PFKFB4 interacts with ICMT and activates RAS/AKT signaling-dependent cell migration in melanoma

Méghane Sittewelle¹², Vincent Kappès¹², Chenxi Zhou¹², Déborah Lécuyer¹², Anne H Monsoro-Burq¹²

Introduction

Cell migration is a complex process, tightly regulated during embryonic development and abnormally activated during cancer metastasis. RAS-dependent signaling is a major nexus controlling essential cell parameters including proliferation, survival, and migration, utilizing downstream effectors such as the PI3K/AKT signaling pathway. In melanoma, oncogenic mutations frequently enhance RAS, PI3K/AKT, or MAP kinase signaling and trigger other cancer hallmarks among which the activation of metabolism regulators. PFKFB4 is one of these critical regulators of glycolysis and of the Warburg effect. Here, however, we explore a novel function of PFKFB4 in melanoma cell migration. We find that PFKFB4 interacts with ICMT, a posttranslational modifier of RAS. PFKFB4 promotes ICMT/RAS interaction, controls RAS localization at the plasma membrane, activates AKT signaling and enhances cell migration. We thus provide evidence of a novel and glycolysis-independent function of PFKFB4 in human cancer cells. This unconventional activity links the metabolic regulator PFKFB4 to RAS-AKT signaling and impacts melanoma cell migration.

DOI 10.26508/lsa.202201377 | Received 20 January 2022 | Revised 4 July 2022 | Accepted 6 July 2022 | Published online 1 August 2022

Reference

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57–70. doi:10.1016/S0092-8674(00)81683-9

1Université Paris-Saclay, Faculté des Sciences d’Orsay, CNRS UMR 3347, INSERM U1021, Orsay, France 2Institut Curie Research Division, PSL Research University, CNRS UMR 3347, INSERM U1021, Orsay, France

Correspondence: anne-helene.monsoro-burq@curie.fr, msittewelle@gmail.com
and synthesize (with kinase activity) or degrade (with phosphatase activity) the fructose-2,6-bisphosphate, the main allosteric activator of PKF1. Thus, increased PFKFB protein kinase activity promotes glycolysis. In human, four distinct genes encode PFKFB isoenzymes 1–4, each one possessing many splicing isoforms and differing in their tissue-specific abundance, kinetics and interaction properties (van Schaftingen et al, 1982; Rider et al, 1992, 2004; Pilakis et al, 1995; Bruni et al, 1999; Manes & El-Maghrabi, 2005). PFKFB proteins are overexpressed in cancer. In particular, increased PFKFB4 levels have been reported in several human tumors, including cutaneous melanoma (Minchenko et al, 2004, 2005a, 2014; Goidts et al, 2012). Moreover, PFKFB4 is induced by hypoxia, is required for survival and proliferation of normal thymocytes (Houdanne et al, 2017) as well as of several cancer cell lines such as lung, breast, and colon adenocarcinomas and prostate and bladder cancer (Ros et al, 2012; Yun et al, 2012; Chesney et al, 2014; Zhang et al, 2016b). So far, most studies have focused on cell metabolism reprogramming by PFKFB4 and have proposed that PFKFB4 is a major driver of Warburg effect (Minchenko et al, 2005a, 2005b, 2014; Goidts et al, 2012; Ros et al, 2012; Yun et al, 2012; Chesney et al, 2014, 2015; Shu et al, 2016; Zhang et al, 2016b; Houdanne et al, 2017; Yao et al, 2019). However, a few recent studies have identified alternative functions of PFKFB4, outside of its canonical control of glycolysis. For example, PFKFB4 regulates small cell lung-cancer chemo-resistance by stimulating autophagy via its interactions with Etk tyrosine kinase (Strohecker et al, 2015; Wang et al, 2018). PFKFB4 also operates as a protein kinase and directly phosphorylates SRC-3, promoting metastatic progression in highly glycolytic breast cancer cells (Dasgupta et al, 2018). During development, PFKFB4 is essential for early embryonic induction and neural crest cells migration through the activation of AKT signaling (Pegoraro et al, 2015; Figueiredo et al, 2017). In cancer, the intriguing relationships between PFKFB4, cell signaling and cell migration remain unexplored.

Here, we have analyzed the importance of PFKFB4 in melanoma cell migration. Using human melanoma cell lines and high PFKFB4 expression (Rambow et al, 2015), we show that PFKFB4 activity is required for active cell migration in several different cellular contexts, without a connection to the rate of glycolysis. Rather, we identify potential interacting proteins by mass spectrometry, among which we validate the protein–protein interactions between PFKFB4 and isoprenylcysteine carboxymethyl transferase (ICMT), an enzyme essential for RAS posttranslational modifications controlling its localization at the plasma membrane. Our study further defines a novel, glycolysis-independent function for PFKFB4, which promotes ICMT–RAS interactions, results in efficient RAS localization at the plasma membrane, activates AKT signaling and enhances melanoma cell migration.

Results

PKF4 controls metastatic melanoma cell migration in vitro in a glycolysis-independent manner

Melanomas present higher expression of PFKFB4 mRNA compared with other tumors (Fig S1). We have previously linked elevated expression of PFKFB4 with embryonic cell migration in vivo (Figueiredo et al, 2017), but in melanoma, whereas PFKFB4 has been linked to promoting the Warburg effect, its role in cell migration remains to be explored. Here we have chosen two human melanoma cell lines expressing high levels of PFKFB4 (MeWo and A375M, Fig 1A and B (Rambow et al, 2015)) to follow the random migration of individual cells by time-lapse video microscopy followed by manual tracking of single cells (Fig 1D, see details in the Materials and Methods section). The MeWo cells are derived from lymph node metastasis of a cutaneous melanoma. They are tumorigenic and metastatic. They bear wild-type alleles at BRafV600 or RasQ61/G12 positions (Rambow et al, 2015; Ranzani et al, 2015) (Fig S2A). The A375M cells are derived from a human amelanotic melanoma. They are also tumorigenic and metastatic. They are mutated for BRafV600 and wild-type for RasQ61 (Rambow et al, 2015). Both cell lines actively migrated on Matrigel. Cells were tracked during 16 h in at least three independent experiments for each cell line (see Supplementary video microscopy). After PFKFB4 depletion using siRNA (Fig 1C), MeWo, and A375M cells migrated in average 33% and 42% slower than control, respectively (n = 27 independent biological replicates; Fig 1E and F and Table S1). Migration distance was also decreased while cell pausing was increased (Fig S2C–F). This defective migration after PFKFB4 depletion was also confirmed by a wound healing scratch assay in Mewo cells (Fig S2G and H). Moreover, to test if non-tumorigenic melanocytes also depended upon PFKFB4 for efficient migration, we used a cell line of spontaneously immortalized wild-type melanocytes (12S2 cells) established from P4/P5 mice primary skin melanocytes (Valluet et al, 2012) and validated a siRNA against mouse PFKFB4 (Fig S7A and B). In this non-tumorigenic cell context, we also observe decreased migration (total distance) due to increased pausing time (Fig S7D–F). Because during Xenopus laevis embryonic development, the migration of melanocytes and melanoma progenitors, the neural crest cells, is also controlled by PFKFB4 (35), we postulated that human and frog protein functions were conserved, allowing us to devise phenotype rescue experiments: Frog pfbfb4 encodes a protein with 95% similarity with the human protein, but the mRNA was not targeted by siRNAs designed against the human mRNA sequence. The frog PFKFB4 expression plasmid was efficiently translated in human melanoma cells and recognized by the antibody designed against human PFKFB4 (Fig 1C). In MeWo cells, the migration phenotype was efficiently rescued by co-transfecting the X. laevis orthologous pfbfb4 sequence (Figs 1E and F and S2C). This rescue showed that the migration phenotype was specific for the depletion of PFKFB4 protein and did not come from off-target effects.

The best-known function for the bi-functional enzymes PKFB1-4 is to regulate glycolysis rate by controlling the second rate-limiting reaction of glycolysis. We investigated if PFKFB4’s role in cell migration was linked with its function as a major activator of the glycolysis rate. To block glycolysis, we first grew the MeWo cells in a culture medium without glucose, which strongly decreased their glycolysis rate (estimated by the diminished lactate production measured in the culture medium, Fig S3B). We did not observe a major decrease of MeWo cells’ average migration speed in the glucose-free medium compared with the complete medium condition (Fig 1G and H). Next, to confirm that MeWo cell migration was unaffected by limiting glycolysis, we added the glucose non-
hydrolysable analog 2-deoxyglucose (2DG) in the complete medium (Fig S3A). Similar to the glucose-free condition, 2DG also decreased glycolysis efficiently (Fig S3C) without affecting MeWo cells’ migration speed (5 mM 2DG, Fig 1H). These results indicated that MeWo cells’ migration was not directly linked to their rate of glycolysis. In contrast, PFKFB4-depleted MeWo cells showed a reduced average migration speed compared with control cells in the glucose-free medium as observed in the complete medium, indicating an action of PFKFB4 on another cellular pathway (Fig 1G). Last, to test if PFKFB4 depletion was altering the overall rate of glycolysis, we performed two complementary approaches: first, we measured lactate production of MeWo cells after PFKFB4 depletion (Fig S3D); second we used the Seahorse XF Analyser to measure real-time extracellular acidification rate (ECAR) and oxygen consumption rate after successive addition of different metabolic inhibitors (first inhibitors of mitochondrial functions rotenone and antimycin A, followed by the
glycolytic inhibitor 2DG) using both MeWo and A375M cell lines (Fig S3E). From these measurements, we were able to assess different parameters of glycolysis such as the total proton efflux rate (PER) and the PER associated to glycolysis only (glycoPER) (Fig S3F and G) from which we evaluated the basal glycolysis and the compensatory glycolysis (Fig S3I and K). We did not observe any significant variation in lactate production (Fig S3D), in PER or glycoPER (Fig S3F–K), nor in compensatory glycolysis (Fig S3I and K). This is in agreement with previous reports indicating that PFKFB4 effect on glycolysis varies according to the cell context (Sakata et al, 1991; Okar et al, 2001; Ros et al, 2012, Ros & Schulze, 2013; Chesney et al, 2014). The characteristics of melanoma cell energy metabolism ensuring their migration in glucose-free conditions remain to be defined. Together, these observations suggested that PFKFB4 levels significantly affect the average speed of melanoma cells migration, independently of the rate of glycolysis.

PFKFB1-4 bi-functional enzymes possess two adjacent large catalytic regions. With their kinase moiety, PFKFB isoenzymes phosphorylate fructose-6-phosphate into fructose-2,6-bisphosphate (Pilkis et al, 1995; Okar et al, 2001) (Fig S3A). With their phosphatase domain, PFKFBs catalyze the reverse reaction. The two catalytic domains are highly conserved: the amino-acid sequence as well as the 3-dimensional protein structure are conserved both between isoenzymes in a given species and between species (Kotoski et al, 2021). To understand if PFKFB4 controlled melanoma cells migration using either its kinase or its phosphatase enzymatic activities, we compared the rescue phenotype of PFKFB4 depletion by various X. laevis PFKFB4 mutants (Fig S2B). The average speed of cells co-transfected with siPFKFB4 and a plasmid encoding a pRfkb4 mutant with two point mutations targeting the kinase and phosphatase enzymatic activities simultaneously (mutations G48A and H258A, [Tauler et al, 1990; Li et al, 1992]) was equivalent to the speed after rescue by wild-type PFKFB4 (xPFKFB4) (Fig 1I). This indicated that PFKFB4-controlled cell migration independently of its enzymatic activities. To identify which region of PFKFB4 protein was involved in this nonconventional effect, we used two complementary deletion constructs. The rescue done with a deletion construct encoding the N-terminal kinase domain (xPFKFB4-Nter) was as efficient as with xPFKFB4 (Fig 1I). In contrast, the migration of cells depleted for PFKFB4 and co-transfected with the deletion construct encoding only the C-terminal phosphatase domain (xPFKFB4-Cter) was not significantly rescued (Fig 1I). This result suggested that PFKFB4 was involved in control of cell migration independently of its kinase or phosphatase activities, but through the N-terminal half of the protein. We next checked if PFKFB4 depletion altered cell cycle or cell death, using FACS analysis. We did not detect variation in cell apoptosis nor in the relative duration of cell cycle phases either in MeWo or in A375M cells (Fig S4). Altogether these results suggest that PFKFB4 regulated the efficiency of melanoma cell migration independently of variations in glycolysis, cell survival rate or cell cycle.

**PFKFB4 interacts with ICMT, a major posttranslational modifier of RAS GTases**

As PFKFB4 protein seemed to control melanoma cell migration independently of its enzymatic activities, we looked for interacting protein partners using mass spectrometry after immunoprecipitation of a FLAG-tagged form of human PFKFB4 expressed in the MeWo cells. In two biological replicates, among 1,556 high confidence hits, we chose 40 candidates with a Mascot score enriched at least ten-times compared with the negative control condition to eliminate the weak hits and limit the nonspecific targets. Moreover, because xPFKFB4 efficiently rescued the PFKFB4 depletion phenotype in human melanoma cells, we postulated that the protein function we looked for was evolutionarily conserved between human and X. laevis PFKFB4. We transfected MeWo cells with the frog xPFKFB4 ortholog followed by immunoprecipitation and mass spectrometry. We then crossed the 40 candidates list obtained with hPFKFB4 with the list of xPFKFB4 targets and sub-selected 22 candidates (Figs 2A and S5). Among these 22 best candidates, we prioritized isoprenylcystein carboxyl methyl transferase (ICMT), a potential modulator of PI3K/AKT signaling pathway, because PFKFB4 was known to affect cell migration via AKT signaling activation during embryogenesis (Pegoraro et al, 2015; Figueiredo et al, 2017). ICMT is an endoplasmic reticulum membrane protein critical for RAS GTases posttranslational modifications. ICMT catalyzes the carboxyl methylation of RAS on its C-terminal CAAX motif. This modification allows RAS protein to be targeted to the plasma membrane, a prerequisite for the coordination by RAS of a variety of signaling pathways, including PI3K/AKT activation ([Dai et al, 1998; Choy et al, 1999; Michaelson et al, 2005; Wright et al, 2009], reviewed in Cansado [2018]). This observation suggested that an interaction between PFKFB4 and ICMT could occur during melanomagenesis and be related to RAS-dependent signaling pathways.

First, we validated the mass spectrometry results by co-immunoprecipitation of ICMT with PFKFB4 in MeWo cells (Fig 2B). Second, we confirmed the protein–protein interaction between PFKFB4 and ICMT by an independent alternative method, a split-ubiquitin two-hybrid approach adapted for mammalian membrane proteins (MaMTH) (Fig 2C) (Petschnigg et al, 2014; Saraon et al, 2017). Briefly, ICMT and PFKFB4 were fused to a portion of the ubiquitin protein, either its N-ter part (Nub, N-ubiquitin) or its C-ter part fused to GAL4 (Cub-GAL4, C-ubiquitin). Constructs were co-transfected in MaMTH-modified HEK293T cells bearing a stable integration of GAL4-binding sites upstream of a luciferase reporter. Upon interaction, the two halves of ubiquitin reunite to form a “pseudo-ubiquitin” which recruits deubiquitinating enzymes (DUBs). The DUBs then cleave the pseudo-ubiquitin, resulting in the release of the GAL4 transcription factor. GAL4 then activates the transcription of GAL4-driven luciferase in the nucleus. As a positive control, co-transfection of PFKFB4-Cub-GAL4 and PFKFB4-Nub strongly increased luciferase expression (by five to thirteen times compared with PFKFB4-Cub-GAL4 alone, for PFKFB4-Nub fusion in N-ter or C-ter, respectively). This denoted a strong and stable interaction, related to the formation of the PFKFB4 homodimer. The co-transfection of ICMT-Cub-GAL4 and PFKFB4-Nub significantly increased luciferase expression compared with ICMT-Cub-GAL4 alone and in a range comparable with the known PFKFB4–PFKFB4 homophilic interaction. Together, these results demonstrated that PFKFB4 and ICMT directly interacted. Last, we tested if the interaction between PFKFB4 and ICMT was important for the known interaction between ICMT and RAS GTPase. When PFKFB4 was depleted in MeWo cells, we observed a decrease of
endogenous RAS immunoprecipitation by ICMT (Fig 2D). In sum, all these results suggested that PFKFB4 direct protein–protein interactions with ICMT impacted ICMT–RAS complex formation in melanoma.

ICMT and PFKFB4 control RAS localization at the plasma membrane and melanoma cell migration

To understand the role of the PFKFB4–ICMT interaction, we first compared PFKFB4 and ICMT depletion phenotypes in MeWo cells, using a validated siRNA against ICMT (Cansado, 2018) (Fig S6A). Parameters of melanoma cell migration were measured as mentioned previously. Compared with control siRNA, cells transfected with siICMT exhibited a decrease in their average speed of migration, as well as altered pausing and distance parameters, similar to cells transfected with siPFKFB4 (Figs 3A and S6B and C). To test the interdependency of PFKFB4 and ICMT, we co-transfected both siRNAs. Melanoma MeWo cells receiving both siPFKFB4 and siICMT did not exhibit a more severe phenotype than with either siRNA alone. This suggested that PFKFB4 and ICMT cooperated in the same pathway to control cell migration and that deleting either one was sufficient for attaining a strong phenotype (Fig 3A). This suggested two alternative (and not exclusive) possibilities: either the need for both proteins simultaneously, cooperating to enhance cell migration, or that one of these two proteins was functional only after being activated by the other. As a whole, this series of results showed that ICMT depletion phenocopied loss of PFKFB4, and that the two protein partners were likely acting in the same pathway impacting melanoma cell migration.

In parallel, we compared PFKFB4 depletion phenotype with the known effect of ICMT depletion, as the major role of ICMT is to modify RAS proteins posttranslationally for their efficient targeting to the plasma membrane (Michaelson et al., 2005). To be free from potential defects in RAS GTPase activity, we have used a constitutively active form of RAS, HA-tagged-RasV12, and tested if PFKFB4 influenced its subcellular localization, by immunofluorescence. After co-transfecting MeWo cells with HA-tagged–RasV12 and either siPFKFB4, siICMT, or control siRNA, we scored RAS localization qualitatively (Fig 3D). On top of the

---

**Figure 2.** PFKFB4 interacts with ICMT, a major posttranslational modifier of RAS GTPases.

(A) Workflow used to select candidates after PFKFB4 immunoprecipitation followed by mass spectrometry analysis (see text for details). (B) Enrichment of ICMT tagged with V5 after immunoprecipitation by FLAG PFKFB4 from MeWo cell extracts. (C) Scheme of the MaMTH strategy used to validate PFKFB4/ICMT protein–protein interactions. Violin plot showing the luciferase activity measured and normalized from MaMTH-modified HEK293T cells extracts (n = 3). The violin represents the probability density at each value; lines are plotted at the median and quartiles (Two-way ANOVA test. **P < 0.01 and ****P < 0.0001). (D) The interaction between tagged ICMT and endogenous RAS was evaluated with or without PFKFB4 depletion (n = 2). Immunoprecipitation of FLAG-ICMT from MeWo cells followed by Western blotting with antibody against V5, FLAG, or endogenous RAS. Source data are available for this figure.
exogeneous RasV12 perinuclear location, the cells transfected with the control siRNA could be categorized into three groups: cells exhibiting a clear and homogeneous RAS membrane enrichment localization (phenotype 1), cells without RAS membrane enrichment (phenotype 3), and cells with an in-between phenotype with discontinuous RAS membrane localization (phenotype 2) (Fig 3D). Whereas phenotype 2 was present in a similar proportion in each condition (siControl, siICMT, or siPFKFB4; 30–37% of the cells), cells with RAS at the plasma membrane represented 51% of the siControl cells, but only 35% of siICMT cells and 17% of siPFKFB4 cells (Fig 3D). The remaining cells displayed phenotype 3. This assay thus showed that PFKFB4 depletion phenocopied ICMT loss for RAS addressing to the plasma membrane in melanoma cells.

Last, to test if RAS was indeed a downstream target of PFKFB4 in the control of cell migration, we measured MeWo and A375M cell migration efficiency after co-transfecting siPFKFB4 with the constitutively active RasV12. In both cell lines, RasV12 rescued the PFKFB4 migration phenotype (Figs 3C and S6D). We noticed that in this case, RasV12 discontinuous membrane localization (Fig 3D) was sufficient to restore cell migration functionally (Fig 3C), probably because of the constitutive and enhanced activity of the RasV12 protein. From this series of results, we concluded that the interaction between PFKFB4 and ICMT was critical for controlling RAS subcellular localization and melanoma cell migration. PFKFB4 thus displays a novel function, important to modulate the activity of a major cell signaling pathway in cancer cells.

PFKFB4 and ICMT control RAS-AKT signaling in melanocytes and in melanoma

RAS signaling is a major hub for the cell to integrate multiple inputs from the extracellular cues as well as from intracellular parameters. Once activated, several downstream pathways are activated in normal as well as cancer cells. The major ones include MAP kinase, PI3 kinase, and Ral signaling cascades (Rodriguez-Viciana et al., 1994; Urano et al., 1996; Peyssonnaux et al., 2000). In melanoma, MAPK/Erk and PI3K/AKT pathways are frequently activated to control cell migration. Here, we next sought to further understand which of these two pathways was modulated by PFKFB4 depletion (Fig 4). Whereas ERK phosphorylation (pERK) remained unchanged after transfection of MeWo cells with siPFKFB4 or siICMT, AKT phosphorylation was strongly decreased both on threonine 308
PFKFB4 interacts with ICMT in melanoma cells migration  
Sittewelé et al.

Figure 4. PFKFB4 and ICMT both control AKT signaling activation in melanoma cells. (A) Protein levels of pAKT T308, pAKT S473, AKT, and vinculin in MeWo cells transfected with siRNA targeting PFKFB4 or ICMT. (B) Normalized pAKT S473/actin levels in MeWo, MNT1, A375, or Lu1205 cells transfected with siRNA targeting PFKFB4 and analyzed as in A; one point represents one biological replicate. (C) Protein levels of pERK, ERK, and vinculin in MeWo cells transfected with siRNA targeting PFKFB4. (D) Protein levels of pAKT S473 in MeWo cells treated with different concentrations of 2DG for 24 h. (E) Average speed of MeWo cells transfected with siRNA targeting PFKFB4 or ICMT, siPFKFB4+xenopus PFKFB4 wild-type, siPFKFB4+caAKT, and siPFKFB4+ca-PI3K. In (A, C, D, E): a representative experiment is shown, n > 3. Graphs show the mean ± SEM. P-values were calculated using the Mann–Whitney test. n.s. P > 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Source data are available for this figure.

We then wondered if PFKFB4 might affect AKT signaling as an indirect effect of glycolysis regulation. We blocked glycolysis with 2DG and tested AKT activation in MeWo cells. Although decreased lactate levels indicated an efficient block of glycolysis (Fig S3), the treatment with 2DG did not affect AKT phosphorylation on S473 (Fig 4D). As observed above for the cell migration phenotype (Fig 1), AKT phosphorylation phenotype after depleting PFKFB4 was thus not likely due to a reduction of glycolysis. Last, we expressed constitutively active forms of either AKT (caAKT) or its upstream regulator PI3 kinase (caPI3K) in PFKFB4-depleted MeWo cells. Defective cell migration parameters observed after PFKFB4 depletion were rescued either by caAKT or by caPI3K (Figs 4E and S6H and I). All these data together indicated that PFKFB4 controlled human melanoma cell migration via a novel nonconventional function controlling the RAS/PI3K/AKT pathway.

Discussion

PFKFB proteins are long-known major regulators of the rate of glycolysis in normal and cancer cell types (Rider et al, 2004). They have been involved in mediating the Warburg effect in many different tumors, and have been shown to be activated in response to hypoxic conditions often found at the heart of primary tumors (Minchenko et al, 2005a, 2005b; Yun et al, 2012; Chesney et al, 2014; Zhang et al, 2016a). In particular, elevated levels of PFKFB4 expression have been described in melanoma (Fig S1, [Minchenko et al, 2005b]). Using a survey of human melanoma cell lines transcriptomes (Rambow et al, 2015), we have selected cells with high PFKFB4 levels, and explored PFKFB4 function in the biology of those cells, focusing on their migration in vitro. We first showed that PFKFB4 enhanced cell migration irrespective of the cells’ glycolysis levels (Figs 1 and S2). Moreover, neither PFKFB4 kinase nor its phosphatase activity was required for this effect, suggesting alternative molecular mechanisms, such as protein–protein interactions. Using PFKFB4 immunoprecipitation followed by mass spectrometry, we have identified a partner of PFKFB4 which was selected for further analysis: ICMT, an enzyme embedded into the endoplasmic reticulum membrane, which adds the terminal methyl group to RAS GTPases posttranslationally. This modification is required for anchorage of RAS GTPTases at the plasma membrane, where RAS activates several downstream signaling events (Cox & Der, 2010). Strikingly, both PFKFB4 and ICMT depletion displayed similar phenotypes including RAS mislocalization, decreased AKT activation and reduced cell migration (Figs 2–4). Migration phenotypes were rescued by a constitutively active form of RAS or by the constitutive activation of AKT signaling (Fig 5). In sum, our study has demonstrated a novel, glycolysis-independent function of PFKFB4, promoting the interaction between ICMT and RAS, resulting in active migration of both melanoma cells and melanocytes (Figs 5 and S3).

Our main thought-provoking finding is that the regulation of cell migration by PFKFB4 does not depend on its kinase activity and that PFKFB4 depletion affects migration independently of experimental modulations of the high/low glycolysis status of the cells (Figs 1 and S2). Our results thus uncouple the classical action of PFKFB4 in glycolysis from its role in cell migration. This undermines current
strategies to counteract PFKFB4 in cancer, which involves developing pharmacological drugs to interfere with PFKFB4 kinase activity, assuming that PFKFB4's major function in cancer is to promote glycolysis and Warburg effect (Chesney et al, 2015). Our data show that a kinase-deficient form of PFKFB4 still retains important cancer-promoting functions outside of glycolysis regulation. Further biochemical and crystallographic analyses, beyond the scope of this cell biology study, would provide details on the protein–protein interacting subdomains interfacing PFKFB4 and ICMT. The disruption of this interaction could be an additional strategy to block PFKFB4 in cancer.

ICMT is a major posttranslational modifier of RAS GTPases. ICMT catalyzes RAS terminal methylation on the ER membrane, needed to address RAS to the plasma membrane (Dai et al, 1998; Choy et al, 1999; Michaelson et al, 2005). One-third of human cutaneous melanoma are mutated on RAS, PI3 kinase, or other partners of this pathway, enhancing its signaling activity (Ali et al, 2013; Cancer Genome Atlas Network, 2015). However, here we showed that PFKFB4 promoted RAS signaling and cell migration in two cell lines with wild-type NRAS. This implied that, even in the absence of a RAS activating mutation, an increase in PFKFB4 cellular levels might also allow enhanced RAS-linked signaling and cell migration. Increase in PFKFB4 gene expression can be achieved by hypoxia, a general feature of growing tumors. HIF1α-responsive elements have been identified to promote pfkfb4 transcription (Minchenko et al, 2004). Whereas in normal cells, there is a fine-tuned, dynamic, and tissue-specific expression of PFKFB genes during development and cell homeostasis (Minchenko et al, 2003; Pegoraro et al, 2013, 2015), it is likely that tumor progression enables a hypoxia-induced broader and sustained expression of PFKFB4, which would in turn promote tumor cell migration in parallel to its activation of Warburg effect.

However, in melanoma cell lines, a tumor type with generally high PFKFB4 levels, we did not observe a strict correlation between PFKFB4 expression levels and the metastatic characteristics of the cells (Fig S2). This indicates the importance of yet unknown additional cell-specific cues.

We have focused on the function of RAS-AKT signaling in the control of melanoma cell migration. This study makes a further parallel between melanoma cell features and the behavior of their parent cells in embryos, the neural crest cells. PFKFB4 was first identified as a regulator of cell migration in neural crest, and as a general patterning regulator during neural and neural crest early development (Pegoraro et al, 2015; Figueiredo et al, 2017). Melanoma initiation and progression involves the reactivation of elements belonging to the neural crest developmental program (Kaufman et al, 2016). We here extend the parallel between the two models, showing increased expression of PFKFB4 in development and cancer as predicted by our previous WGCNA analyses (Plouhinec et al, 2017). Moreover, in the embryonic cells, which rely on yolk breakdown for their energy metabolism rather than on glycolysis, we have revealed the first indications for a nonconventional function of PFKFB4. This function involved enhancing AKT signaling and cell migration (Pegoraro et al, 2015; Figueiredo et al, 2017). It remained unclear whether this novel function of PFKFB4, found in a nonmammalian in vivo model, was also important in mammalian cells. Here, human melanoma cells present similar regulations by PFKFB4, implicating AKT signaling and the control of cell migration. This study thus further emphasizes the importance of PFKFB4 “moonlight” or nonconventional signaling functions, a term naming a function which is revealed when the major “sunlight” function is masked (here, the key control of glycolysis rate by PFKFB proteins).
Although we stress the importance of the PFKFB4–ICMT–RAS–AKT signaling pathway, we do not exclude parallel important functions for other signaling proteins that modulate cell migration: our mass spectrometry screen for PFKFB4 partners has provided about 20 other strong interaction candidates, which could also be involved in cell trafficking or cell migration (Figs 2 and S3). In conclusion, our study highlights a novel and unsuspected link between three major treatment and relapse, will be important targets for future therapeutic options.

Materials and Methods

Cloning, plasmids

All plasmids used are listed in Table S2. For testing protein–protein interactions, a two-hybrid-like assay adapted for mammalian membrane-bound proteins (MaMTH) was used (Petschnigg et al, 2014; Saraon et al, 2017). Cloning used Gibson method ([Gibson et al, 2009], primers listed in Table S3), hICMT (clone Origene no. RC207000) and hPFKFB4 (clone Origene no. RC201573) were inserted in-frame into the MaMTH bait destination vector, which contains ubiquitin C-terminal half fused to the yeast GAL4 DNA-binding domain, or into the C-tagged or N-tagged MaMTH prey destination backbone vector, which contains ubiquitin N-terminal half.

Cell lines, cell culture, cell treatments, and cell transfection

The well-characterized human melanoma cell lines MeWo (Kerbel et al, 1984; Ishikawa et al, 1988), A375M (Sriramarao & Bourdon, 1996), MNT1 (Cuomo et al, 1991), Lu1205 (Juhasz et al, 1993) were kindly provided by Dr L Larue (Rambow et al, 2015). Their mutagenic status for key driver mutations in melanoma is summarized in Fig S2 (Rambow et al, 2015; Ranzani et al, 2015). Cells were cultured in RPMI (Gibco) supplemented with 10% SVF and 1% penicillin/streptomycin (Invitrogen). HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% SVF and 1% penicillin/streptomycin (Invitrogen). The wild-type mouse melanocytes 12S2 cells were kindly provided by Dr S Druillennec. They were established from the skin of wild-type mouse with a mixed background (C57Bl/6&129/SV) which were previously washed in lysis buffer were added and incubated with agitation overnight at 4°C. Beads were then washed five times in
Curves were plotted using RStudio; superplots and statistical analysis were generated using SuperPlotsofData (Goedhart, 2021).

Two-dimensional random cell migration assayed by time-lapse video microscopy

We dispensed 40,000 or 50,000 cells per well into twelve-well plates coated with Matrigel (Thermo Fisher Scientific) or 40 ng/μl of fibronectin (#F1141; Sigma-Aldrich) (for 1252 or MeWo/A375M, respectively). Two-dimensional (2D) random cell migration was monitored by time-lapse video microscopy under bright white light, with an inverted phase-contrast microscope (Leica MM AF) equipped with a cell culture chamber (37°C, humidified atmosphere containing 5% CO₂), an x–y–z stage controller, and a charge-coupled device CoolSnap camera (Photometrics). Images were acquired at 8-min intervals during 16 h, with the Metamorph software (Molecular Devices). Movies were reconstructed with the ImageJ software (http://rsbweb.nih.gov/ij/). Cells were tracked manually by using the center of the nucleus as guide and parameters were calculated with ImageJ Manual Tracking plug-in. Individually tracked cells were chosen to be alive by eye monitoring over the entire duration of the movie. Briefly, the manual tracking plug-in is recording x and y positions of each cells tracked and then generate statistical values as the mean overall velocity (in μm/h), total distance travelled (in μm) or % pausing (which is the cumulative fraction of time of the total duration of the movie where the cells are not changing position).

Wound healing/scratch assay

We dispensed 60,000 MeWo cells per well in a 96-well plate and incubated it in a humidified incubator at 37°C and 5% CO₂. When the cells reached confluence, a wound was made using Incucyte WoundMaker (Essen Bioscience) followed by three washes to remove debris.

The healing was monitored every 3 h for 90 h by Incucyte LiveCell Imaging Systems (Essen Bioscience) with a ×10 objective. The relative cell density at the wound was calculated by Incucyte analyzer after training on a subset of images, providing real-time cellular confluence data, based on segmentation of high-definition phase-contrast images. The relative wound cell density is defined as the percent of confluence in the wound area compared with the confluence outside of this region. Raw data were then exported to Prism software for plotting and statistical analysis using ANOVA test.

FACS sorting, cell cycle, and cell death analysis

Standard protocols were used. Briefly, for cell death analysis, cells were trypsinized and resuspended in PBS containing 0.25 μg/ml of 7-aminoactinomycin D (7-AAD). Cell suspension was analyzed on FACSCANTO II cell sorter. Percent of stained (dead) cells was calculated. For cell cycle analysis, cells were trypsinized, rinsed in PBS, fixed in 70% ethanol, and stored at −20°C overnight. After several PBS washes, cells were resuspended in 3.5 mM Tris HCl, pH 7.6, 10 mM NaCl, 1 μg/ml 7-AAD, 0.1% NP-40, 40 U/μl RNase A, and incubated in the dark for 30 min. The cell suspension was sorted on
FACS CANTO II cell sorter. The relative importance of each phase of the cell cycle was automatically calculated using standard modeling algorithms of the FlowJo software.

Immunofluorescence

MeWo cells were plated 24 h before transfection on glass coverslips. At 48 h after transfection, cells were rinsed with PBS, and fixed with paraformaldehyde 4% for 15 min. After PBS wash, cells were permeabilized and nonspecific protein binding blocked in 10% SVF and 0.1% Triton in PBS for 1 h at room temperature. Then, cells were incubated at room temperature for 1 h with primary antibodies diluted in blocking buffer (Table S4), rinsed with PBS, incubated 1 h at room temperature in dark in secondary antibody at a 1:1,000 dilution in blocking buffer (Alexa Fluor 647/555/488-conjugated goat anti-rabbit/mouse/rat). The actin cytoskeleton was stained with Alexa Fluor 647 or 488 Phalloidin (Invitrogen). Cell nuclei were stained by DAPI at 1 μg/ml in PBS for 10 min at room temperature. Coverslips were then mounted using ProLong Diamond (Molecular Probes), and imaged with 63× or 100× oil immersion objective of a wide-field microscope (DM RXA, Leica; camera CoolSNAP HQ, Photometrics), using Metamorph software.

Data Availability

This study does not include data to be deposited in external repositories. The mass spectrometry data included in the source data associated to this article.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.202201377.

Acknowledgements

The authors are very grateful to Drs. C Pouponnot, M Akobtawi, A Eychène, S Saule, and L Larue for insightful scientific discussions during this study and to Dr. V Petit for guidance in the culture of melanoma cell line. We deeply thank S Seal, C Pouponnot, and M Akobtawi for their proofreading of the manuscript. We thank all the Monsoro-Burq team members for their constant support and Drs M Perron, P Gilardi, and C Pouponnot for acting as thesis committee advisors. Dr. A Eychène, Dr. S Duillencet, and Dr. L Larue kindly provided reagents and cell lines. We thank F Maczkowiak for mutagenesis of the X. laevis pfkfb4. We also acknowledge the valuable help from MN Soler and L Besse on Institut Curie Imaging platform facility PICT-IBiSA (Orsay), of C Lasg to FACS analysis (Orsay), of L Barrio-Cano on Pasteur Institute Biomarkers platform facility (Paris), and members of the mass spectrometry platform at Jacques Monod Institute (Paris). We also acknowledge Dr. Staglar who provided the plasmid backbones and cell lines for the MaMTH experiment. This work was supported by grants to AH Monsoro-Burq from Université Paris Saclay, Centre National de la Recherche Scientifique (CNRS), Agence Nationale pour la Recherche (ANR–15–CE13–0012-01, ANR-21–CE13–0028-01), Fondation pour la Recherche Médicale (DEQ20150317337), and Institut Universitaire de France (IUF). M Sittewelle was supported by doctoral fellowships from Fondation pour la Recherche Médicale (FRM ECO20160736105, FRM FDT201904007974). C Zhou was supported by IUF and ANR-21-CE13-0028-01 funding to AH Monsoro-Burq.

Author Contributions

M Sittewelle: conceptualization, data curation, validation, investigation, visualization, methodology, and writing—original draft, review, and editing.

V Kappès: validation, investigation, visualization, methodology, and writing—review and editing.

C Zhou: validation, investigation, visualization, methodology, and writing—review and editing.

D Lécuyer: validation, investigation, visualization, and methodology.

AH Monsoro-Burq: conceptualization, supervision, funding acquisition, validation, investigation, visualization, project administration, and writing—original draft, review, and editing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References

Ali Z, Yousaf N, Larkin J (2013) Melanoma epidemiology, biology and prognosis. EJC Suppl 11: 81–91. doi:10.1016/j.ejcsup.2013.07.012

Bertolotto C (2013) Melanoma. From melanocyte to genetic alterations and clinical options. Scientifica (Catania) 2013: 635203. doi:10.1155/2013/635203

Bruni P, Vandoolaeghe P, Rousseau GG, Hue L, Rider MH (1999) Expression and regulation of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase isozymes in white adipose tissue. Eur J Biochem 259: 756–761. doi:10.1046/j.1432-1327.1999.00104.x

Cancer Genome Atlas Network (2015) Genomic classification of cutaneous melanoma. Cell. 161: 1681–1696. doi:10.1016/j.cell.2015.05.044

Cansado J (2018) To finish things well: Cysteine methylation ensures selective GTPase membrane localization and signalling. Curr Genet 64: 341–344. doi:10.1007/s00294-017-0576-x

Chesney J, Clark J, Klarer AC, Imbert-Fernandez Y, Lane AN, Telang S (2016) Fructose-2, 6-Bisphosphate synthesis by 6-Phosphofructo-2-Kinase / Fructose-2, 6-Bisphosphatase a (PFKFB4) is required for the glycolytic response to hypoxia and tumor growth. Oncotarget 5: 6670–6686. doi:10.18632/oncotarget.2213

Chesney J, Clark J, Lanceta L, Trent JQ, Telang S (2015) Targeting the sugar metabolism of tumors with a first-in-class 6-phosphofructo-2-kinase (PFKFB4) inhibitor. Oncotarget 6: 18001–18011. doi:10.18632/oncotarget.4534

Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, Ivanov IE, Philips MR (1999) Endomembrane trafficking of ras: The CAAX motif targets proteins to the ER and Golgi. Cell 98: 69–80. doi:10.1016/s0092-8674(00)80607-8

Cox AD, Der CJ (2010) Ras history. Small GTPases 1: 2–27. doi:10.4161/sgtp.1.1.12178

Cuomo M, Nicotra MR, Apolloni C, Fraioli R, Giacomini P, Natali PG (1991) Production and characterization of the murine monoclonal antibody 2G10 to a human T4-tyrosinase epitope. J Invest Dermatol 96: 446–451. doi:10.1111/1523-1747.ep12470092

Dai Q, Choy E, Chiu V, Romano J, Silvia SR, Steitz SA, Michaelis S, Philips MR (1998) Mammalian prenyltransferases for carboxyl methyltransferase. in
GIPLFB4 interacts with ICMT in melanoma cells migration

Sittewelle et al.

https://doi.org/10.26508/lsa.202201377

vol 5 | no 12 | e202201377

12 of 13
Rider MH, Bertrand L, Vertommen D, Michels PA, Rousseau GG, Hue L (2004) PFKFB4 interacts with ICMT in melanoma cells migration. Biochem J 381: 561–579. doi:10.1042/BJ20040752

Rider MH, Vandamme J, Lebeau E, Vertommen D, Vidal H, Rousseau GG, Sakata J, Abe Y, Uyeda K (1991) Molecular cloning of the DNA and expression of bovine heart 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase. Head-To-Head with a bifunctional enzyme that controls glycolysis. Biochem J 285: 405–411. doi:10.1042/bj2850405

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Roussos ET, Condeelis JS, Patsialou A (2011) Chemotaxis in cancer. Nature Rev Cancer 11: 573–587. doi:10.1038/nrc31078

Sakata J, Abe U, Uyeda K (1991) Molecular cloning of the DNA and expression and characterization of rat testes 6-phosphofructo-2-kinase, 6-phosphatase. J Biol Chem 266: 15764–15770. doi:10.1016/s0021-9258(18)98475-9

Saraon P, Grozavu I, Lim SH, Snider J, Yao Z, Stagljar I (2017) Detecting membrane protein-protein interactions using the mammalian membrane two-hybrid (MaMTH) assay. Curr Protoc Chem Biol 9: 38–54. doi:10.1002/cpcp.15

Shu Y, Lu Y, Pang X, Zheng W, Huang Y, Li J, Li J, Zhang C, Shen P (2016) Phosphorylation of PPARY at Ser98 promotes glycolysis and cell proliferation in hepatocellular carcinoma by targeting PFKFB4. Oncotarget 7: 76984–76994. doi:10.18632/oncotarget.12764

Sriramaaro P, Bourdon MA (1996) Melanoma cell invasive and metastatic potential correlates with endothelial cell reorganization and tenascin expression. Endothelium 4: 85–97. doi:10.3109/1062339690024685

Strohecker AM, Joshi S, Possemato R, Abraham RT, Sabatini DM, White E (2015) Identification of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase as a novel autophagy regulator by high content shRNA screening. Oncogene 34: 5662–5676. doi:10.1038/onc.2015.23

Tauler A, Lin K, Piliks S (1990) Hepatic 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase. Use of site-directed mutagenesis to evaluate the roles of His-258 and His-392 in catalysis. J Biol Chem 265: 15617–15622. doi:10.1016/s0021-9258(18)54442-9

Theveneau E, Mayor R (2016) Neural crest cell migration. Neural Crest Cells, pp 73–88. doi:10.1007/B978-0-12-401730-6.00004-1

Uranz T, Emkey R, Feig LA (1996) Raf-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. EMBO J 15: 810–816. doi:10.1093/EMBOJ/15.5.810

Valluet A, Durollenne S, Barbotin C, Coriat C, Monsoro-Burq AH, Larcher M, Pouponnot C, Baccarini M, Larue L, Eychene A (2012) 2-Raf and C-raf are required for melanocyte stem cell self-maintenance. Cell Rep 2: 774–780. doi:10.1016/j.celrep.2012.08.020

van Schaftingen E, Lederer B, Bartrons R, Hers H-G (1982) Kinase of 6-phosphofructo-2, 6-bisphosphatase. J Biol Chem 257: 1832–1833. doi:10.1016/0021-9258(82)90139-9

Wang Q, Zeng F, Sun Y, Qiu Q, Zhang J, Huang W, Huang J, Huang X, Guo L (2018) Etk interaction with PFKFB4 modulates chemoresistance of small-cell lung cancer by regulating autophagy. Clin Cancer Res 24: 950–962. doi:10.1158/1078-0432.ccr-17-1475

Yao L, Wang L, Cao Z-G, Hu X, Shao Z-M (2019) High expression of metabolic enzyme PFKFB4 is associated with poor prognosis of operable breast cancer. Cancer Cell Int 19: 195. doi:10.1186/s12935-019-0882-0

Yun SJ, Jo SW, Ha YS, Lee OJ, Kim WT, Kim YJ, Lee SC, Kim WJ (2012) PFKFB4 as a prognostic marker in non-muscle-invasive bladder cancer. Urol Oncol 30: 893–899. doi:10.1016/j.urolonc.2010.08.018

Zhang H, Lu C, Fang M, Yan W, Chen M, Ji Y, He S, Liu T, Chen T, Xiao J (2016a) 2-HIF-1α activates hypoxia-induced PFKFB4 expression in human bladder cancer cells. Biochem Biophys Res Commun 476: 146–152. doi:10.1016/j.bbrc.2016.05.026

Zhang X, Yang X, Yang C, Li P, Yuan W, Deng X, Cheng Y, Li P, Yang H, Tao J, et al (2016b) Targeting protein kinase CK2 suppresses bladder cancer cell survival via the glucose metabolic pathway. Oncotarget 7: 87361–87372. doi:10.18632/oncotarget.13571

CC BY License: This article is available under a Creative Commons License Attribution 4.0 International, as described at https://creativecommons.org/licenses/by/4.0/