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

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Abstract.

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 such as proliferation, survival and migration using downstream effectors among which the PI3K/AKT signaling. In melanoma, oncogenic mutations frequently enhance RAS, PI3K/AKT or MAP kinase signaling, in addition to other cancer hallmarks including the activation of metabolism regulators such as PFKFB4, a critical regulator of glycolysis and Warburg effect. Here, we explore a novel function of PFKFB4 in melanoma cell migration. We find that instead of acting as a kinase as recorded in glycolysis, PFKFB4 interacts with ICMT, a post-translational modifier of RAS. PFKFB4 promotes ICMT/RAS interaction, controls RAS addressing at the plasma membrane, activates AKT signaling and enhances cell migration. We thus evidence a novel 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.

Highlights.

- PFKFB4, a known regulator of glycolysis, displays an unconventional role in melanoma cell migration.
- PFKFB4 interacts with ICMT by protein-protein interactions and promotes RAS addressing at the plasma membrane.
- PFKFB4 and ICMT cooperation modulates AKT signaling and controls melanoma cell migration.

Introduction.

Cell migration is one of the critical processes involved in the formation and maintenance of multicellular organisms. When they acquire motility, cells activate complex properties such as cytoskeleton remodeling, inhibition of cell-cell contacts, remodeling the extracellular matrix and response to chemo-attractants (*1*, *2*). To orchestrate these processes, numerous redundant and complementary signaling pathways cooperate. While many of these pathways have been studied separately, the crosstalk between signaling and other parameters such as cell cycle or cell metabolism only begins to be explored in stem cells, normal development and cancer(*3*). Cell motility and invasiveness properties are reactivated during cancer progression, following the aberrant activation of multiple cellular programs, such as growth factor-independent signaling, metabolic and epigenetic reprograming, which cooperate to sustain growth, proliferation, and survival properties in the primary tumor (*3*). Metastasis and formation of secondary tumors are the major cause of death for aggressive cancers such as cutaneous melanoma (*4*). A clinical strategy to target melanoma is to develop therapies targeted against identified driver mutations. The numerous genetic alterations involved in cutaneous melanoma development can be classified into four subtypes: BRAF, RAS (N/H/K), NF1 and Triple-WT (*5*). The mitogen-activated protein kinase (MAPK) pathway is the most frequently altered with about 50% of tumors mutated in *BRAF* gene, followed by about 25% with mutations in NRAS and 14% in NF1, leaving about 10% of the tumors with no identified driver mutation and more complex genetic landscapes (*5*, *6*). Although RAS signaling acts upstream of both MAPK and PI3K/AKT signaling (*7*), it is interesting to note that the two main *NRAS* activating mutations in cutaneous melanoma, the hotspots Q61 (80% of *NRAS* subtype tumors) and G12 (15%) drive a different activation of the downstream pathways with preferential activation of MAPK or PI3K/AKT respectively, suggesting a complex modulation of the structure and activity of oncogenic proteins (*8*, *9*). In all cases, these alterations in signaling lead to increased cell proliferation, survival and migration.

In addition to mutations activating signaling pathways, metabolism rewiring allows cancer cells to promote active cell proliferation. In particular, enhanced glycolysis rate even under normal oxygen conditions, called Warburg effect, drives many parallel biosynthesis pathways to provide cell building blocks together with bioenergy (*10*). Moreover, the hypoxic environment often found in early primary tumors prior to vascularization, also stimulates the activation of metabolic regulators induced by the hypoxia-inducing factor HIF1. This is the case for the family of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatases enzymes (PFKFB1-4) which are major regulators of glycolysis, controlling the rate of the second irreversible and rate-limiting reaction of glycolysis catalyzed by the phospho-fructokinase 1 (PFK1). PFKFB enzymes are bi-functional and synthesize (with kinase activity) or degrade (with phosphatase activity) the fructose-2,6-biphosphate which is the main allosteric activator of PFK1. Thus, increased PFKFB protein kinase activity promotes glycolysis. In human, four distinct genes encode PFKFB isoenzymes 1 to 4, each one possessing many splicing isoforms and differing in their tissue-specific abundance, kinetics and regulation properties (*11–16*). PFKFB proteins are overexpressed in cancer. In particular, PFKFB4 increased levels have been reported in several human tumors including cutaneous melanoma (*17–20*). Moreover, *PFKFB4* is induced by hypoxia, is required

for survival and proliferation of normal thymocytes (*21*) as well as of several cancer cell lines such as lung, breast and colon adenocarcinomas, prostate and bladder cancer (*22–25*). So far, most studies have focused on cell metabolism reprogramming by PFKFB4 and have proposed that PFKFB4 is a major driver of Warburg effect (*17*, *19*, *20*, *22–30*). However, five recent studies have identified alternative functions of PFKFB4, outside of its canonical control of glycolysis. For example, PFKFB4 regulates small lung-cancer chemoresistance by stimulating autophagy, via its interactions with Etk tyrosine kinase (*31*, *32*). PFKFB4 also operates as a protein kinase and directly phosphorylates SRC-3, promoting metastatic progression in highly glycolytic breast cancer cells (*33*). During development, PFKFB4 is essential for early embryonic inductions and neural crest cells migration by the activation of AKT signaling (*34*, *35*). In cancer, the intriguing relationships between PFKFB4, cell signaling and cell migration remain unexplored.

Here we have explored the importance of PFKFB4 in melanoma cell migration. Using human metastatic melanoma cell lines with high *PFKFB4* expression, 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, among which we validate the protein-protein interactions between PFKFB4 and isoprenylcystein carboxymethyl transferase (ICMT), an enzyme essential for RAS post-translational modifications controlling its addressing to 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.

PFKFB4 controls metastatic melanoma cell migration in vitro in a glycolysis-independent manner.

Melanomas present higher expression of *PFKFB4* compared to other tumors (Figure S1). We have previously linked elevated expression of PFKFB4 with embryonic cell migration *in vivo* (*35*), but its role in melanoma had not been explored before. Here we have chosen two human melanoma cell lines with relative high levels of PFKFB4 (Figure 1Aa,b) to follow the random migration of individual cells by time-lapse video microscopy (Figure 1B). The Mewo cells are tumorigenic and metastatic, derived from a metastasis of cutaneous melanoma in lymph node, and bear wild-type alleles at BRafV600 or RasQ61 positions ((*36*), Figure S2A). The A375M cells are tumorigenic and metastatic, derived from human amelanotic melanoma. They are mutated for BRafV600 and wild-type for RasQ61 (*36*). Both cell lines actively migrated on Matrigel. Cells were tracked during 16 hours in at least three independent experiments for each cell line. After PFKFB4 depletion using siRNA, both Mewo and A375M cells migrated twice slower than control (Figure 1A-B a,b,c,; Table S1). Migration distance and cell pausing parameters were also affected (Figure S2C). This migration phenotype was efficiently rescued by co-transfecting the *Xenopus laevis* orthologous *pfkfb4* sequence (Figure 1B-S2B,C). Frog *pfkfb4* encodes a protein with 95% similarity with the human protein, but the mRNA was not targeted by siRNAs designed against the human mRNA sequence. This rescue showed that the migration phenotype was specific for the depletion of PFKFB4 protein and did not come from off-target effects.

We then investigated if PFKFB4 played a role in cell migration in link with its function as a major activator of the glycolysis rate. To block glycolysis, we first cultivated the Mewo cells in a medium without glucose, which strongly decreased their glycolysis rate (estimated by the diminished lactate production measured in the culture medium, Figure S2D,E). We did not observe a major decrease of the Mewo cells average speed of migration in the glucose-free medium compared to the complete medium conditions (Figure 1Bd). To confirm that the Mewo cell migration was unaffected by limiting glycolysis, we then added the glucose non-hydrolysable analog 2-deoxyglucose (2DG) in the complete medium (Figure S2D, F). Similarly to the glucose-free condition, 2DG also decreased glycolysis efficiently (Figure S2D) without affecting Mewo cell speed of migration (5 mM 2DG, Figure 1Be). These results indicated that Mewo cells migration was not closely linked to their rate of glycolysis. Furthermore, PFKFB4-depleted Mewo cells showed a reduced average migration speed compared to control cells in the glucose-free medium as observed in the complete medium (Figure 1B). Together, these observations suggested that PFKFB4 levels significantly affect the average speed of cells migration, independently of the rate of glycolysis.

PFKFB4 is a bi-functional enzyme controlling the second rate-limiting reaction of glycolysis. With its kinase moiety, PFKFB4 phosphorylates fructose-6-phosphate into fructose-2,6-bisphosphate, the allosteric activator of PFK1 ((*14*, *37*), Figure S2D). With its phosphatase domain, PFKFB4 catalyzes the reverse reaction. To understand if PFKFB4 controlled melanoma cells migration using either of these enzymatic activities, we compared the rescue phenotype after PFKFB4 depletion by various *X. laevis* PFKFB4 mutants (Figure S2B). The average speed of cells co-transfected with siPFKFB4 and a *pfkfb4* mutant with two point mutations inactivating both kinase and phosphatase

enzymatic activities simultaneously (mutations G48A and H258A; called xPFKFB4-DBM; (*38*, *39*)) was equivalent to the speed after rescue by wild-type PFKFB4 (xPFKFB4) (Figure 1B). This indicated that PFKFB4 controlled cell migration independently of its enzymatic activities. To identify which part of PFKFB4 protein was involved in this non-conventional 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 (Figure 1B). 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 (Figure 1B). This result suggested that PFKFB4 was involved in control of cell migration independently of its kinase or phosphatase activities, but through its N-terminal part.

PFKFB4 interacts with ICMT, a major post-translational modifier of RAS GTPases.

As PFKFB4 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 1556 high confidence hits, we chose the 41 ones with a Mascot score enriched at least ten-times compared to the negative control condition in order to eliminate the weak hits and limit the non-specific targets. Finally we crossed this list with targets obtained in Mewo cells transfected with the frog PFKFB4 ortholog following the reasoning that the protein function we looked for was evolutionary conserved between human and *x. laevis* PFKFB4 (Figure 2A-S3). Among the 23 remaining best candidates, we prioritized isoprenylcystein carboxyl methyl transferase (ICMT), a potential modulator of PI3K/AKT signaling pathway, because PFKFB4 is known to affect cell migration via AKT signaling activation during embryogenesis (*34*, *35*) ICMT is an endoplasmic reticulum membrane protein critical for RAS GTPases post-translational modifications. ICMT catalyzes the carboxyl methylation of RAS on its C-terminal CAAX motif. This modification allows RAS protein to be addressed to the plasma membrane, a prerequisite for the coordination by RAS of variety of signaling, including PI3K/AKT activation ((*40–43*), reviewed in (*44*)). This observation suggested that an interaction between PFKFB4 and ICMT could occur during melanomagenesis and be related to RAS-dependent signaling pathways.

First, we confirmed the mass spectrometry results by co-immunoprecipitation of ICMT with PFKFB4 in Mewo cells (Figure 2B). Secondly, to confirm the protein-protein interaction between PFKFB4 and ICMT with an alternative method, we used a two-hybrid assay adapted to mammalian cells (Figure 2C). ICMT and PFKFB4 were cloned in-frame either to GAL4 DNA-binding domain (DBD, pBind plasmid) or VP16 activation domain (pAct plasmid) and co-transfected with a luciferase reporter driven by GAL4 binding sites (pG5) into HEK293T cells. When candidate proteins interact, they link the GAL4-DBD to the strong transcriptional activator VP16 resulting in the transcriptional activation of GAL4-driven luciferase. As negative controls, PFKFB4 fused to GAL4-DBD (pBind-PFKFB4) was co-transfected with an empty pAct backbone or ICMT was fused to VP16 activation domain (pAct-ICMT) and co-transfected with empty pBind vector. Both negative controls drove background-level luciferase signals, as did the co-transfection of the empty pAct and pBind alone. In contrast, serving as a positive control, the co-transfection of pBind-PFKFB4 and pAct-PFKFB4 increased luciferase expression by

six-times compared to the negative controls, which denotes a strong and stable interaction, related to the formation of the PFKFB4 homodimer. Finally, the co-transfection of pBind-PFKFB4 and pAct-ICMT significantly increased luciferase expression compared to controls, albeit at a lower level than the PFKFB4-PFKFB4 positive control, suggesting weaker or more transient protein-protein interaction. Together, these results demonstrated that PFKFB4 and ICMT directly interact in melanoma cells. Finally, 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 (Figure 2D). In sum, all these results suggested that PFKFB4 direct protein-protein interactions with ICMT impact ICMT-RAS complex formation in melanoma.

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

To understand the role of the interaction between PFKFB4 and ICMT, we first compared PFKFB4 and ICMT depletion phenotypes in Mewo cells, using a validated siRNA against ICMT (*45*). Parameters of cell migration were measured as previously. Compared to control siRNA, cells transfected with siICMT exhibited a decrease in their average speed of migration similar to cells transfected with siPFKFB4 (Figure 3A, Figure S5). To test the interdependency of PFKFB4 and ICMT, we co-transfected both siRNAs. Melanoma Mewo cells receiving both siPFKFB4 and siICMT do not exhibit a more severe phenotype than with either siRNA alone. This suggested that PFKFB4 and ICMT cooperate in the same pathway to control cell migration and that depleting either one is sufficient for attaining a strong phenotype (Figure 3A). To further test this hypothesis, we tested the epistasis between PFKFB4 and ICMT by combining depletion of one factor and gain-of-function of the other, in order to see if increased activity of either one of these proteins could compensate for the loss of the other, as could be the case if they were acting in parallel and redundant pathways: the siPFKFB4 was co-transfected with the *Icmt* expression plasmid, or the siICMT with the *pfkfb4* expression plasmid. When compared to the speed migration of Mewo cells transfected with a control siRNA and the corresponding siRNA alone, neither ICMT nor PFKFB4 gain of function rescued the phenotype of PFKFB4 or ICMT depletion respectively (Figure 3A). This suggested two alternatives: 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 to the known effect of ICMT depletion, as the major role of ICMT is to modify RAS proteins post-translationally for their efficient targeting to the plasma membrane (*42*). In order to differentiate between potential defects in RAS GTPase activity and defective subcellular localization, 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 a control siRNA, we scored RAS localization to the plasma membrane qualitatively (Figure 3B). The cells transfected with the control siRNA could be categorized in three groups: cells exhibiting a clear and homogeneous

RAS membranous localization (phenotype 1), cells without RAS membrane enrichment (phenotype 3), and cells with an in-between phenotype with discontinuous RAS membrane localization (phenotype 2) (Figure 3C). While this phenotype 2 was represented in approximately the same 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 (Figure 3B). The remaining cells displayed the phenotype 3. This assay thus showed that PFKFB4 depletion phenocopied ICMT loss for RAS addressing to the plasma membrane of melanoma cell.

Finally, we reasoned that if the main function of the PFKFB4-ICMT interaction was indeed to promote RAS localization at the plasma membrane, a form of RAS with constitutive plasma membrane addressing independently of ICMT maturation should rescue the migration phenotype of PFKFB4 depletion. For example, myristoylated RAS does not depend upon processing at the ER membrane and methylation by ICMT contrarily to prenylated (CAAX) proteins ((*46*), Figure S4). In order to eliminate a potential interference with the GTPase function of RAS and distinguish solely defects in protein addressing, we selected two forms of RAS with constitutive GTPase activity (ca) due to an activating mutation on the V12 residue (*46*). We thus repeated the above experiments and measured cell migration efficiency, after co-transfecting siPFKFB4 with either a myristoylated form of constitutively active RAS, which is also mutated on C186 to avoid additional prenylation (myr-RASV12C186S) or a form of ca-RAS needing modification by ICMT to be correctly addressed (RASV12). Only the myr-RASV12C186S form rescued the migration phenotype of PFKFB4 (Figure 3A). Altogether, we concluded that the interaction between PFKFB4 and ICMT was critical for controlling RAS subcellular localisation 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 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 (*47–49*). 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. The phosphorylation of ERK (pERK) remained unchanged in cells transfected with siPFKFB4 or with siICMT (Figure 4C). In contrast, we observed a strong decrease in AKT phosphorylation on both serine 473 and threonine 308, the two major modifications leading to AKT full activation (Figure 4A, Figure S5). To extend this finding to other melanoma cell contexts, we examined AKT activation in three other cell lines after PFKFB4 depletion. In all cell lines, siRNA against PFKFB4 reduced target protein levels efficiently (Figure S5). PhosphoAKT levels were significantly decreased in A375M and MNT1 cells while there was no significant decrease in Lu1205 (Figure 4B). This suggested that PFKFB4 influenced AKT signaling in several but not all melanoma cell contexts. Although it remains unclear why MAP kinase signaling remains unaffected while AKT pathway is decreased in this particular context, other similar examples have been described in the literature (*8*, *9*).

We then wondered if PFKFB4 might affect AKT signaling as an indirect effect of glycolysis regulation. We thus blocked glycolysis with 2DG and tested AKT activation in Mewo cells. Although decreased lactate levels indicated an efficient block of glycolysis (Figure S2F), the treatment with 2DG did not affect AKT phosphorylation on S473 (Figure 4D). As observed before for the cell migration phenotype (Figure 1), the effect of depleting PFKFB4 on AKT activation was thus not due to a reduction of glycolysis rate. Finally, 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 (Figure 4E). All these data together indicated that PFKFB4 controlled human melanoma cell migration via the RAS/PI3K/AKT pathway.

Discussion.

PFKFB proteins are long-known major regulators of the rate of glycolysis in normal and cancer cell types (*11*). They have been involved in mediating the Warburg effect in many different tumors (*17*, *19*, *22*, *25*, *30*, *50*). In particular, elevated levels of *PFKFB4* expression have been described in melanoma (Figure S1, (*30*)). Using transcriptomics analysis to identify human melanoma cell lines with high *PFKFB4* levels (*36*), we have explored the function of PFKFB4 in the biology of those cells, focusing on their migratory capacity. We first showed that PFKFB4 enhanced cell migration irrespective of the cells glycolysis levels (Figure 1, 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 selected for further analysis: ICMT, an enzyme embedded into the endoplasmic reticulum membrane, which adds the terminal methyl group to RAS GTPases post-translationally. This modification is required for RAS GTPases anchorage at the plasma membrane, where RAS activates several downstream signaling events (*7*). Strikingly, both PFKFB4 and ICMT depletion displayed similar phenotypes including RAS mislocalization, decreased AKT activation and reduced cell migration (Figures 2, 3, 4). Migration phenotypes were rescued by a modified RAS protein targeted to the plasma membrane independently of the processing by ICMT or by the constitutive activation of AKT signaling (Figure 5). In sum, our study has demonstrated a novel, glycolysis-independent function of PFKFB4, promoting the interaction between ICMT and RAS, resulting in enhanced migration of melanoma cells (Figure 5).

Importantly, the regulation of cell migration by PFKFB4 did not depend on PFKFB4 kinase activity (Figure 1). In addition, PFKFB4 depletion affected migration independently of experimental modulations of the high/low glycolysis status of the cells (Figures 1, S2). Our results thus uncouple the generally known PFKFB4 action in glycolysis from its role in cell migration. This would lead to re-examine the strategy generally chosen to target PFKFB4 activity in cancer. Indeed, assuming that PFKFB4 played a major function to promote glycolysis and therefore Warburg effect, a few pharmacological drugs were recently developed to interfere with PFKFB4 kinase activity (*28*). Our data suggested that a kinase-deficient form of PFKFB4 still retained 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 (*40–42*). One third of cutaneous melanoma are mutated in RAS, PI3 kinase or other partners of this signaling pathway (*5*, *6*). 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, increasing PFKFB4 cellular levels might allow enhanced RAS-linked signaling and cell migration. Increase in *PFKFB4* gene expression can be achieved by hypoxia, a general feature of growing tumors. HIF1a-responsive elements have been identified to promote *pfkfb4* transcription (*18*). While in normal cells, there is a fine-tuned, dynamic and tissue-specific expression of PFKFB genes during development and cell homeostasis (*34*, *51*, *52*), 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 addition 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 (Figure 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 (*34*, *35*). Melanoma initiation and progression involves the reactivation of elements belonging to the neural crest developmental program (*53*). 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 (*54*). 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 non-conventional function of PFKFB4. This function involved enhancing AKT signaling and cell migration (*34*, *35*). It remained unclear whether this novel function of PFKFB4, found in a non-mammalian *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 non-conventional signaling functions, a term naming a function which is revealed when the major "sunlight" function masked (here, the key control of glycolysis rate by PFKFB proteins).

While we stress the importance of the PFKFB4-ICMT-RAS-AKT signaling pathway, we do not exclude important functions for other RAS-regulated signaling proteins that modulate cell migration, such as Rac1 Rho GTPase, which also happens to be processed by ICMT (*55*). Further studies will compare the relative functions and potential cooperation of these alternative pathways. Finally, our mass spectrometry screen for PFKFB4 partners has provided two dozen other candidates for interacting with PFKFB4. This list is enriched in proteins involved in cell trafficking or cell migration (Figures 2, S3). This is striking, as increasing evidence shows the importance of RAS signaling linked

to endomembranes such as ER or Golgi apparatus, in addition to its prominent function at the plasma membrane (*56–58*). Hence, although we have not detected direct interactions between PFKFB4 and RAS GTPase, this raises the intriguing possibility that PFKFB4 also plays roles in RAS signaling at these levels. The functions of PFKFB4 in these disparate locations remain to be explored.

In conclusion, our study highlights an unsuspected link between major hallmarks of cancer cells, namely cell metabolism, signaling and migration. The crosstalk between key regulators of glycolysis and Warburg effect (PFKFB4) and a pleiotropic cell signaling pathway (ICMT-RAS) further increases the complexity of the network known to promote melanoma cancer progression. Those intricate relationships, which might also act as re-wiring options upon cancer 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, the Check-Mate Mammalian Two-Hybrid System (Promega) was employed. Cloning used Gibson method ((*59*), primers used in Table S3). hICMT (clone Origene n°RC207000) and hPFKFB4 (clone Origene n°RC201573) were inserted in-frame into the pBIND backbone vector, which contains the yeast GAL4 DNA-binding domain, or into the pACT backbone vector, which contains the herpes simplex virus VP16 activation domain and a nuclear localization sequence.

Cell lines, cell culture, cell treatments, cell transfection.

The well-characterized human melanoma cell lines Mewo (*60*, *61*), A375M (*62*), MNT1 (*63*), Lu1205 (*64*) were kindly provided by Dr. L. Larue (*36*). Their mutagenic status for key driver mutations in melanoma is summarized in Figure S2 (*36*). Cells were cultured in RPMI (Gibco) supplemented with 10% SVF and 1% penicillin/streptomycin (Invitrogen). HEK293T cells were cultured DMEM (Gibco) supplemented with 10% SVF and 1% penicillin/streptomycin (Invitrogen). All cell lines were incubated at 37°C with 5% CO2. At 24 hours before transfection, cell lines were plated at 200,000 cells per well (A375M and HEK293T) or 300,000 cells per well (MNT1, Mewo, Lu1205) into six-well plates. For siRNA experiments, cells were transfected either with a control siRNA (Stealth Negative Control Medium GC Duplex, Invitrogen) or with siPFKFB4 (Dharmacon Smartpool siGenome D-006764-01/02/04/17, siPFKFB4 (1); Invitrogen #HSS107863, siPFKFB4 (2)), or with siICMT (Dharmacon Smartpool siGenome #M-005209-01-0010) (Figure S7) using lipofectamine RNAimax (Invitrogen) according to the manufacturer's instructions. For gain-of-function experiment, cells were transfected with a total of 0,5 to 1µg of DNA using lipofectamine 2000 (Invitrogen). For Check-Mate experiments, 0.5 µg of each plasmid (pBind/pG5 or pAct/pG5) were transfected using lipofectamine 2000. For glycolysis blockade, a RPMI glucose-free medium was used (Gibco). Alternatively, 2-5mM of 2-deoxy-glucose (SIGMA) was added to the normal medium. All experiments were analyzed 48h after transfection (Figures 1-4).

Luciferase assay.

A luciferase reporter driven by five GAL4 binding sites (pG5-luc) was co-transfected into HEK293T cells together with the plasmids to be tested and a control plasmid coding Renilla luciferase for normalization of the signals. Cell medium was changed 24h after transfection. At 48 hours, cells were rinsed with PBS and lysed with passive lysis buffer 1X (Promega) for 15 minutes with agitation at room temperature. Firefly and Renilla luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). For each condition, signal intensity was normalized by the ratio between Firefly and Renilla luminescence. Transfections were performed in triplicate, each with technical duplicates.

Protein extraction and Western-blotting.

Cells were washed in PBS and lysed in RIPA buffer (10mM Tris-HCL pH 8, 150 mM NaCl, 1% NP-40, 0,1% SDS, 0,5M sodium deoxycholate) supplemented with phosphatases inhibitor (Sigma) and proteases inhibitors (Sigma) at 4˚C. Protein samples were resolved on 12 % SDS-PAGE gels and transferred to PVDV membranes (Biorad). After blocking in 5% skimmed milk diluted in TBS - 0,1% Tween (TBS-T), membranes were probed with primary antibody diluted in the blocking buffer overnight at 4°C (dilutions are indicated in Table S4). After three washes in TBS-T, membranes were probed with HRP-conjugated goat anti-rabbit or anti-mouse (1:20000) 1 hour at room temperature. ECL signal was quantified by densitometric analyses using Image J software (http://rsb.info.nih.gov/ij/).

Co-Immunoprecipitation and mass-spectrometry.

At 48h after transfection, total proteins were extracted using a mild lysis buffer (100mM NaCl, 0,5% NP40, 20mM Tris-HCl pH 7,5, 5mM $MgCl_2$, protease inhibitors and phosphatases inhibitors). Cells were lysed by mechanical passages through a 26-gauge syringe. For each sample, 20 µl of FLAG-M2 magnetic beads (Sigma M8823) previously washed in lysis buffer were added and incubated with agitation overnight at 4°C. Beads were then washed five times in lysis buffer. For mass spectrometry analysis, beads were further washed twice with H_2O. Proteins were digested with trypsin, desalted using ZipTip C18 and analyzed using a nanoESI-Orbitrap Fusion (ThermoScientific). Data were analyzed using Mascot (Matrix Science). Mascot scores of the negative control, here tagged-V5 PFKFB4, were compared to the sample tagged-FLAG PFKFB4 protein, as advised by the platform. For analysis by Western-blotting, the co-immunoprecipitated proteins were eluted by boiling 10 min in Laemmli buffer (50mM Tris pH 6.8, glycerol, 2% SDS, 3% DTT, bromophenol blue), or eluted by competition with Flag peptide at 200ug/ml (five incubations of 5 minutes, P4799, Sigma). Samples were finally concentrated using 3KDa columns (Millipore).

RNA extraction and RT-qPCR.

Total RNA was extracted from cell lysed in Trizol® (Thermofisher), and then purified by chloroform extraction and isopropanol precipitation. We used M-MLV reverse transcriptase (Promega) for reverse transcription and SYBR Green mix (Biorad) for quantitative PCR. Results were normalized against reference genes *tbp* and *18S* (see Table S5 for sequences).

L-lactate dosage.

Cell supernatant was collected 24h after changing cell medium then immediately filtered by centrifugation for 15 minutes at 4°C on a 10KDa column (Abcam or Millipore). This step eliminates proteins, including Lactate Deshydrogenase (LDH) to avoid non-specific L-Lactate degradation in the sample. Extracellular L-Lactate concentration was measured from the filtered medium using L-Lactate kit (Abcam ab65330).

Two-Dimensional random cell migration assayed by time-lapse video microscopy.

We dispensed 50,000 cells per well into twelve-well plates coated with Matrigel (FisherScientific). 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 (CCD) CoolSnap camera (Photometrics). Images were acquired at 8-minute intervals during 16 hours, with the Metamorph software (Molecular Devices). Movies were reconstructed with the ImageJ software (http:// rsbweb.nih.gov/ij/). Cells were tracked manually and parameters were calculated with ImageJ Manual Tracking plug-in.

Immunofluorescence.

Mewo cells were plated 24h before transfection on glass-coverslips. At 48h after transfection, cells were rinsed with PBS, fixed with paraformadehyde 4% for 15 minutes. After PSB wash, cells were permeabilized and non-specific protein binding blocked in 10% SVF, 0,1% Triton in PBS for 1h at room temperature. Then, cells were incubated at room temperature for 1 hour with primary antibodies diluted in blocking buffer (Table S4), rinsed with PBS, incubated 1h at room temperature in dark in secondary antibody at a 1:1000 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 1µg/ml in PBS for 10 minutes at room temperature. Coverslips were then mounted using ProLong Diamond (Molecular Probes), and imaged with 63X or 100X oil immersion objective of a widefield microscope (DM RXA, Leica; camera CoolSNAP HQ, Photometrics), using Metamorph software.

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Figure Legends.

Figure 1. PFKFB4 controls in vitro cell migration in metastatic melanoma in a glycolysis-independent manner.

A) Quantification of PFKFB4 mRNA and protein levels in four human melanoma cell lines: MNT1, A375M, Mewo and Lu1205. a) Relative *PFKFB4* mRNA levels measured by RT-qPCR and normalized by the expression of 18S and TBP housekeeping genes. Error bars: S.E.M. b) PFKFB4 protein levels detected by Western-blotting, with PFKFB4/actin relative quantification (optical density). c) PFKFB4 protein levels in Mewo and A375M cells 48h after transfection with siRNA targeting PFKFB4. B) Starting 48h after transfection, cell migration was tracked for 16 hours from phase contrast images. Each point corresponds to the average speed of one cell. Mewo (b,d,e,f) or A375M (c) cells were co-transfected either with siControl/empty plasmid, siPFKFB4/empty plasmid or with siPFKFB4 together with a *X. laevis* PFKFB4 plasmid in its wild-type form (b, c, f) or mutant forms (f). The average speed was also measured when cells were cultured in glucose-free medium (d) or complete medium supplemented with 2DG (e). In each panel, a representative experiment is shown (n>3), and displays mean ± SEM. p-values were calculated using the Mann-Whitney test. n.s.: $p > 0,05$; *: $p < 0,05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Figure 2. PFKFB4 interacts with ICMT, a major post-translational 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 two-hybrid strategy used to validate PFKFB4/ICMT protein-protein interactions. Luciferase activity was measured and normalized from HEK293T cells extracts. Two representative biological replicate are shown with mean ± SEM (Mann-Whitney test. *: $p < 0,05$). **D**) The interaction between tagged ICMT and endogenous RAS was evaluated with or without PFKFB4 depletion. Immunoprecipitation of FLAG-ICMT from Mewo cells followed by Western-blotting with antibody against either V5, FLAG or endogenous RAS.

Figure 3. ICMT and PFKFB4 both control RAS addressing at the plasma membrane and melanoma cell migration.

A) Average speed of Mewo cells transfected with siRNA (siCtrl, siPFKFB4 or siICMT) and plasmid (empty vector, hICMT-myc-Flag, hPFKFB4-myc-Flag, RasV12 or myr-RasV12C186S). Graphs show the mean calculated in one experiment performed at least in triplicate with at least 30 cells in each condition, Error bars are calculated with SEM. p-values are calculated using the Mann-Whitney test. n.s.: $p > 0,05$; *: $p < 0,05$; **: $p < 0.01$; ***: $p < 0.001$ and ****: $p < 0.0001$.

B) Detection of RAS subcellular localization by immunostaining on Mewo cell transfected with RasV12-HA (yellow). Nuclei were stained with DAPI (blue). At the plasma membrane, RAS was found distributed according to three main phenotypes: either a clear and homogeneous membrane localization (Phenotype 1), or the absence of signal (Phenotype 3), or an intermediate phenotype with intermittent RAS expression at the membrane (Phenotype 2). Insets show enlargements of the areas framed in red. The proportion of each phenotypes was quantified after transfection of either siControl,

or siICMT or siPFKFB4 (n=3).

Figure 4. PFKFB4 and ICMT both control AKT signaling activation in melanoma cells.

A) Protein levels of pAKT T308, pAKT S473, AKT and actin 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 hours. **E)** Average speed of Mewo cells transfected either with siControl+empty vector, siPFKFB4+empty vector, siPFKFB4+xenopus PFKFB4 wild-type, siPFKFB4+ca-AKT, 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 **** $p < 0.0001$.

Figure 5: Model of cell migration control by a non-canonical function of PFKFB4, modulating RAS signaling.

We propose that the interaction between PFKFB4 and ICMT would promote the ICMT/RAS interaction needed for RAS trafficking to the plasma membrane, where RAS would activate PI3K-mediated AKT phosphorylation on T308. In turn, AKT activation would then modulate cell migration. PTEN, Phosphatase and Tensin homolog; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PDK1, Phosphoinositide-dependent kinase-1; PP2A, Protein phosphatase 2; PHLPP, PH domain and Leucine rich repeat Protein Phosphatase ; mTORC2, mTOR complex 2; Rce1, Ras converting enzyme 1 ; ICMT, isoprenylcysteine carboxyl O-methyltransferase.

Supplementary Materials

Supplementary Figure Legends

Figure S1: Melanoma exhibit high expression of *PFKFB4 mRNA.*

Compared to other tumors, melanoma express high levels of *PFKFB4*, followed by gliomas. *https://portals.broadinstitute.org/ccle/*

Figure S2: PFKFB4 controls metastatic melanoma cell migration in vitro in a glycolysis-independent manner.

A) Metastasis properties and mutational status of the cell lines used in this study. Sequencing results for key melanoma driver mutations BRafV600E and RasQ61 locus were retrieved from [34] and ATCC database). NA: non-available data. **B**) Schematic structure of the *Xenopus laevis* PFKFB4 wild-type protein (xPFKFB4) and V5-tagged xPFKFB4 mutants. The *x*PFKFB4-DBM-V5 (G48A;H258A mutant is both kinase-dead and phosphatase-dead. The xPFKFB4-Nter-V5 is a truncated form of PFKFB4 with the kinase domain conserved. xPFKFB4-Cter-V5 is a truncated form of PFKFB4 with the domain phosphatase domain conserved. **C**) Travel distance and pausing were also measured Mewo cells: 48h after transfection either with siControl+empty vector, siPFKFB4+empty vector or siPFKFB4+xenopus PFKFB4 wild-type. Graphs show the mean ± SEM. Each graph represents one experiment performed more than three times. **D**) Summary of glycolysis. The rate-limiting step of glycolysis is the second irreversible reaction, catalyzed by phosphofructokinase-1 (PFK1). PFK1 activity depends on the availability of its allosteric regulator, fructose 2,6-bisphosphate. PFKFB4 catalyses the synthesis or degradation of fructose 2,6-bisphosphate. The 2-deoxyglucose (2DG) is a glucose analog that cannot be metabolized. 2DG blocks glycolysis by competition with cellular glucose. At the end of the glycolysis, pyruvate is transformed into lactate, which is secreted in the extracellular medium. Lactate levels are a readout for the rate of glycolysis. **E**) Relative extracellular L-Lactate levels in Mewo cells cultured either in a complete medium (reference) or in glucose-free medium. **F**) Relative level of extracellular L-lactate of Mewo cells treated with different concentrations of 2DG.

Figure S3: Results of IP PFKFB4 followed by mass spectrometry

A) List of the top-41 candidate interactants of PFKFB4. We divided the result list into two parts. On the left, common targets found when the immunoprecipitation is done with xenopus or human PFKFB4 (23 targets in light green). On the right, targets only found with human PFKFB4 (18 targets in dark green). **B, C**) Gene ontology analysis of potential partners of PFKFB4 – EnrichR results. **B)** GO Biological process 2018 of EnrichR datas, sorted by p-value ranking. **C**) GO Cellular component 2018 of EnrichR datas, sorted by p-value ranking.

Figure S4. ICMT-dependent and -independent post-translational modifications of RAS.

RAS proteins contain a CAAX motif. They are translated in the cytoplasm and directly prenylated by specific enzymes such as Protein Farnesyltransferases (PFTases) to be anchored in the endoplasmic reticulum (ER) membrane. In the ER membrane, the CAAX motif is first cleaved by Rce1 followed by

methylation by ICMT. Once methylated, RAS can be targeted at the plasma membrane. After methylation, KRAS is directly sent to the plasma membrane (independently of further processing in the Golgi apparatus), while NRAS and HRAS are sent to the Golgi where they are palmitoylated and targeted to the plasma membrane. At the plasma membrane, RAS proteins coordinate a variety of signaling cascades, such as the MAPK, Ral and PI3K/AKT pathways. In contrast, myristoylation targets proteins to the plasma membrane without ER processing. Adding a myristoylation signal on RAS N-terminal part leads to its modification by a N-myristoyltransferase (NMT) and its anchorage to the plasma membrane. The additional C186S mutation is necessary to prevent further RAS prenylation, which has been shown to block its GTPase enzymatic activity (Cadwallader, 1994).

Figure S5: PFKFB4 and ICMT control AKT signaling in melanoma

A) Control of the efficiency of siRNAs targeting PFKFB4 in different melanoma cell lines by Western-blot 48h after transfection. B) Average speed of Mewo cells 48h after transfection either with siControl+empty vector or siCtrl+ca-AKT. This experiment shows that ca-AKT alone does not increase the average speed of Mewo cells. Graphs show the mean ± SEM. p-values calculated using the Mann-Whitney statistical test. n.s. $p > 0,05$. C) Protein levels pAKT S473, AKT and actin in Mewo cells transfected with two different siRNA targeting PFKFB4. D) Control of the efficiency of siRNA targeting ICMT in Mewo cells.

Supplementary Tables

Table S1: small interfering RNAs.

siRNA Reference	Sequences	Targeted Exon
siPFKFB4 (1) Dharmacon Smartpool D-006764-01/02/04/17	GAGCGACCATCTTTAATTT	5
	CATCGTATATTACCTCATG	8
	GAAATGACCTACGAGGAAA	10
	GGGACAGGCCTCAGAACGT	13-14
siPFKFB4 (2) - Invitrogen #HSS107863	GACCTACGAGGAAATTCAGGATAAT	10
siICMT- Dharmacon Smartpool #M-005209-01-0010	N/A (Court et al., 2017)	

Table S2: Plasmids

Plasmid Name	Reference
Xenopus pcDNA3-PFKFB4wt-V5	this work-Gateway cloning
Xenopus pcDNA3-PFKFB4-DBM-V5 (G48A;H258A)	this work-Gateway cloning
Xenopus pcDNA3-PFKFB4-Nter-V5 (aa1-251)	this work-Gateway cloning
Xenopus pcDNA3-PFKFB4-Cter-V5 (aa 251-470)	this work-Gateway cloning
Xenopus pCS107-PFKFB4-Flag-HA	this work.
Human pcDNA3-HA-Myr-AKT ; « ca-AKT »	Given by Eychene A.
Human pcDNA3-p110-caax ; « ca-PI3K »	Given by Eychene A.
Human PFKFB4-Flag-myc	Origene RC201573
Human ICMT-Flag-myc	Origene RC207000
Human HRasV12-HA	Given by Eychene A.
Human pCS107-ICMT-V5	This work Cloned from hICMT-Flag-myc
pG5-luc ; pBind ; pAct	Promega E2440; Checkmate Mammalian two-hybrid System

Table S3: Primers used for Gibson cloning.

Final Plasmid	5'-3' primer sequence
pAct-ICMT	Fwd: ccgggatcgccagggatccgtcgacGTCGACTGGATCCGGTACCG Rev: gatgatatcaacgcgtcaagtcgacTCAGGTCCACCTTGACCCCC
pBind-ICMT	Fwd:ccggaattcccggggatccgtcgacGTCGACTGGATCCGGTACCG Rev:gatgatatcaacgcgtcaagtcgacTCAGGTCCACCTTGACCCCC
pACT-PFKFB4	Fwd:ccgggatcgccagggatccgtcgacCCATGGCGTCCCCACGGGA Rev:gatgatatcaacgcgtcaagtcgacTCTGGTGAGCAGGCACCGTG
pBind-PFKFB4	Fwd: ccggaattcccggggatccgtcgacCCATGGCGTCCCCACGGGA Rev:gatgatatcaacgcgtcaagtcgacTCTGGTGAGCAGGCACCGTG

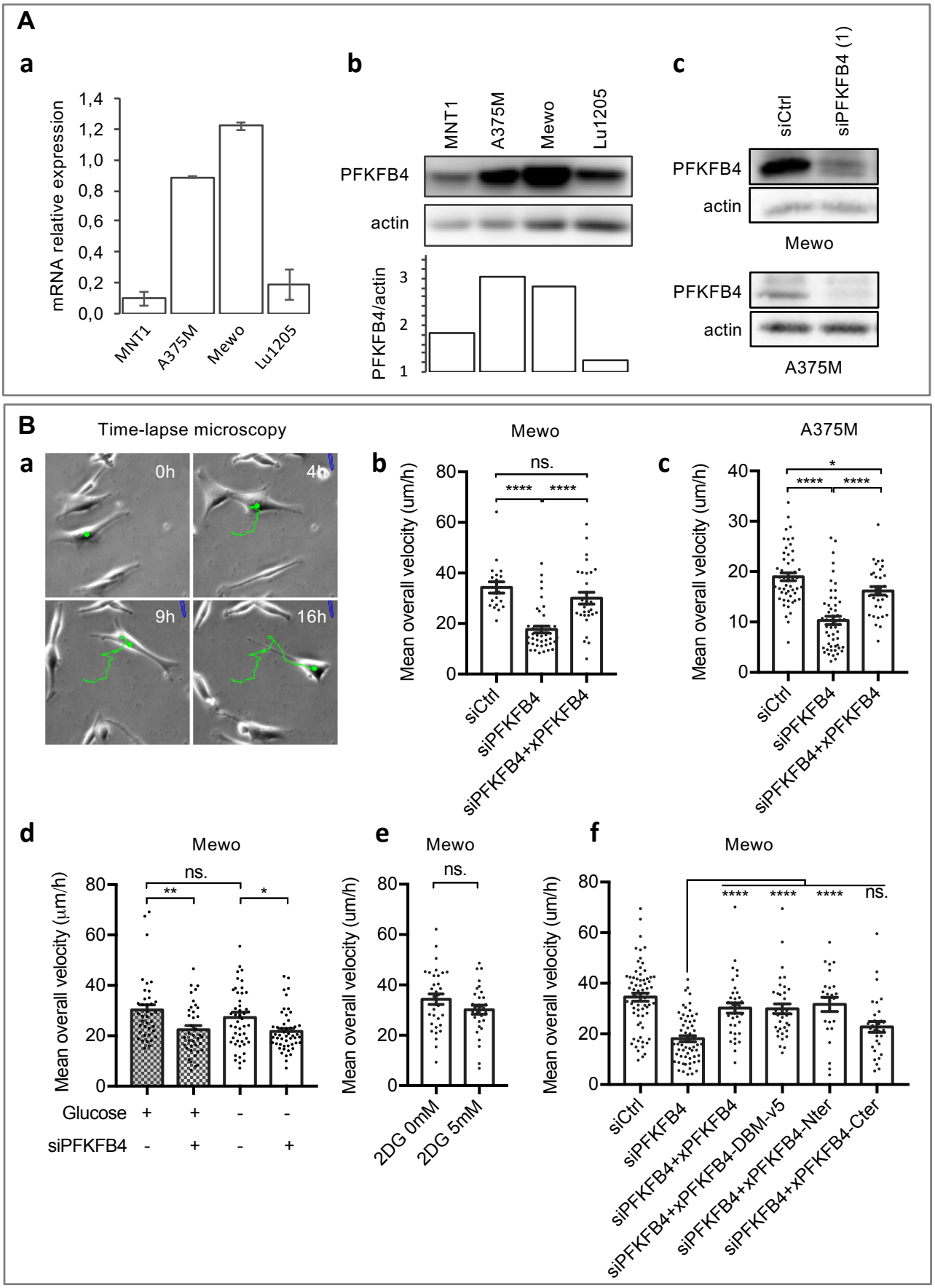
Table S4: Primary antibodies.

Antibody name /species	Dilution	Provider	Reference
PFKFB4 Rabbit	1/2000	Abcam	ab137785
PFKFB3 Rabbit	1/1000	Abcam	Ab181861
Phospho-AKT (Thr308) Rabbit	1/1000	Cell signaling	4056
Phospho-AKT (Ser473) Rabbit	1/1000	Ozyme	4060
AKT Rabbit	1/1000	Cell signaling	9272
Actin Rabbit	1/2000	Sigma	A2066
Vinculin HVINI Mouse	1/5000	Sigma	V9131
Phospho-ERK 1&2 Mouse	1/1000	Sigma	M9692
ERK Rabbit	1/1000	Santacruz	Sc-93
Phospho-PDK1 (Ser241) Rabbit	1/1000	Cell signaling	3061
PDK1 Rabbit	1/1000	Cell signaling	3062
PTEN Rabbit	1/1000	Cell signaling	9559
Phospho-GSK-3beta (Ser9) Rabbit	1/1000	Ozyme	9336
ICMT Rabbit	1/1000	Proteintech	51001-2-AP
HA Rat	1/1000 for WB; 1/500 for IF	Roche	Clone 3F10
Flag Rabbit	1/1000	Sigma	F7425

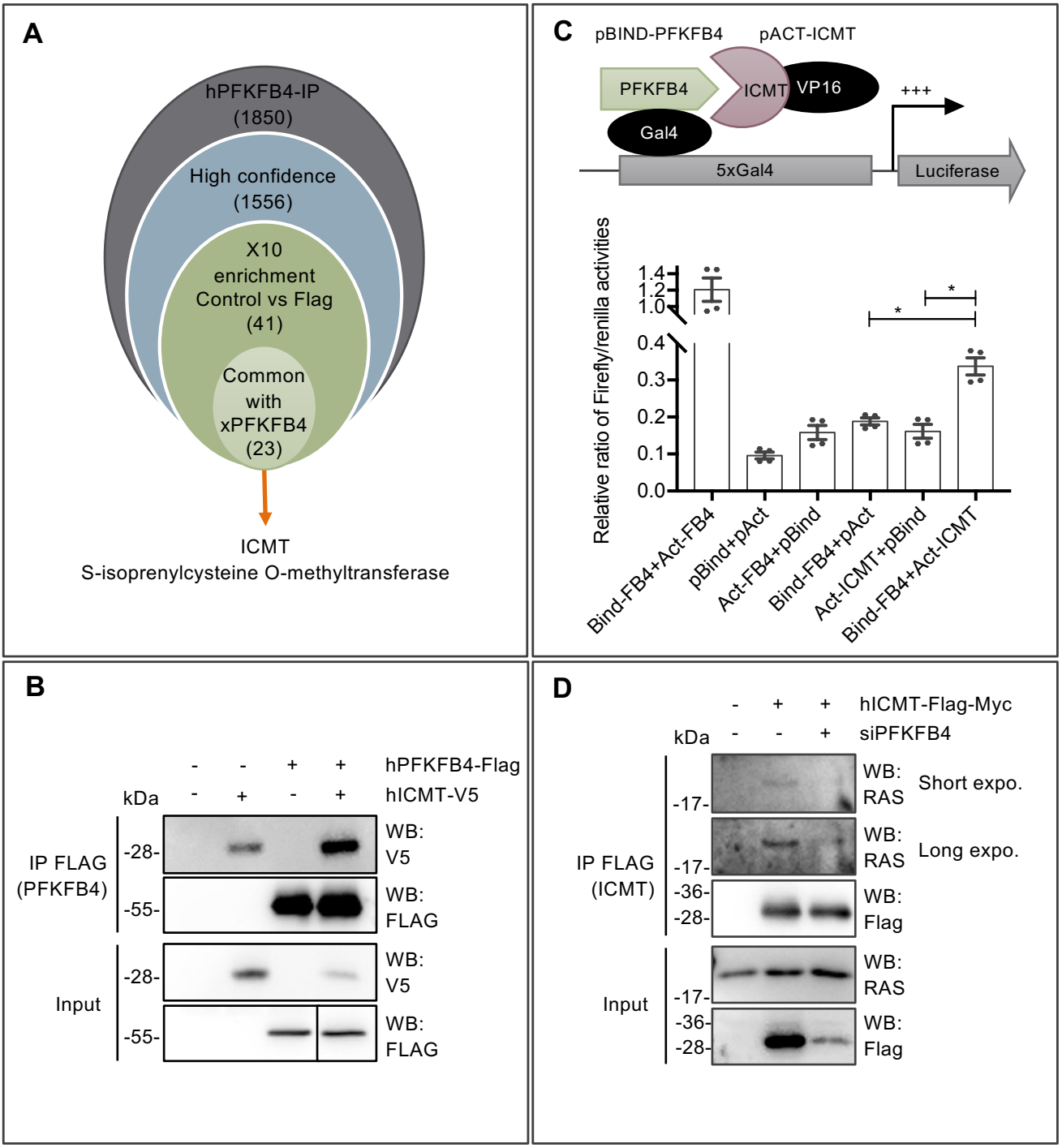
Table S5: PCR primers.

Primers Name	5'-3' Sequence
hTBP	Fwd: 5’-CACGAACCACGGCACTGATT-3’ Rev : 5’-TTTTCTTGCTGCCAGTCTGGAC-3’
h18S	Fwd:5’-GTAACCCGTTGAACCCCATT-3’ Rev : 5’-CCATCCAATCGGTAGTAGCG-3’
hpfkfb4 (exon 3-6)	Fwd:5’-TTTTTCTCCCCGACAATGAAGAG-3’ Rev : 5’-CACACAGATGGACRCGACAAA-3’

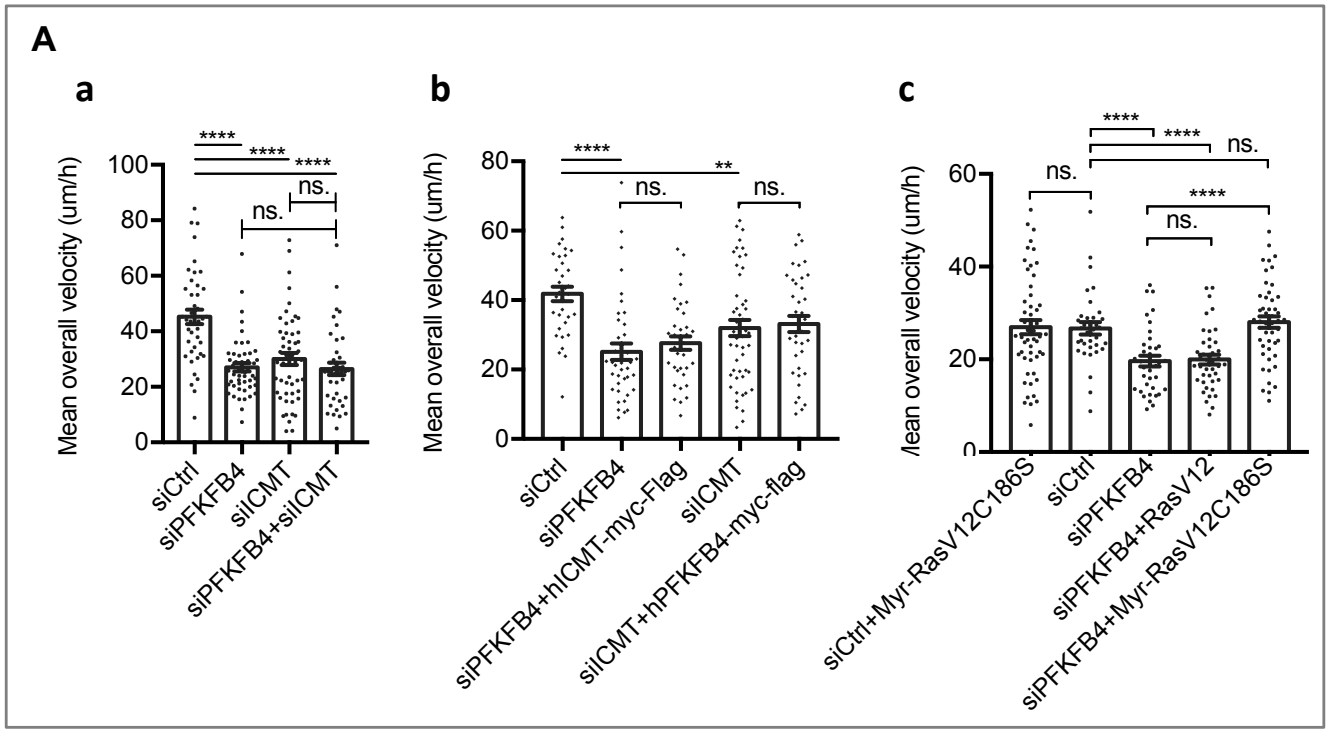
Sittewelle et al., Figure 1

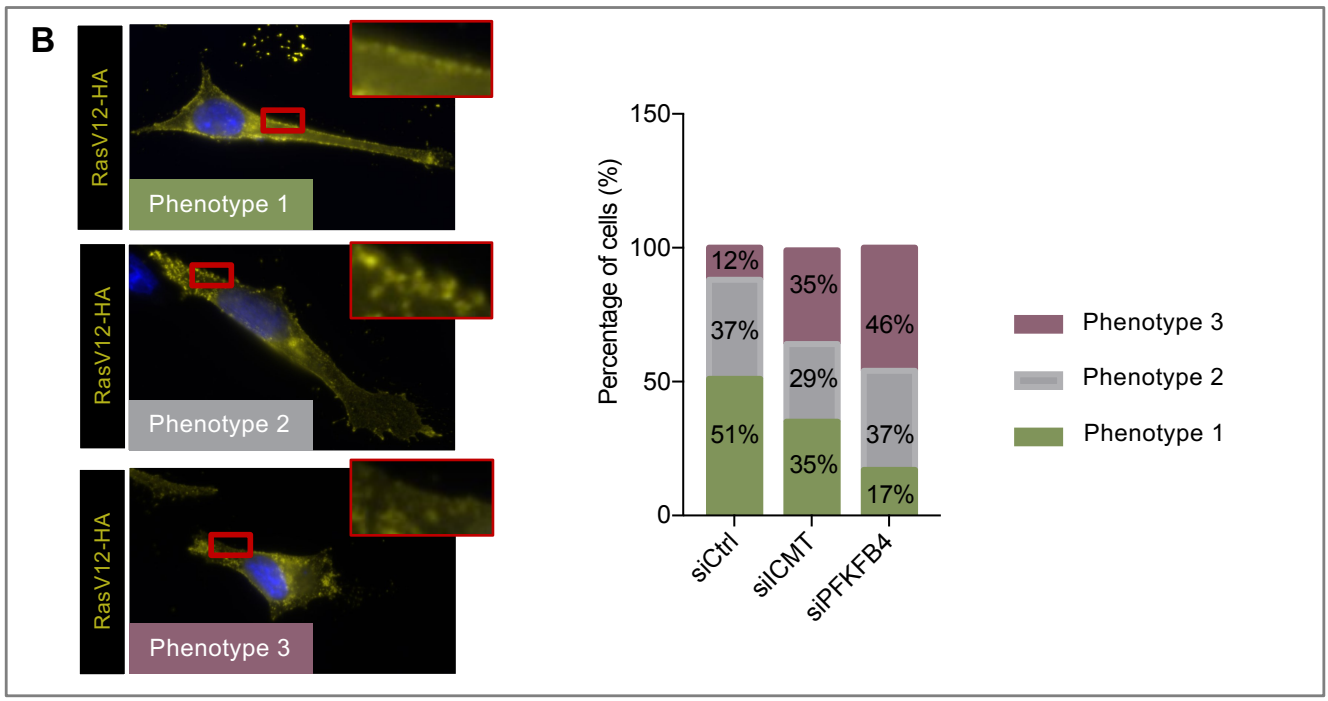


Sittewelle et al., Figure 2

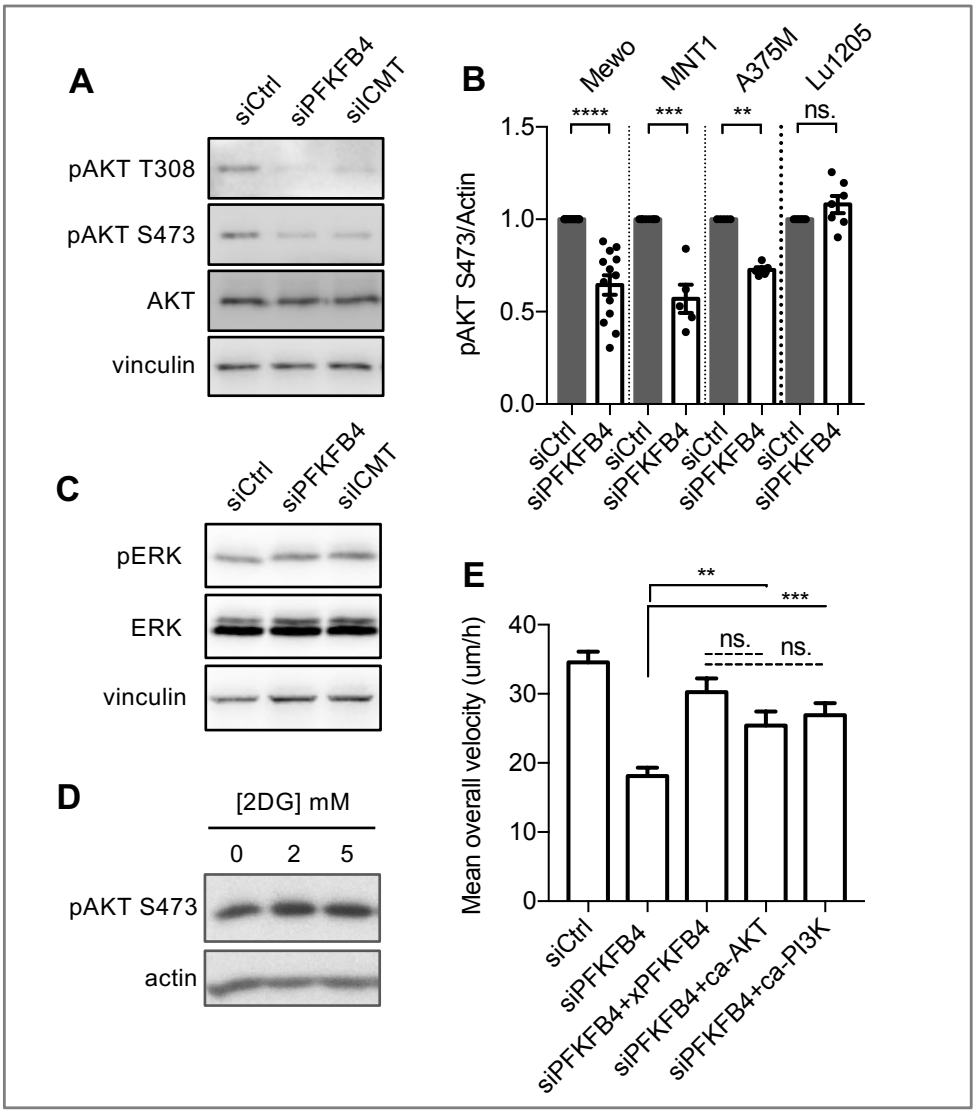


Sittewelle et al., Figure 3

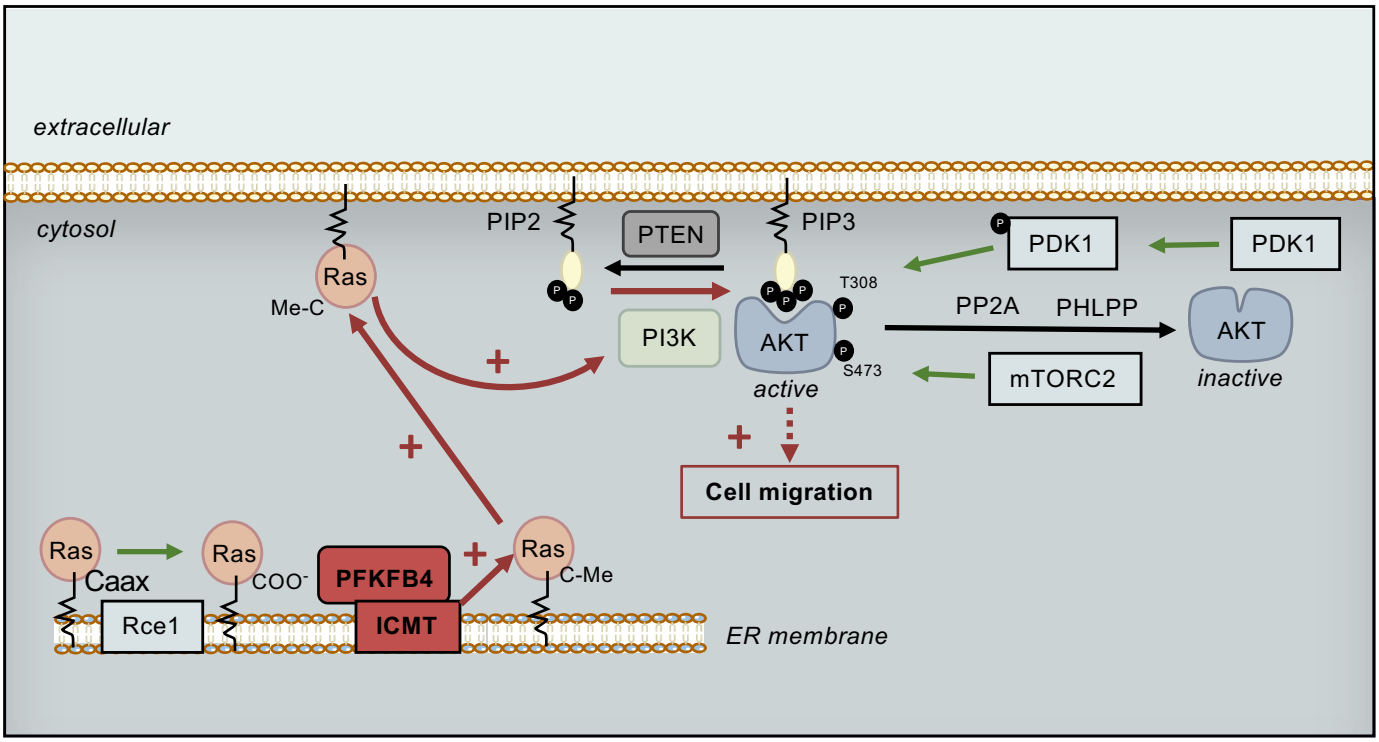




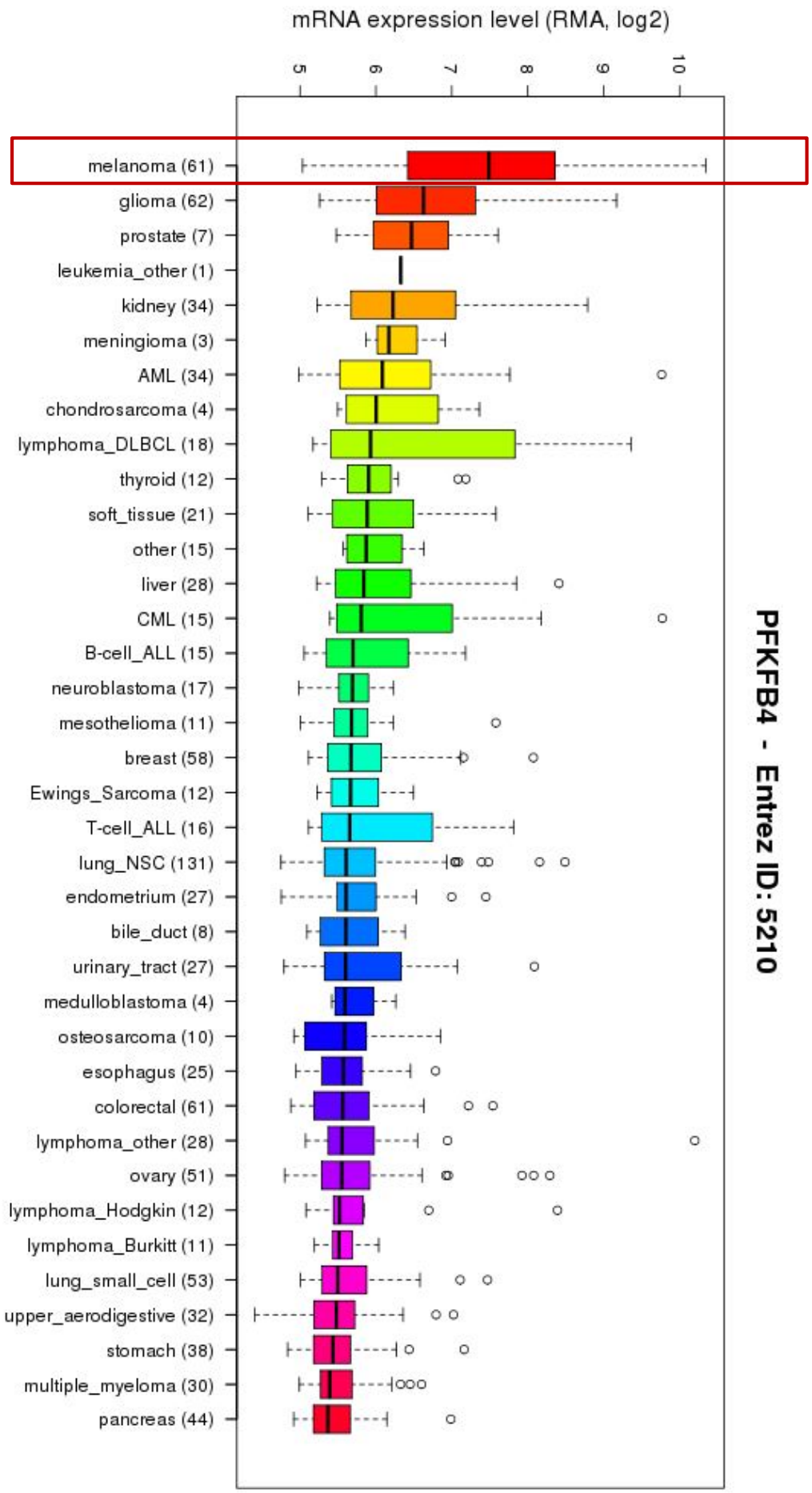
Sittewelle et al., Figure 4



Sittewelle et al., Figure 5



Sittewelle et al., Figure S1

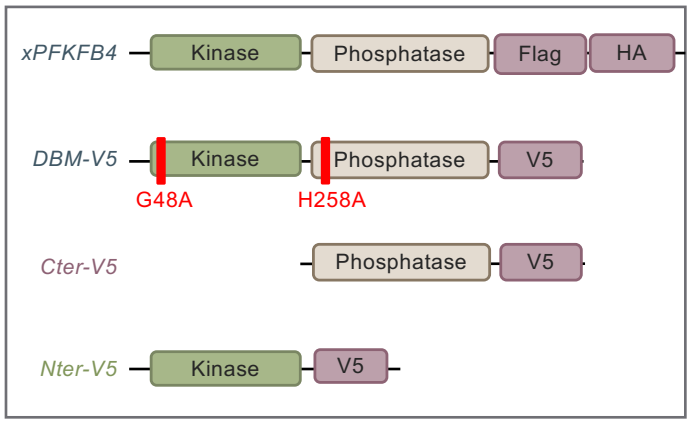


Sittewelle et al., Figure S2

A

Cell line	Metastatic properties	Mutated BRAFV600E	Mutated RASQ61	Mutated p53	Mutated CDKN2A	Mutated CDK4	Mutated FGFR1	Mutated MAPK3
MNT1	-	No	No	NA	NA	NA	NA	NA
Mewo	+	No	No	Yes	Yes	NA	Yes	Yes
A375M	+	Yes	No	NA	Yes	NA	NA	NA
Lu1205	+	Yes	No	NA	NA	Yes	NA	NA

B



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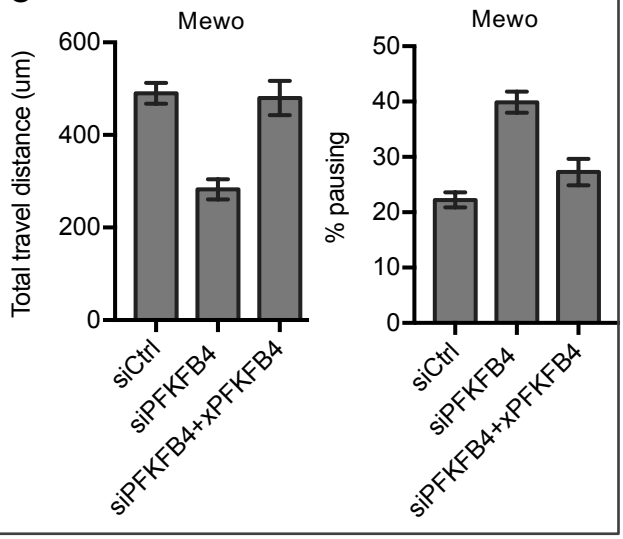

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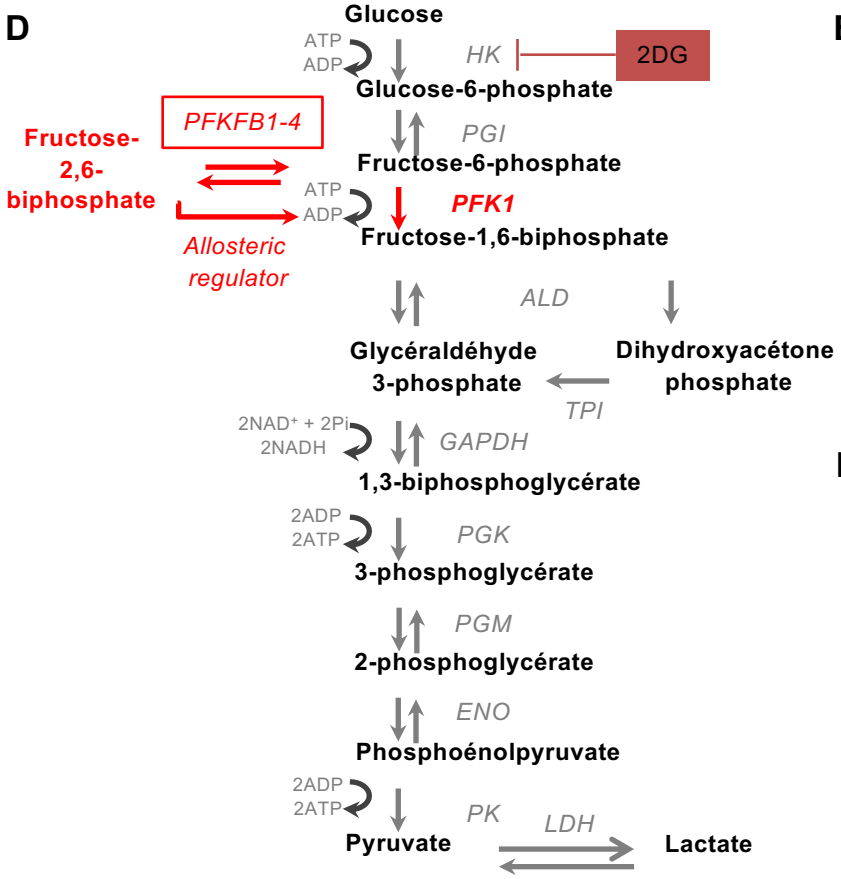

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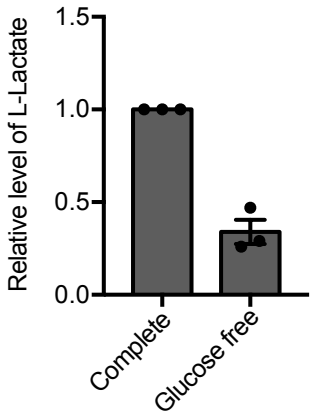

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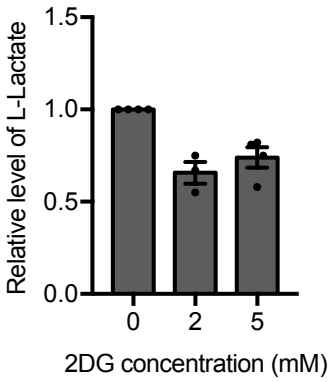


Sittewelle et al., Figure S3

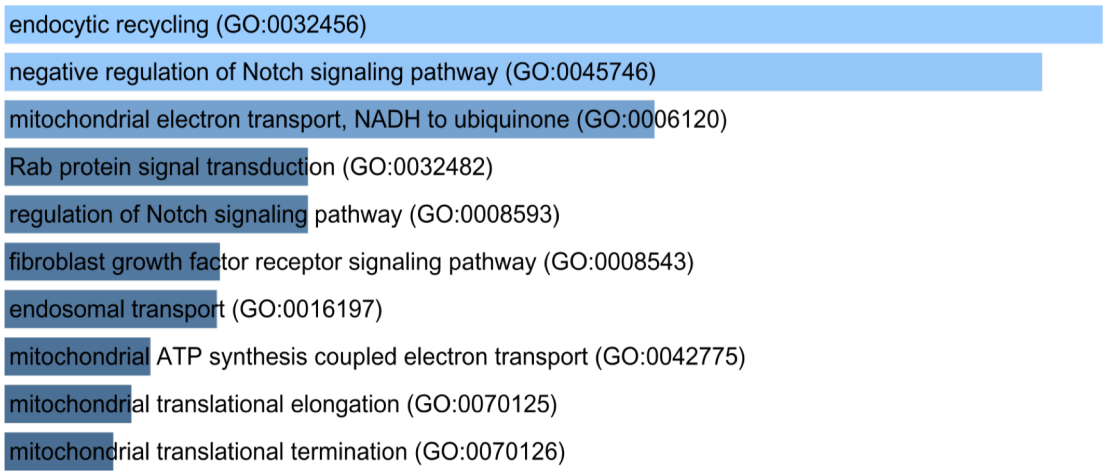
A

Gene	Description	Score Ctlr V5	Score FB4-Flag
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4	267	11649
ZNF593	Zinc finger protein 593	0	62
TMEM109	Transmembrane protein 109	0	49
VDAC2	Voltage-dependent anion-selective channel protein 2	0	46
RAB14	Ras-related protein Rab-14	0	44
NDUFA4	Cytochrome c oxidase subunit NDUFA4	0	43
MMP14	Matrix metalloproteinase-14	0	42
NDUFS7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7	0	42
C8orf59	Uncharacterized protein C8orf59	0	41
MRPL22	39S ribosomal protein L22, mitochondrial	0	40
PIN4	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 4	0	39
CCNT2	Cyclin-T2	0	38
HDGFRP2	Hepatoma-derived growth factor-related protein 2	0	33
CAPRIN1	Caprin-1	0	33
RPS27A	Ubiquitin-40S ribosomal protein S27a	0	32
ICMT	**Protein-S-isoprenylcysteine O-methyltransferase**	**0**	**31**
PLS3	Plastin-3	0	31
TRPT1	tRNA 2'-phosphotransferase 1	0	29
KIF22	Kinesin-like protein KIF22	0	28
MRPL54	39S ribosomal protein L54, mitochondrial	0	28
CAP1	Adenylyl cyclase-associated protein 1	0	23
PURB	Transcriptional activator protein Pur-beta	0	21

Gene	Description	Score Ctlr V5	Score FB4-Flag
NPM3	Nucleoplasmin-3	0	78
MSL2	E3 ubiquitin-protein ligase MSL2	0	55
DAZAP1	DAZ-associated protein 1	0	42
AGPAT1	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	0	39
GNB1L	Guanine nucleotide-binding protein subunit beta-like protein 1	0	39
RPS12	40S ribosomal protein S12	0	34
BOP1	Ribosome biogenesis protein BOP1	0	33
SLC1A2	Excitatory amino acid transporter 2	0	29
ZC3H4	Zinc finger CCCH domain-containing protein 4	0	28
JMJD6	Bifunctional arginine demethylase and lysyl-hydroxylase	0	28
UPF3B	Regulator of nonsense transcripts 3B	0	26
ATP5I	ATP synthase subunit e, mitochondrial	0	25
RAB13	Ras-related protein Rab-13	0	24
PDLIM4	PDZ and LIM domain protein 4	0	23
SELK	Selenoprotein K	0	22
INO80B	INO80 complex subunit B	0	21
ZNF187	Zinc finger and SCAN domain-containing protein 26	0	19
HSPBP1	Hsp70-binding protein 1	0	18

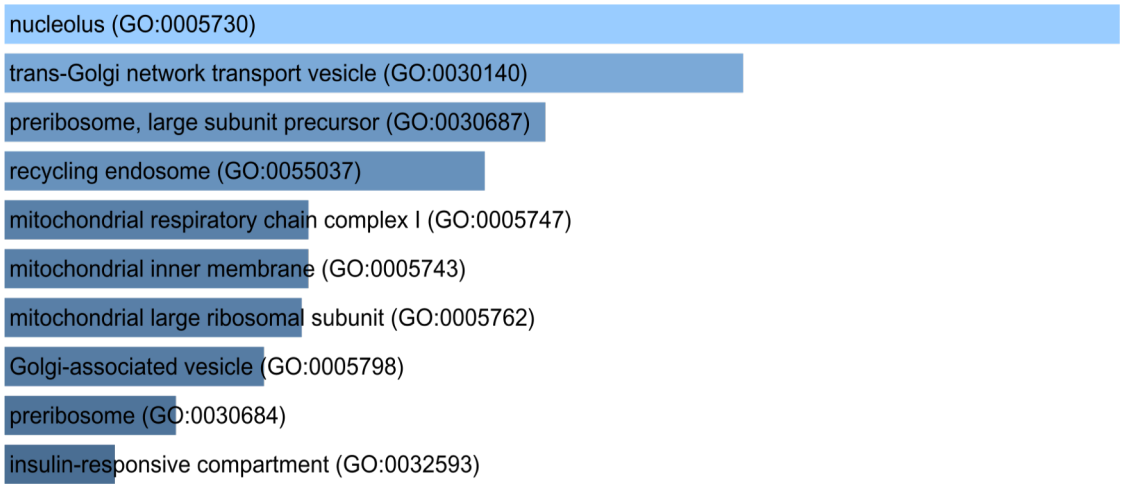
B

GO Biological Process

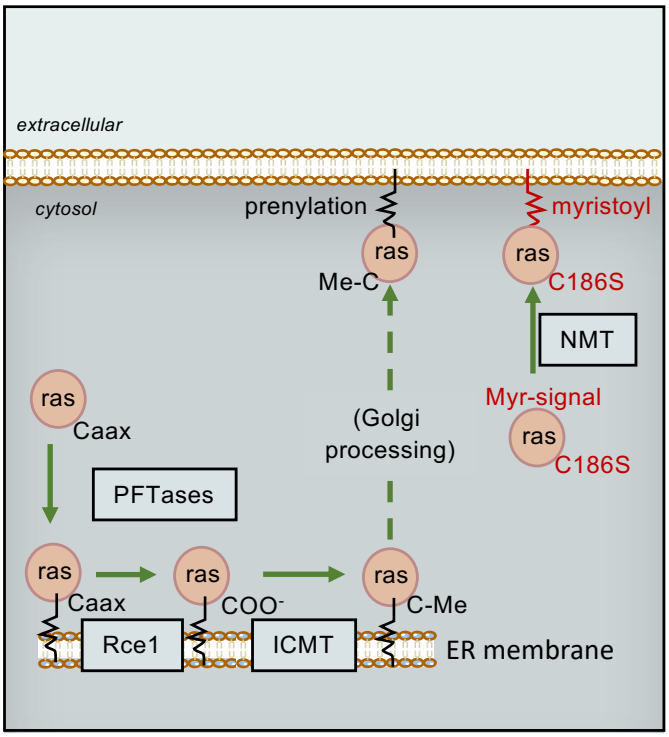
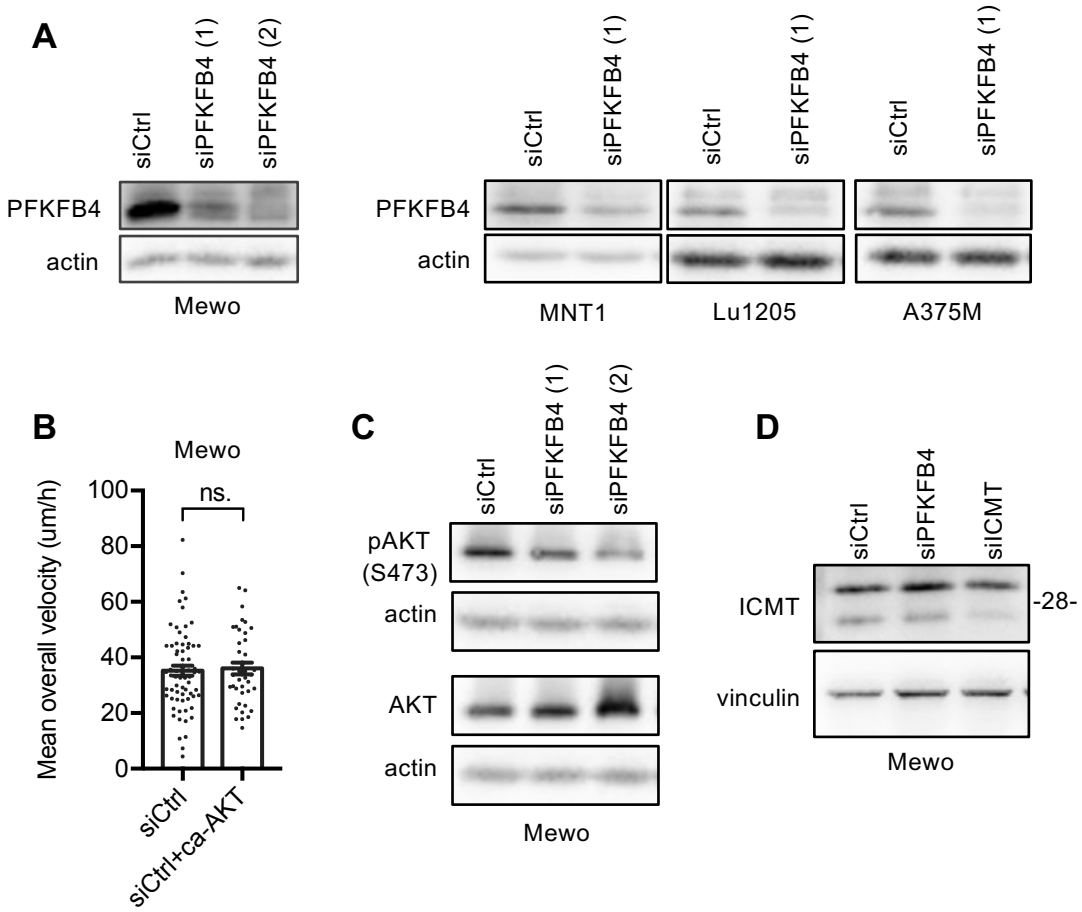


C

GO Cellular Component



Sittewelle et al., Figure S4



Sittewelle et al., Figure S5