upper chamber and allowed to migrate towards the lower chamber with complete medium for 2 h. Values shown were averaged from three different experiments (mean ± standard deviation). Statistically significant differences between groups are indicated (*** $p < 0.001$; n = 3). C: Section from summary model in Fig. 9 showing how PI3K inhibition prevents CAV1 from connecting to PDK1/Akt precluding thereby migration.

family kinase inhibition reduce CAV1-induced metastasis of B16-F10 (CAV1) cells in C57BL6 mice [15,19]. Here, we analyzed the effect of a selective Akt inhibitor (Akti, 1 μM) on the CAV1-induced metastatic potential. Indeed, Akt inhibition reduced CAV1-induced migration (Fig. 8A and B). No effect of Akti at this concentration on cell viability was observed by Trypan Blue staining (Supplementary Table 1). To evaluate the role of Akt *in vivo* in this sequence, B16-F10 cells overexpressing or not CAV1 were pre-treated with Akti for 24 h. Then, the cells were collected and injected into the tail vein of C57BL/6 mice. After 21 days, the metastatic lung tumor mass was determined (Fig. 8C). These experiments revealed that pre-treatment with Akti for 24 h reduced the ability of CAV1-overexpressing B16-F10 cells to metastasize to the lung *in vivo* (Fig. 8C-E). These data confirm that Akt signaling is required for CAV1-enhanced lung metastasis of B16-F10 cells.

Rac1, a small GTPase implicated in actin cytoskeleton remodeling, lies downstream of Akt [27,28]. Moreover, CAV1 promotes Rac1 activation and migration in cancer cells [17]. With this in mind, we anticipated that CAV1 overexpression might activate Rac1 to modify the actin cytoskeleton in cells and that these effects could be prevented by blocking Akt. Thus, we evaluated whether a selective inhibitor of Akt (Akti) reduced Rac1 activation, cell migration and metastasis, and in doing so mimicked the effects of glycolytic restriction. Indeed, Rac1 activity was elevated in B16-F10(CAV1) cells compared to the B16-F10 (Mock) cells. Moreover, Akti reduced CAV1-enhanced Rac1 activation, confirming that indeed Rac1 is activated in CAV1-expressing cells as an effector downstream of Akt (Fig. 8F and G).

4. Discussion

CAV1 overexpression regulates metabolism and increases the migration of cancer cells. Here, we demonstrate for the first time that glycolytic inhibition reduces CAV1-enhanced migration via a Src family kinase/PDK1/Akt/Rac1-dependent pathway. Thus, our findings highlight the importance of CAV1-dependent metabolic reprogramming in promoting cancer cell metastasis and point towards possible therapies to prevent metastatic disease by modulating metabolism, CAV1 expression and the downstream signaling pathway.

Since inhibition of glycolysis reduces CAV1-enhanced migration and metastasis [19], we sought to uncover the signaling pathway involved. Using non-cytotoxic concentrations of the glycolysis inhibitor 2-DG, we demonstrate here that glycolysis is necessary for Akt activation and CAV1-induced migration. Moreover, inhibition of PI3K/Akt signaling mimicked the effect of glycolytic restriction, thereby reducing migration *in vitro* and metastasis *in vivo*. Thus, the PI3K/Akt signaling pathway is required for CAV1 to develop its metastatic potential. Furthermore, CAV1 overexpression was associated with Akt phosphorylation on Threonine 308. As glycolytic restriction could prevent this phosphorylation in cells overexpressing wildtype CAV1 but failed to do so in cells overexpressing phosphomimetic CAV1/Y14E, we suggest that glycolytic restriction inhibits mechanisms related to CAV1 phosphorylation and downstream signaling pathways involving Akt.

The effects of CAV1 expression in cancer cells have been studied for decades. In prostate cancer, caveolae-associated G-proteins have been implicated in androgen receptor and PI3K signaling activation [41].

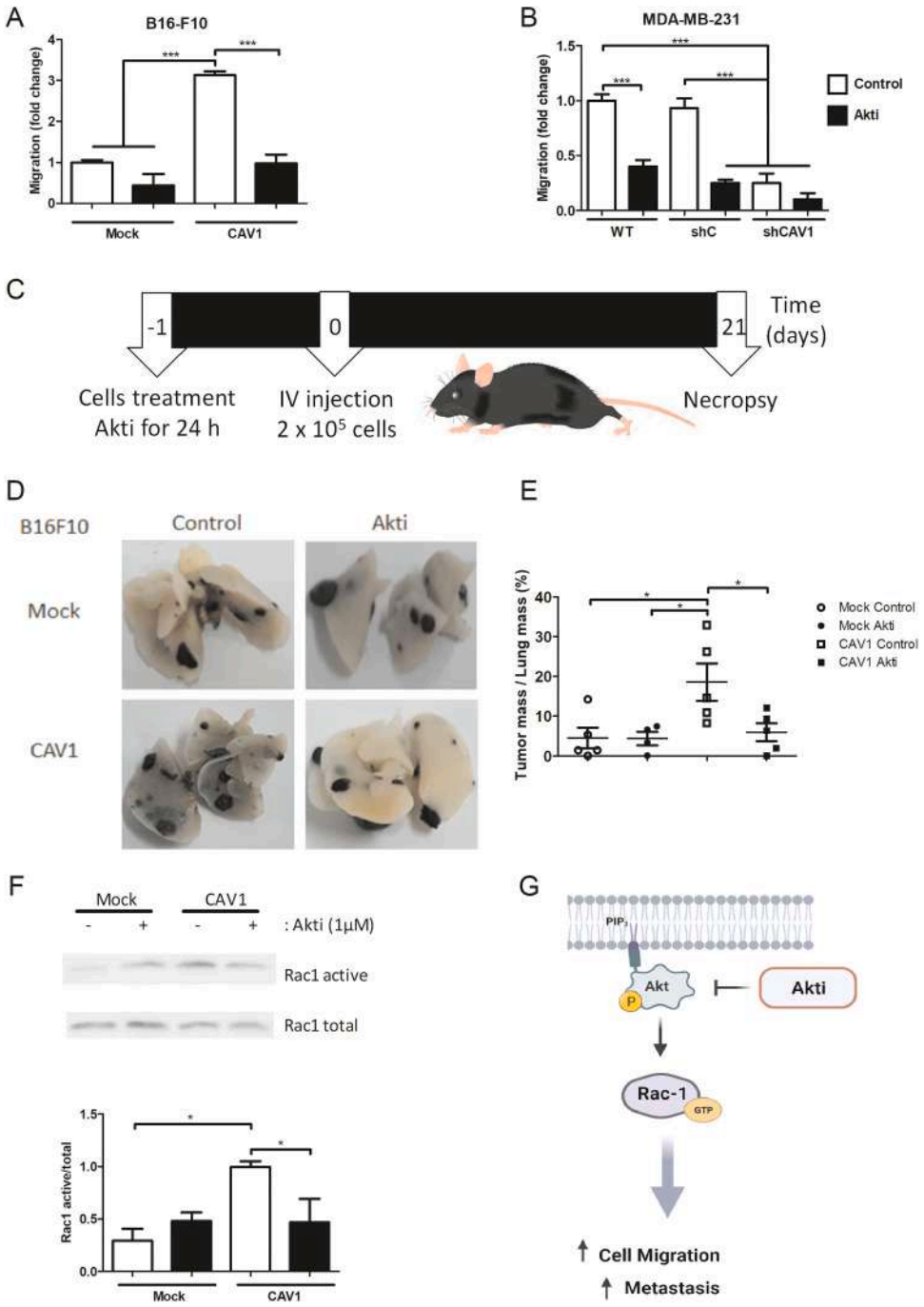

Fig. 8. Inhibition of the Akt pathway reduced migration *in vitro* and metastasis *in vivo*. A: B16-F10(Mock) and (CAV1) cells were treated for 24 h with Akti (1 μM). Then, cells (2×10^5) were seeded in Boyden chambers coated on the lower side of the membrane with fibronectin (2 μg/ml) and allowed to migrate for 2 h. Cells that migrated through the pores were stained and counted. B: MDA-MB-231 (WT), (shC), and (shCAV1) cells (1×10^5 cells) were treated with Akti, seeded in Boyden chambers, allowed to migrate for 2 h, stained and counted. Values shown were averaged from three independent experiments. Statistically significant differences are indicated (*** $p < 0.001$; n=3). C: Schematic representation of the experimental design for the metastasis assays. B16-F10(Mock) and (CAV1) cells were pre-treated or not for 24 h with Akti (1 μM) and injected intravenously (2×10^5 cells) into the tail vein of C57BL/6 mice. After 21 days, mice were euthanized, lungs were fixed, and total lung and metastasis nodule mass were determined. D: Representative images of the lung metastasis in each condition are shown. E: Average values (mean ± SEM) are shown for lung occupation by tumors in C57BL/6 mice inoculated with B16-F10(Mock) and (CAV1) cells, pre-incubated or not with the Akti. Statistically significant differences are indicated (* $p < 0.05$; N=5). F: Pull-down assay for active Rac1 in extracts from B16-F10(Mock) and (CAV1) cells treated or not with Akti (1 μM). Values shown were obtained by scanning densitometry and averaged from three independent experiments. Statistically significant differences between groups are indicated (* $p < 0.05$; n=3). G: Section from summary model in Fig. 9 showing how Akt inhibition prevents Rac1 activation, migration and metastasis.

Moreover, it has been shown that CAV1 promotes the epithelia to mesenchyme transition (EMT) through PI3K/Akt/Slug activation in bladder cancer [42]. On the other hand, inhibiting CAV1 expression by microRNA-203 reduces PI3K/Akt activation and thereby EMT [43]. In addition, CAV1 promotes anoikis resistance through the activation of the epithelial growth factor receptor/integrinβ1/Src, PI3K/Akt and MEK/ERK signaling [44]. Also, a FAK/Src/PI3K/Akt signaling pathway activated downstream of CAV1 is implicated in MCF7 breast cancer cell migration [45]. Thus, a considerable body of evidence links CAV1-induced migration/invasion to the activation of signaling

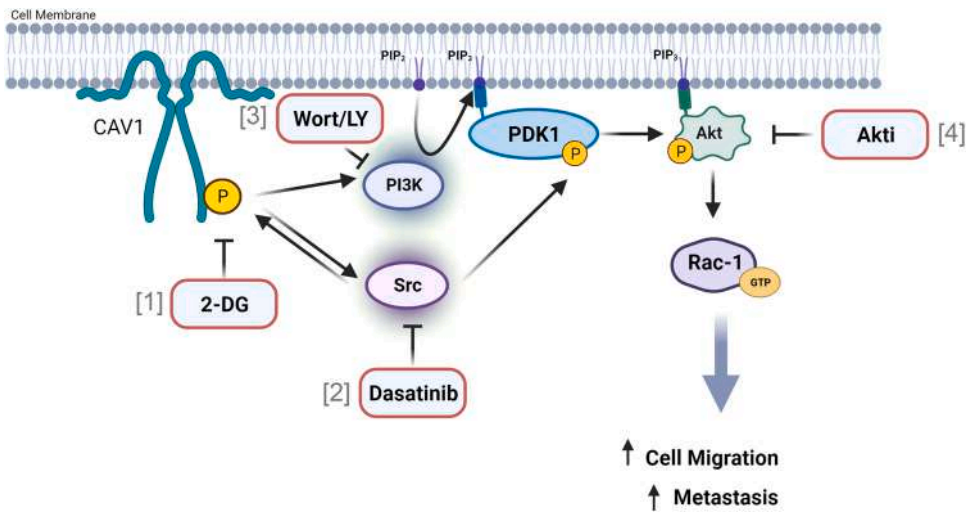

Fig. 9. Proposed model. Phosphorylated CAV1 promotes Src phosphorylation, which phosphorylates and activates PDK1. Following PI3K-dependent recruitment to the plasma membrane, Akt is phosphorylated and activated by PDK1-dependent phosphorylation on Threonine 308. PDK1-dependent phosphorylation of Akt promotes Rac1 activation, cell migration and metastasis. In addition, 2-DG reduces glycolysis [1] [19] and CAV1 phosphorylation, thereby preventing the recruitment and activation of Src. Moreover, inhibiting Src with Dasatinib prevents CAV1 phosphorylation, migration and metastasis [2] [15]. Downstream, Akt is not phosphorylated, thereby preventing cell migration and metastasis. Similar effects were observed following PI3K inhibition by Wort or LY [3], Src-family kinase inhibition by Dasatinib [2], PDK1 inhibition by BX-912, or Akt inhibition by Akti [4]. Thus, in this study, we identified a novel CAV1/Src/PDK1/Akt/Rac1 cascade [1–4]. Our previous studies showed that preventing glycolysis [1] [19], or inhibiting Src [2] [15] reduced metastasis. In our current study, we show how reducing Akt signaling [3,4] prevents CAV1-induced metastasis.

pathways converging on Akt. However, neither how precisely CAV1-enhanced glycolysis connects to these events, nor the consequences of their inhibition have been elucidated. Here, we provide for the first-time evidence indicating that inhibition of the PI3K/Akt signaling pathway blocks CAV1-induced metastasis, in a manner similar to that observed with the glycolysis inhibitor 2-DG.

Previous work by our group highlighted the relevance of CAV1 phosphorylation on tyrosine-14 for migration, invasion and metastasis [16]. Moreover, others have shown that pCAV1 can recruit Src to the plasma membrane and promote its activation [29]. On the other hand, inhibition of Src family kinases with PP2 or Dasatinib prevents CAV1 phosphorylation and reduces the ability of CAV1 overexpressing cells to promote migration, invasion and metastasis [15]. Here we demonstrate that overexpression of wildtype and phosphomimetic CAV1 were associated with Src activation. Treating CAV1-overexpressing cells with Dasatinib suppressed the ability of wildtype CAV1 overexpressing cells to activate the Akt signaling pathway but failed to prevent Akt activation by overexpression of the phosphomimetic CAV1/Y14E. These results indicate that tyrosine-14 phosphorylation of CAV1 promotes Src/Akt activation, thereby enhancing migration/invasion *in vitro* and metastasis *in vivo*, and that these effects of CAV1 may be blocked by either glycolytic restriction or Src inhibition.

It has been shown that CAV1 binds to and inhibits PP1 and PP2A phosphatases, thereby activating PDK1 and Akt [46]. In addition, Src reportedly promotes PDK1 and downstream Akt activation [30]. Here, we observed that an inhibitor of PDK1 (BX-912) was able to prevent CAV1-induced migration, suggesting that PDK1 is involved in the CAV1-dependent signaling pathway downstream of Src.

Previous results obtained by our group demonstrated that phosphorylated CAV1 induces migration by activating a p85α/Rab5/Rac1 signaling pathway. p85α, the regulatory subunit of PI3K and a Rab5 GTPase-activating protein, is sequestrated by phosphorylated CAV1 at the plasma membrane. In this way, CAV1 promotes endosomal Rab5-GTP loading, activation of Rac1, local remodeling of actin cytoskeleton and migration [17,47]. Moreover, we suggest that CAV1 recruits PI3K, possibly via the p85 subunit, to favor p110 activation at the membrane, as do 14–3–3 proteins [48], or the insulin receptor substrate (IRS [49]). In fact, CAV1 promotes migration by regulating several proteins. For instance, the presence of CAV1 in cancer cells is associated with lamellipodia formation mediated by Akt [24]. Moreover, Akt has been implicated in Rac1 activation through the PI3K/PDK1/Akt axis [27,28]. Here, we demonstrated that CAV1 promotes Rac1 activation via this sequence, thereby contributing to remodeling of the actin cytoskeleton required for migration. Importantly, these effects were blocked by Akt inhibition.

PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). Then, PIP3 acts as a lipid second messenger, which recruits proteins, such as Akt and PDK1, thereby facilitating Akt phosphorylation on Threonine 308 (by PDK1) and Serine 473 (by PDK2, mTORC2 complex) [50–52]. In addition, mechanosensitive CAV1 activation induces CAV1 phosphorylation, p85 recruitment to caveolae, PI3K/Akt activation and migration in breast cancer cells [53]. Indeed, here we demonstrate that PI3K activation was required for CAV1-enhanced migration. Moreover, inhibiting PI3K mimicked the effect of glycolytic restriction. Additionally, pCAV1 interacts with and recruits Src to the plasma membrane [29], which promotes Akt activation downstream of PI3K through PDK1 and subsequent Akt phosphorylation [30,54]. With this in mind, we propose a model in which CAV1 recruits Src to the plasma membrane, thereby facilitating its activation. In fact, in cells overexpressing CAV1, Src activation is elevated. Furthermore, Src increased the activation of Akt, downstream of PI3K, by promoting PDK1-dependent phosphorylation on T308. Then, active Akt induces Rac1 activation, migration and metastasis. Moreover, all these effects can be prevented by either glycolytic restriction and/or Src/PDK1/Akt inhibition (Fig. 9).

5. Conclusions

In conclusion, our results highlight the relevance of the CAV1-induced glycolytic switch for metastasis and elucidate the mechanism involved. Our findings suggest that CAV1 promotes Src/PDK1/Akt activation, thereby increasing Rac1 activity, migration and metastasis. Thus, the metastatic potential of CAV1-expressing cells can be reduced by glycolytic restriction and/or inhibition of the Src/PDK1/Akt signaling axis.

Ethics approval and consent to participate

Not applicable.

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CRediT authorship contribution statement

Layla Simón: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Keila Torres:** Writing – review & editing, Investigation. **Lisette Leyton:** Supervision, Resources. **Andrew F. G. Quest:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Pamela Contreras:** Methodology. **Natalia Díaz-Valdivia:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data analysed during this study are included in this published article and its supplementary information files.

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Consent for publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116841.

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