Citrullination of pyruvate kinase M2 by PADI1 and PADI3 regulates glycolysis and cancer cell proliferation

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Chromodomain helicase DNA binding protein 4 (CHD4) is an ATPase subunit of the Nucleosome Remodelling and Deacetylation (NuRD) complex that regulates gene expression. CHD4 is essential for growth of multiple patient derived melanoma xenografts and for breast cancer. Here we show that CHD4 regulates expression of PADI1 (Protein Arginine Deiminase 1) and PADI3 in multiple cancer cell types modulating citrullination of arginine residues of the allosterically-regulated glycolytic enzyme pyruvate kinase M2 (PKM2). Citrullination of PKM2 R106 reprogrammes cross-talk between PKM2 ligands lowering its sensitivity to the inhibitors Tryptophan, Alanine and Phenylalanine and promoting activation by Serine. Citrullination thus bypasses normal physiological regulation by low Serine levels to promote excessive glycolysis and reduced cell proliferation. We further show that PADI1 and PADI3 expression is up-regulated by hypoxia where PKM2 citrullination contributes to increased glycolysis. We provide insight as to how conversion of arginines to citrulline impacts key interactions within PKM2 that act in concert to reprogramme its activity as an additional mechanism regulating this important enzyme.

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A hallmark of cancer cells is the high glycolysis and lactic acid production under aerobic conditions, a metabolic state known as the Warburg effect\(^1\). Tumour tissues accumulate increased amounts of glucose used not only for energy production, but also for anabolic reactions. Glycolytic intermediates are notably used for de novo synthesis of nucleotides or amino acids like glycine and serine produced in large amounts to sustain high rates of cancer cell proliferation\(^2,3\). Coupling of energy production via glycolysis to the availability of the intermediates required for nucleotide and amino acid synthesis is controlled in large part by an alternatively spliced isoform of the enzyme pyruvate kinase called PKM2 expressed in proliferating embryonic and cancer cells\(^4,5\). Unlike the PKM1 isoform that is constitutively active, PKM2 activity is positively regulated by serine (Ser), fructose 1,6-biphosphate (FBP) an intermediate of the glycolytic pathway and succinylaminomimidazole-carboxamide riboside (SAICAR), an intermediate in de novo purine nucleotide synthesis\(^6,7\). High levels of these molecules stimulate PKM2, but when their levels are lowered through excessive glycolysis, PKM2 activity is inhibited by amino acids such as tryptophan (Trp), alanine (Ala) and phenylalanine (Phe) that compete with Ser to allosterically regulate PKM2 activity\(^8-10\). Through this complex feedback loop, PKM2 couples glycolytic flux to the level of critical intermediate metabolites. PKM2 activity is also regulated by post-translational modifications, such as tyrosine phosphorylation or proline hydroxylation under hypoxia\(^11,12\).

Melanoma cells are no exception to the Warburg effect, showing high levels of aerobic glycolysis induced by transformation with oncogenic BRAF or NRAS\(^13\). Treatment with the oncogenic BRAF inhibitor vemurafenib down-regulates aerobic glycolysis that is regained in resistant cells\(^14\). Transcription factor MITF (Microphthalmia associated transcription factor) regulates many parameters of melanoma cell physiology including metabolism\(^15\). MITF directly regulates PPARGC1 and cells with high MITF expression show higher oxidative phosphorylation compared to cells with low MITF\(^16,17\).

Bossi et al\(^18\) performed an shRNA dropout screen to identify proteins involved in epigenetics and chromatin functions essential for patient derived melanoma xenograft (PDX) growth. This screen identified the ATPase subunit of the PBAF chromatin remodelling complex BRG1 along with CHD4, the catalytic ATPase subunits of the Nucleosome Remodelling and Deacetylation (NuRD) complex, as essential for tumour formation by all tested melanoma PDX. NuRD, is an epigenetic regulator of gene expression, acting in many, but not all\(^19\), contexts as a co-repressor that remodels chromatin through its ATPase subunits and deacetylates nucleosomes through its HDAC1 and HDAC2 subunits\(^20-24\). CHD4 has also been reported to be essential in breast cancer\(^25\). Here, we describe a pathway where CHD4 regulates expression of the PADD1 (Protein Arginine Deiminase 1) and PADD3 enzymes (hereafter designated as PADI1 and PADI3) that convert peptidyl-arginine to citrulline\(^26\). Increased PADI1 and PADI3 expression enhances citrullination of the key glycolytic enzyme PKM2 leading to excessive glycolysis, lowered ATP levels and slowed cell growth. In human cancers, PADI1 and PADI3 expression is regulated by hypoxia stimulating PKM2 activity and contributing to the increased glycolysis seen under hypoxic conditions.

### Results

**CHD4 regulates PADI1 and PADI3 expression in melanoma cells.** We performed siRNA CHD4 silencing in a collection of melanoma cells. Silencing was specific for CHD4 and did not affect CHD3 expression as measured by RT-qPCR and confirmed by immunoblot (Fig. 1a, b). CHD4 silencing also did not appreciably affect expression of the lineage-specific transcription factors MITF or SOX10. In agreement with the results of the previous shRNA dropout screen, siRNA-mediated CHD4 silencing reduced colony forming capacity, increased the proportion of slow proliferating cells (Fig. 1a–d, and Supplementary Fig. 1), but did not induce apoptosis (Fig. 1e). Analogous results were seen when we saw following CHD3 silencing. CHD4 silencing had a less dramatic effect than silencing of MITF that induces a potent cell cycle arrest and senescence\(^17,27,28\). RNA-seq following CHD4 silencing in melanoma cells identified more than 1000 up-regulated genes compared to 364 down-regulated genes showing that CHD4 acted primarily as a transcriptional repressor (Fig. 1f and Supplementary Dataset 1). Up-regulated genes were enriched in several signalling pathways (Fig. 1g).

De-regulated expression of selected genes upon CHD4 silencing was confirmed by RT-qPCR on independent RNA samples in 501Mel, MM117 and SK-Mel-28 melanoma cells (Fig. 1h–j). While RAC2 and CNTFR showed comparable up-regulation in the 3 lines, many other genes were differentially regulated. For example, THY1 was potently induced in 501Mel cells, but it expression was mildly down-regulated in Sk-Mel-28 cells, whereas MAP2 that was down-regulated in 501Mel cells was up-regulated in MM117 cells. In contrast, expression of PADI1 and PADI3 was strongly induced in all tested melanoma lines (Fig. 2a, b). PADI3 expression was almost undetectable and potently activated by CHD4 silencing, whereas some lines had low basal PADI1 levels also strongly stimulated by CHD4 silencing. RNA-seq further showed that expression of PADI2 and PADI4 was low to undetectable and their expression was not induced by siCHD4 silencing (Supplementary Dataset 1).

The PADI1 and PADI3 genes are located next to each other (Supplementary Fig. 2). ChIP-seq in melanoma cells revealed that CHD4 occupied an intronic regulatory element in PADI1 immediately adjacent to sites occupied by transcription factors CTCF and FOSL2 (API). This element is predicted to regulate both the PADI1 and PADI3 genes (Supplementary Fig. 2) and is further marked by H2AZ, H3K4me1, BRG1 and ATAC-seq, but is not by MITF and SOX10.

Analyses of the Cancer Cell Line Encyclopedia (CCLE) showed a strong correlation of PADI1 and PADI3 expression indicating their co-regulation was not restricted to melanoma cells (Supplementary Fig. 3). Remarkably, expression of both PADI1 and PADI3 was negatively correlated with CHD4 (Supplementary Fig. 2b–c). These data support the idea that CHD4 repressed PADI1 and PADI3 in many cancer cell lines (see also below).

**PADI1 and PADI3 citrullinate glycolytic enzymes and stimulate glycolysis.** To identify potential PADI1 and PADI3 substrates in melanoma cells, we made protein extracts from siC and siCHD4 cells, performed immunoprecipitation (IP) with a pan-citrulline antibody and analysed precipitated proteins by mass-spectrometry (Fig. 2c and Supplementary Dataset 2). A statistical analysis of the data using the Perseus software revealed an increased number of total peptide spectral matches (PSMs) and PSMs for citrullinated peptides following CHD4 silencing. A set of 520 proteins was enriched in the pan-citrulline IP from siCHD4 cells (Fig. 2c and Supplementary Dataset 2). Ontology analyses showed strong enrichment in proteins involved in translation, proteasome, spliceosome and multiple aspects of metabolism, including glycolysis and the pentose phosphate pathway (Supplementary Fig. 4a). Comparison with public datasets of citrullinated human proteins indicated that around half of the citrullinated proteins identified in rheumatoid arthritises were present in our data set while around 30% of the proteins
Fig. 1 CHD3 and CHD4 are required for normal melanoma cell proliferation. **a**, **b** 501MEL cells were transfected with the indicated siRNAs and CHD3 and CHD4 expression evaluated by RT-qPCR or by immunoblot along with that of MITF and SOX10. **c** The indicated cell lines were transfected with siRNA and after reseeding the number of colonies counted after 10 days. **d** The indicated cell lines were transfected with siRNAs and cell proliferation evaluated by cell trace violet assay. **e** The indicated cell lines were transfected with siRNA and apoptosis detected by FACs after labelling with Annexin-V. Silencing of MITF known to induce cell cycle arrest and senescence was included as a control. **f** Volcano plot showing changes in gene expression following CHD4 silencing. Genes up or down-regulated based on Log2 fold-change >1/-1 with an adjusted p-value <0.05 were identified. **g** Ontology analyses of CHD4 regulated genes. Shown are the enrichment scores for GSEA, as well as David functional enrichment and KEGG pathway categories. **h–j** Verification of deregulated expression of selected genes following siCHD4 in independent RNA samples from 501Mel, MM117 and Sk-Mel-28 cells. In all experiments n = 3 biological replicates and unpaired t-test with two tailed p-value analyses and confidence interval 95% were performed by Prism 5. p-Values: *p < 0.05; **p < 0.01; ***p < 0.001. Data are mean ± SEM.
found here were previously found in human tissues[29,30] (Supplementary Fig. 4b, c).

Glycolysis was amongst the pathways strongly enriched in siCHD4 cells with enhanced citrullination of multiple enzymes (Fig. 2d). We focussed on PKM2, a highly regulated enzyme playing a central role in integrating control of glycolysis with cellular metabolic status and cell cycle[31]. PKM2 converts phosphoenolpyruvate (PEP) to pyruvate then converted to lactic acid. To investigate PKM2 citrullination, melanoma cells were transfected with siC, siCHD4 or vectors allowing ectopic expression of PAD1 and PAD3 (Fig. 2e). While CHD4 silencing or PAD1/3 expression did not alter overall PKM2 levels (Fig. 2f), strongly increased amounts of PKM2 were detected in the pan-citrulline IP following siCHD4 compared to siC in both 501Mel and MM117 melanoma cells and after ectopic PAD1 and PAD3

![Image](https://example.com/image.png)
expression, particularly upon co-expression of both enzymes (Fig. 2g–h). Previous studies in human or mouse cells\(^{29,32}\) identified at least 3 PKM2 arginine residues that were subject to citrullination R106, R246 and R279 (Supplementary Fig. 5a). The above mass-spectrometry detected R106 and R279 citrullination, whereas that of R246 could not be unambiguously determined as it was located the C-terminus of the peptide (Supplementary Fig. 5b–d). A similar situation was also seen for R489 that had not been found in previous studies (Supplementary Fig. 5e). To confirm their citrullination, we generated antibodies against synthetic peptides corresponding to citrullinated R106 and R246, residues predicted to play a role in regulating PKM2 activity (see below). In dot-blot assays, each of these antibodies showed strong signal for the citrullinated peptide, but little for the equivalent wild-type peptide with arginine (Supplementary Fig. 6a–d). Similarly, citrullinated R106 antibody did not recognize citrullinated R246 and vice-versa, and signal for each antibody was lost after siPKM2 silencing (Supplementary Fig. 6e). Immunoblots on extracts from cells transfected with siCHD4 or PAD1 and PAD3 expression vectors showed enhanced signal for PKM2 compared to the control transfected cells indicating increased citrullination of these two arginine residues (Supplementary Fig. 6g, h). The R489 peptide was too hydrophobic to obtain soluble peptide for antibody production and thus its citrullination could not be confirmed.

To determine if siCHD4 silencing and enhanced PKM2 citrullination altered glycolysis, we profiled melanoma cell metabolism in real-time. CHD4 silencing increased the basal OCR (oxygen consumption rate) and ECAR (extracellular acidification rate), markedly increased maximum OCR and ECAR and decreased the OCR/ECAR ratio due to the increased ECAR values (Fig. 3a–d). ECAR was blocked using 2-deoxy-D-glucose confirming that it was due to increased glycolysis (Fig. 3c). Increased glycolysis and lactic acid production diverts pyruvate from oxidative metabolism a more efficient ATP source. Consequently, excessive glycolysis following CHD4 silencing led to decreased intracellular ATP levels (Fig. 3e). Similarly, CHD4 silencing increased PKM2 activity that mirrored the increased glycolysis of the living cells (Fig. 3f and Supplementary Fig. S7a, b). In contrast, no increased PKM activity was seen upon siCHD4 silencing in primary WI-38 fibroblasts (Supplementary Fig. 7a, b) that expressed PKM1 and not PKM2 (Supplementary Fig. 6h) and no increase in glycolysis was seen upon ectopic PAD1 and PAD3 expression (Supplementary Fig. 7d).

The increased glycolysis seen upon CHD4 silencing was strongly diminished when PAD1 and PAD3 were additionally silenced (Fig. 3g). In contrast, exogenous expression of PAD1, PAD3 or both stimulated glycolysis (Fig. 3h). Consistent with increased glycolysis, PAD1/3 expression led to reduced intracellular ATP levels (Fig. 3i) and reduced cell proliferation (Fig. 3j). PAD1 and PAD3 were therefore necessary and sufficient for increased glycolysis accounting for the effect seen upon CHD4 silencing. Similar to CHD4 silencing, expression of PAD1, PAD3 or both stimulated PKM2 enzymatic activity (Fig. 3f and Supplementary Fig. 7a–c). Increased glycolysis after siCHD4 or PAD1/3 expression could therefore be attributed to increased PKM2 activity in keeping with its previously described role as the key regulatory enzyme.

It has previously been shown that treatment of melanoma cells with BRAF inhibitors induces metabolic reprogramming, strongly reducing glycolysis\(^{14}\) Moreover, dependence on glycolysis sensitizes melanoma cells to the effects of BRAF inhibition\(^{33}\). Consistent with these observations, CHD4 silencing or ectopic PAD1/3 expression that increased glycolysis sensitised Sk-Mel-28 cells to the effects of the BRAF inhibitor vemurafenib (Supplementary Fig. 8). Hence, by regulating glycolysis, CHD4 silencing or PAD1 and PAD3 expression acts to modulate melanoma cell sensitivity to BRAF inhibition. Nevertheless, the effect of CHD4 silencing had more potent effects on vemurafenib sensitivity that ectopic PAD1 and PAD3 expression suggesting additional pathways are affected.

**PAD1 and PAD3 stimulate glycolysis in a variety of cancer cell types.** As mentioned above, **PAD1** and **PAD3** were coordinately regulated in multiple types of cancer cells and their expression was inversely related to that of **CHD4**. To test this idea, we silenced **CHD4** in a variety of cancer cell lines. CHD4 silencing in SiHa cervical carcinoma cells strongly diminished their colony forming capacity (Fig. 4a), potently increased **PAD3** expression (Fig. 4b) and stimulated glycolysis (Fig. 4c, d). Moreover, glycolysis was stimulated by ectopic **PAD1/3** expression leading to reduced OCR/ECAR ratio and ATP levels (Fig. 4e–g). In HeLa cells, CHD4 silencing reduced colony forming capacity and activated **PAD1** and **PAD3** expression (Fig. 4h, i). Glycolysis was stimulated by both CHD4 silencing and ectopic **PAD1/3** expression (Fig. 4j). CHD4 silencing strongly stimulated **PAD1** and **PAD3** expression in MCF7 breast cancer cells and increased glycolysis (Fig. 4k, l). Analogous results were observed in two different types of renal cell carcinoma cell lines, UOK-109 translocation renal cell carcinoma cells (Fig. 4m–q) and A498 clear cell renal carcinoma cells (Fig. 4r–v). Therefore, in cell lines from distinct cancer types, CHD4 silencing or ectopic **PAD1/3** expression increased glycolysis and negatively impacted cell proliferation.

**Citrullination reprograms PKM2 allosteric regulation.** As described in the introduction, PKM2 isoform activity is positively regulated by Ser and FBP and negatively by Trp, Ala and Phe, thus coupling glycolytic flux to the level of critical intermediate metabolites\(^{4–6}\). PKM2 allosteric regulation involves three distinct enzyme conformations\(^{8,9,34}\) (Supplementary Fig. 9a). In the apo (resting) state, in absence of small molecules and ions, the PKM2 N-terminal and A domains adopt an active conformation, but the B domain is in an inactive conformation. In the activated R-state, binding of FBP or Ser and magnesium, stabilises the N and A domains in their active conformation, and rotates the B domain.
Fig. 3 CHD4 silencing regulates glycolysis and cell proliferation. a Effect of CHD4 silencing on basal and maximal OCR values in 501Mel cells. b Effect of CHD4 silencing on the basal OCR/ECAR ratio in the indicated cell types. c, d Effect of CHD4 silencing on basal and maximal ECAR values in 501Mel cells and basal ECAR values in the indicated cell types. e CHD4 silencing reduces intracellular ATP levels in the indicated cell lines. f Stimulation of PKM2 enzymatic activity in extracts from cells under the indicated conditions. g, h ECAR values in 501Mel cells following transfection with indicated siRNAs or expression vectors. i Intracellular ATP levels following CHD4 silencing or PAD1/3 expression. EV = empty expression vector control. j Reduced cell proliferation following PAD1/3 expression. k-m ECAR values and intracellular ATP levels in MM117 cells following transfection with indicated siRNAs or expression vectors. ECAR and OCR and ATP values were determined from \( n = 3 \) biological replicates with 6 technical replicates for each N in the case of OCR and ECAR, \( n = 3 \) biological replicates and unpaired t-test with two tailed \( p \)-value analyses and confidence interval 95% were performed by Prism 5. \( p \)-Values: * \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \). Data are mean ± SEM. Values for PKM2 enzymatic activity were determined by Prism 5 using a 2-way ANOVA test.
towards the A domain that together form the active site. In the inactive T-state, upon binding of inhibitory amino acids (Trp, Ala and Phe), the B domain adopts a partially active conformation, but the N and A domains undergo structural changes and disorganise the active site. The structural changes observed between the different PKM2 states are reinforced allosterically by organisation into a tetramer that is essential for enzyme function.

In previous studies and as described here PKM2 can be citrullinated at 3 arginine residues R106, R246, R279, and potentially also at R489. Citrullination of R106 and R246 was confirmed by immunoblot (Supplementary Fig. 6f, g). In the apo state, R246 forms hydrogen bonds between its guanidino group and the main chain carbonyls of V215 and L217 at the pivotal point where the B domain moves between its active and inactive
Citrullination regulates glycolysis and proliferation in multiple types of cancer cells. a Diminished colony forming capacity of SiHa cells following CHD4 silencing. b PADI1 and PADI3 expression in SiHa cells following CHD4 silencing. c, d Basal and maximal glycolysis in SiHa cells following CHD4 silencing. e Glycolysis in SiHa cells following PADI1/3 expression. f OCR/ECAR ratio in SiHa cells following CHD4 silencing or PADI1/3 expression. g Intracellular ATP levels in SiHa cells following CHD4 silencing. h–j Colony forming capacity, PADI1, PADI3 expression and glycolysis in HeLa cells following CHD4 silencing or PADI1/3 expression as indicated. k, l PADI1, PADI3 expression and glycolysis in MCF7 cells following CHD4 silencing or PADI1/3 expression as indicated. m–q Colony forming capacity, PADI1, PADI3 expression and glycolysis and proliferation of A498 clear cell renal carcinoma cells following CHD4 silencing or PADI1/3 expression as indicated. For RT-qPCR, ATP levels and Phe that compete for binding to the pocket.

A diversity of cell lines from the CCLE showed coordinate expression and increased colony forming capacity of SiHa cells following CHD4 silencing.

Citrullination stimulates glycolysis in hypoxia. The above analyses of the CCLE showed coordinate PADI1 and PADI3 expression in multiple cancer cell lines. Analyses of TCGA human tumour datasets showed strongly positively correlated co-expression of Phe and Trp on glycolysis in MCF7 cells following CHD4 silencing or PADI1/3 expression as indicated. For RT-qPCR, ATP levels and Phe that compete for binding to the pocket.

As no consequence of R279 citrullination on PKM2 activity could be readily predicted, this residue was not further studied.

Transition between the R- and T-states is finely regulated by changes in the relative concentrations of Ser versus Trp, Ala and Phe that compete for binding to the pocket. Loss of R106 positive charge upon citrullination will diminish its ability to interact with the carboxylate group of the free amino acids. Due to its extended network of hydrogen bonds within the pocket and as it does not modify the active conformations of the N and A domains, it is possible that binding of Ser is less affected than that of the hydrophobic amino acids that induce important structural changes within the N and A domains. Consequently, R106 citrullination could reduce the inhibitory effect of Trp, Ala and Phe thereby shifting the equilibrium towards activation by Ser.

To test the above hypotheses, we asked if citrullination modulated glycolysis under different conditions. When cells were grown in absence of Ser, basal glycolysis was reduced and was no longer stimulated upon siCHD4 or PADI1/3 expression (Fig. 5b).

In agreement with this idea, increasing concentrations of exogenous FBP had little effect on basal glycolysis, but blocked stimulation by siCHD4 (Fig. 6b). Addition of exogenous Ser at low FBP concentration (0.5 mM) augmented basal glycolysis and re-established stimulation by siCHD4. In contrast, at higher FBP concentration (2.0 mM), little increase in basal or siCHD4-stimulated glycolysis was seen in presence of exogenous Ser. Increasing FBP therefore inhibited Ser and citrullination-dependent stimulation of glycolysis.

Deciphering the effects of citrullination on PKM2 enzymatic activity. Addition of exogenous Phe or Trp strongly inhibited PKM2 activity in control cell extracts, whereas this inhibition was partially overcome in extracts from siCHD4 or PADI1/3 expressing cells (Fig. 5h, i and Supplementary Fig. 7e–g). At higher inhibitor concentrations, PKM2 activity was most efficiently restored by PADI1/3 overexpression. Thus, citrullination attenuated PKM2 inhibition by Phe and Trp to restore both its enzymatic activity and glycolysis in living cells.

Phe and Trp on glycolysis were not seen in WI-38 fibroblasts that expressed PKM1 and not PKM2 consistent with previous results showing that PKM1 is not allosterically regulated by these ligands and hence that the effects were principally mediated via PKM2.
PADI1 and PADI3 in multiple tumour types such as cutaneous melanoma, uveal melanoma, bladder, lung and head and neck, with PADI1 often being the gene showing strongest correlation with PADI3 (Supplementary Fig. 10a–f). Despite this strong co-regulation, no negative correlation with CHD4 was seen suggesting there may be alternative mechanisms regulating PADI1 and PADI3 co-expression in tumours.

PAD expression is up-regulated under hypoxic conditions, for example in glioblastoma. In support of this, CCLE interrogation with DepMap revealed a positive correlation of PADI1 and PADI3 expression with several hypoxia signatures (Supplementary Fig. 10g, h). Interrogation of the TCGA showed PADI1 and/or PADI3 expression in several solid tumours, bladder, pancreatic, cervical, head and neck and clear cell renal cancers with
known hypoxic character. We examined the correlation between the hypoxic signatures of several cancer types with \( PADI1 \) and \( PADI3 \) expression. In pancreatic adenocarcinoma, ccRCC, and lung adenocarcinoma, \( PADI1 \) and \( PADI3 \) expression was positively correlated with the hypoxia signature of the different patient samples (Fig. 7a–c). This correlation was lower in cancers, such as bladder or cervical that had higher intrinsic \( PADI1 \) and \( PADI3 \) expression. These data indicated hypoxia rather than CHD4 as a major regulator of \( PADI1 \) and \( PADI3 \) expression in human tumours.

Glycolysis is increased under hypoxic conditions suggesting that increased PKM2 activity due to higher PAD1 and PAD3 expression may contribute to this effect. To test this hypothesis, we grew 501Mel melanoma cells in hypoxic conditions where \( PADI1 \) and \( PADI3 \) expression was induced concomitantly with the hypoxia responsive gene VEGF (Fig. 7d). Similarly, VEGF, \( PADI1 \) and \( PADI3 \) expression was induced in pseudo-hypoxic conditions in presence of 300 μM dimethylxalylglycine (DMOG) and when grown as 3D melanospheres where cells within the sphere are in hypoxia (Fig. 7e, f). As a consequence of induced \( PADI1/3 \) expression, citrullination of PKM2 R106 was increased in presence of DMOG, while that of R246 was unchanged (Fig. 7g, h). Glycolysis was also increased in presence of DMOG (Fig. 7i) and this increase was attenuated after siRNA knockdown of PAD1 and PAD3 as was the increased citrullination of R106. Hence the increased glycolysis seen in cells grown with DMOG where PAD1 and PAD3 expression was induced was at least in part mediated by PKM2 citrullination.

**Discussion**

Here we describe a regulatory pathway by which PKM2 citrullination regulates glycolysis and cancer cell proliferation. PKM2 is an allostatic regulator integrating a finely balanced feedback mechanism that modulates its activity over a wide range of absolute and relative amino acid concentrations.

FBP and Ser each stimulate PKM2 activity by stabilising the active R-state. Our data showed that exogenous FBP did not stimulate glycolysis in agreement with the report of Macpherson et al., that intracellular FBP concentrations are sufficient to saturate PKM2. They also reported that FBP and Phe can simultaneously bind PKM2 with Phe preventing maximal activation of the FBP bound tetramer maintaining PKM2 in a lower activity state as seen in tumours. Glycolysis was stimulated by exogenous Ser. Stabilisation of the active state by Ser, whose binding is mutually exclusive with Phe/Trp/Ala, would therefore lead to higher PKM2 activity compared to FBP. Ser depletion lowered basal glycolysis consistent with a dynamic equilibrium between a Ser-bound PKM2 and a less active FBP-Phe form that limits glycolysis and allows its dynamic regulation by changing this equilibrium (Fig. 6e). In agreement with this, exogenous Ser stimulated glycolysis, pushing the equilibrium towards Ser bound PKM2, whereas FBP that is already saturating and antagonised by Phe did not (Fig. 6e).

While we detected citrullination of multiple enzymes of the glycolytic pathway, our data on the effects of Ser, Phe/Ala/Trp and FBP on glycolysis and/or PKM2 enzymatic activity all converge on PKM2 as being the central target. This was further supported by the observation that glycolysis in primary WI-38 fibroblasts that express PKM1 was not affected by ectopic \( PADI1/3 \) expression or addition of Phe and Trp and that PKM1 activity was not affected by siCHD4 silencing. However, we cannot exclude that citrullination of other glycolytic enzymes is also important. It has been reported that activity of ENO can be modulated by citrullination. Nevertheless, the many publicly available structures of PKM2 in different states allowed us to make and test predictions as to how citrullination of key arginines affects its activity. It will be interesting to assess how citrullination affects structure and function of the other glycolytic enzymes and to understand how this contributes to cellular metabolism. Similarly, although we cannot exclude that other genes deregulated by CHD4 silencing contribute to the observed cellular phenotypes, the de-regulated expression of \( PADI1 \) and/or \( PADI3 \) was a common feature in all the cell types tested and correlated with observed increase in glycolysis.

Our data provide insight as to how conversion of arginine to citrulline impacts their key interactions. Unlike other post-translational modifications such as phosphorylation, or methylation, and to some extent acetylation, that often act positively to
create new interactions with proteins that specifically recognise the modified amino acids, citrullination acts negatively due to loss of side chain charge and weakened hydrogen bonding ability. In the case of PKM2, our data illustrate how weakening of interactions paradoxically translated into a positive reprogramming and stimulation of glycolysis.

PKM2 has been shown to be regulated by other post-translational modifications, the best characterised of which are tyrosine phosphorylation\cite{12}, lysine acetylation on K305\cite{36} and K433\cite{37} and oxidation of C358\cite{38,39}. In each case, these modifications result in inhibition of PKM2 enzymatic activity. Moreover, most of the above studies concentrated on how post-translational
modifications affected PKM2 activity after cell lysis or PKM2 immunoprecipitation overlooking that PKM2 activity in cells is regulated by a dynamic and complex crosstalk amongst its different ligands. Measuring glycolysis in the living cells was essential to assess how citrullination impacted cross-talk by multiple ligands to stimulate PKM2 and glycolysis. Citrullination is therefore a physiological mechanism that has an effect analogous to synthetic small molecules that increase PKM2 activity and stimulate excessive glycolysis resulting in Ser autotrophy and reduced cell proliferation.

Under most normal conditions, expression of PADI1 and PADI3 is tightly regulated with low or no expression. In cancer cell lines, experimental CHD4 silencing and correlative expression over the CCLE both showed that CHD4 negatively regulated their expression in human tumours. We confirmed up-regulation of PADI1 and PADI3 in cells grown under hypoxic conditions and we further showed that the increased glycolysis under hypoxic conditions was attenuated by PAD1 and PAD3 silencing. Hence, PKM2 citrullination is enhanced under hypoxic conditions and contributes to the increased glycolysis.

PAD enzyme expression is also de-regulated in pathological situations such as rheumatoid arthritis (RA) where the production of antibodies against aberrantly citrullinated proteins contributes to the chronic inflammatory state. Moreover, citrullination of glycolytic enzymes including PKM2 was observed in RA. This study also showed that PAD1 and PAD3-mediated citrullination stimulated PKM2 enzymatic activity in vitro independent of FBP, Ser and inhibitory amino acids. We therefore cannot exclude that PKM2 citrullination has an intrinsic stimulatory activity in addition to modulating regulation by activator or inhibitor ligands. Moreover, the RA-associated environment is characterised by hypoxia and heterogeneous availability of nutrients, resembling that of some tumours. Thus, PAD1 and PAD3 expression and the subsequent PKM2 citrullination seen in RA may account for the increased glycolysis seen in activated RA-associated fibroblast-like synoviocytes, another hallmark of the disease.

In conclusion, we identify a pathway regulating cancer cell proliferation where PAD1 and PAD3 citrullinate key arginines in PKM2 involved in its allosteric regulation to modulate glycolysis and cell proliferation. PAD1 and PAD3-mediated PKM2 citrullination also contributes to the increased glycolysis seen under hypoxic conditions, a hallmark of many cancers and RA and may be active in other pathological contexts associated with increased glycolysis.

Methods

A list of oligonucleotides, antibodies and resources can be found in Supplementary Dataset 3.
Fig. 7 PKM2 citrullination contributes to increased glycolysis in hypoxia. a–c Correlation of PADI1 and PADI3 expression with that of the HARRIS_HYPOXIA signature in the indicated cancer types using TCGA data sets. The Spearman correlations and p-values are indicated. d, e Expression of the indicated genes assessed by RT-qPCR in cells grown under hypoxia (1% O₂) (d) or pseudo-hypoxia with 300 μM DMOG (e). n = 3 biological replicates. f Induction of PAD1 and PAD3 expression in 501Mel cells grown as 3D melanospheres for 7 days. g Immunoblots showing enhanced PKM2 R106Cit when grown under increasing concentrations of DMOG. h Immunoblots showing expression of the indicated proteins in transfected cells in presence of 300 μM DMOG. i Glycolysis in transfected cells in presence of 300 μM DMOG. ECAR values were determined from n = 4 biological replicates with 6 technical replicates for each N and unpaired t-test with two tailed p-value analyses and confidence interval 95% were performed by Prism 5. p-Values: *p < 0.05; **p < 0.01; ***p < 0.001. Data are mean ± SEM.
analyses and con
duplicate or triplicate and analysed by Prism 5 using a 2-way ANOVA test. All
with 6 technical replicates for each N. Unpaired
Seahorse experiments were derived from a minimum of
2-deoxyglucose (2-DG). After measurement, cells were washed with PBS,
mRNA expression (RNA Seq V2 RSEM).
The ECAR and OCR were measured in an XF96 extracellular analyzer
was added to all RPKM
expression were
was con-
comparisons between the genes. For Figure S10g
for the indicated tumours were first
PKM2 enzymatic assays were performed in
PKM2 enzymatic assays were performed in
Motif enrichment analyses were performed using
RNA preparation, quantitative PCR and RNA-seq analysis. RNA isolation was performed according to standard procedure (Qiagen kit), qRT-PCR was carried out
SYBR Green 1 (Qiagen) and Multiscribe Reverse Transcriptase (Invitrogen) and monitored using a LightCycler 480 (Roche). RPLPO gene expression was used to
primer sequences for each cDNA were designed using
PCR primers. RNA-seq was performed essentially as described.46. Gene ontology
analyses were performed with the Gene Set Enrichment Analysis software GSEA v3.0 using the hallmark gene sets of the Molecular Signatures Database v6.2 and the functional annotation clustering function of DAVID.
Analysis of oxygen consumption rate (OCR) and glycolytic rate (ECAR) in living cells. The ECAR and OCR were measured in an XF96 extracellular analyzer
Seahorse analyzer. A total of 20,000 cells per well were seeded and transfected
sRNA or expression vector 72 h and 24 h respectively prior the experiment. The
cells were incubated in a CO2-free incubator at 37 °C and the medium was changed to
XF medium supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose for an hour before measurement. For OCR profiling, cells were sequentially exposed to 2 µM oligomycin, 1 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), and 0.5 µM rotenone and antimycin A. For
the road ahead.
1. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. Cell 144, 646–674 (2011).
2. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324, 1029–1033 (2009).
3. Rosenzweig, A., Blenis, J. & Gomes, A. P. Beyond the Warburg effect: how do cancer cells regulate one-carbon metabolism? Front Cell Dev. Biol. 6, 90 (2018).
4. Christofk, H. R. et al. The M2 splice isoform of pyruvate kinase is important for tumour metabolism and tumour growth. Nature 452, 230–233 (2008).
5. Dayton, T. L., Jacks, T. & Vander Heiden, M. G. PKM2, cancer metabolism, and the road ahead. EMBO Rep. 17, 1721–1730 (2016).
6. Chaneton, B. et al. Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature 491, 458–462 (2012).
7. Keller, K. E., Tan, J. I. & Lee, Y. S. SAICAR stimulates pyruvate kinase isoform M2 and promotes cancer cell survival in glucose-limited conditions. Science 338, 1069–1072 (2012).
8. Morgan, H. P. et al. M2 pyruvate kinase provides a mechanism for nutrient sensing and regulation of cell proliferation. Proc. Natl Acad. Sci. USA 110, 5881–5886 (2013).
9. Macpherson, J. A. et al. An allosteric mechanism for M2 pyruvate kinase as an aminoc
carbon sensor. Biochem. J. 475, 1821–1837 (2018).
10. Macpherson, J. A. et al. Functional cross-talk between allosteric effects of activating and inhibiting ligands underlies PKM2 regulation. Elife 8, https://doi.org/10.7554/elife.45068 (2019).
11. Liu, W. et al. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 145, 732–744 (2011).
12. Hitosugi, T. et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumour growth. Sci. Signal. 2, ra73 (2009).
13. Hall, A. et al. Dysfunctional oxidative phosphorylation makes malignant melanoma cells addicted to glycolysis driven by the (V600E)BRAF oncogene. Oncotarget 4, 584–599 (2013).
14. Parmenter, T. J. et al. Response of BRAF-mutant melanoma to BRAF inhibition is mediated by a network of transcriptional regulators of glycolysis. Cancer Disc. 4, 423–433 (2014).

Data availability
Source data are provided within this paper and are available from the authors upon request. The CHD4 ChIP-seq and RNA-seq data described here have been deposited in GEO with the accession number GSE134850. The additional ChIP-seq data used for Figure S2 are available in the GEO data database under accession codes: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94488, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94488 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM2842802. Source data are provided with this paper.

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References
1. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. Cell 144, 646–674 (2011).
2. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324, 1029–1033 (2009).
3. Rosenzweig, A., Blenis, J. & Gomes, A. P. Beyond the Warburg effect: how do cancer cells regulate one-carbon metabolism? Front Cell Dev. Biol. 6, 90 (2018).
4. Christofk, H. R. et al. The M2 splice isoform of pyruvate kinase is important for tumour metabolism and tumour growth. Nature 452, 230–233 (2008).
5. Dayton, T. L., Jacks, T. & Vander Heiden, M. G. PKM2, cancer metabolism, and the road ahead. EMBO Rep. 17, 1721–1730 (2016).
6. Chaneton, B. et al. Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature 491, 458–462 (2012).
7. Keller, K. E., Tan, J. I. & Lee, Y. S. SAICAR stimulates pyruvate kinase isoform M2 and promotes cancer cell survival in glucose-limited conditions. Science 338, 1069–1072 (2012).
8. Morgan, H. P. et al. M2 pyruvate kinase provides a mechanism for nutrient sensing and regulation of cell proliferation. Proc. Natl Acad. Sci. USA 110, 5881–5886 (2013).
9. Macpherson, J. A. et al. An allosteric mechanism for M2 pyruvate kinase as an amino-acid sensor. Biochem. J. 475, 1821–1837 (2018).
10. Macpherson, J. A. et al. Functional cross-talk between allosteric effects of activating and inhibiting ligands underlies PKM2 regulation. Elife 8, https://doi.org/10.7554/elife.45068 (2019).
11. Liu, W. et al. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 145, 732–744 (2011).
12. Hitosugi, T. et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. Sci. Signal 2, ra73 (2009).
13. Hall, A. et al. Dysfunctional oxidative phosphorylation makes malignant melanoma cells addicted to glycolysis driven by the (V600E)BRAF oncogene. Oncotarget 4, 584–599 (2013).
14. Parmenter, T. J. et al. Response of BRAF-mutant melanoma to BRAF inhibition is mediated by a network of transcriptional regulators of glycolysis. Cancer Disc. 4, 423–433 (2014).
43. Vossenaar, E. R. et al. Absence of citrulline-specific deiminase in cancer cells. *Nature Commun.* **33**, 650–663 (2016).

44. Bustamante, M. F., Garcia-Carbonell, R., Whisenant, K. D. & Guma, M. C. Citrulline-based protein damage in cancer. *Cancer Discov.* **6**, 936–959 (2016).

45. García-Carbonell, R. et al. Critical role of glucose metabolism in rheumatoid arthritis and fibroblast-like synoviocytes. *Arthritis Rheumatol.* **68**, 1614–1626 (2016).

46. Ahn, I. K. et al. GC/TOF-MS-based metabolic profiling in cultured fibroblast-like synoviocytes from rheumatoid arthritis. *J. Bone Spine* **83**, 707–713 (2016).

47. Tyanova, S. et al. The Perseus computational platform for comprehensive analysis of (pro)teomics data. *Nat. Methods* **13**, 731–740 (2016).

48. Laurette, P. et al. Transcription factor MITF and remodeler BRG1 define chromatin organisation and regulation elements in melanoma cells. *eLife* https://doi.org/10.7554/eLife.06857 (2015).

49. Zhang, Y. et al. Model-based analysis of ChIP-seq (MACS). *Genome Biol.* **9**, R137 (2008).

50. Joshi, S. et al. TEAD transcription factors are required for normal primary myoblast differentiation in vitro and muscle regeneration in vivo. *PLoS Genet.* **13**, e1006600 (2017).

51. Lauurette, P. et al. Chromatin remodelers BrG1 and Bpf1 are required for normal gene expression and progression of oncogenic Braf-driven mouse melanoma. *Cell Death Differ.* https://doi.org/10.1038/s41418-019-0333-z (2019).

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Author contributions

S.F., S.S. performed ChIP-seq, RNA-seq, transfections and metabolism experiments, G.D. and G.G. performed bioinformatics analyses, L.N., performed and analysed mass-spectrometry experiments, S.D. constructed and provided PADI1 expression vector, D. Bennet for the MM117 and MM074 primary melanoma cells, D. Bennet for the HERMES-3A line, all the staff of the IGBMC common facilities in particular the IGBMC mass spectrometry platform and the high throughput screening facility. This work was supported by institutional grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Université de Strasbourg, the Association pour la Recherche contre le Cancer (CR, contract number PJA 20181208268), the Ligue Nationale contre le Cancer, the Institut National du Cancer, the ANR-10-LABX-0030-INRT French state fund through the Agence Nationale de la Recherche under the frame programme Investissements d’Avenir labelled ANR-10-IDEX-0002-02. The IGBMC high throughput sequencing facility is a member of the ‘France Génomique’ consortium (ANR10-INBS-09-08). The mass spectrometry facility is supported by grants from the ARC foundation and from the Cancéropole Grand Est. ID is an ‘équipe labellisée’ of the Ligue Nationale contre le Cancer. SC was supported by a fellowship from the Ligue Nationale contre le Cancer.

Competing interests

The authors declare no competing interests.

Additional information

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