DOI:
10.1128/MCB.00236-16

Document Version
Peer reviewed version

PHD2 is a regulator for glycolytic reprogramming in macrophages

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Running title

PHD2, metabolism and macrophages

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Characters excluding spaces (Abstract, introduction, results, discussion, figure legends): 35.443

Word count (Abstract): 176

Number of references: 37

Abstract

The prolyl-4-hydroxylase domain (PHD) enzymes are regarded as the molecular oxygen sensors. There is an interplay between oxygen availability and cellular metabolism, which in turn has significant effects on the functionality of innate immune cells like macrophages. If and how PHDs affect macrophage metabolism however is enigmatic. We hypothesized that via manipulation of PHD2 macrophage metabolism and function can be controlled. We characterized the metabolic phenotype of PHD2-deficient RAW cells and primary PHD2 knock out bone marrow derived macrophages (BMDM). Both showed typical features of anaerobic glycolysis, which were paralleled by increased pyruvate dehydrogenase kinase (PDK)1 protein levels and a decreased pyruvate dehydrogenase enzyme activity. Metabolic alterations were associated to an impaired cellular functionality. Inhibition of PDK1 or knock out of the Hypoxia inducible factor (HIF)-1α reversed the metabolic phenotype and impaired functionality of the PHD2-deficient RAW cells and BMDM. Taken together we identified a critical role of PHD2 for a reversible glycolytic reprogramming in macrophages with a direct impact on their function. We suggest that PHD2 serves as an adjustable switch to control macrophage behavior.

Introduction

Macrophages are an essential component of innate immunity and well recognized to play a critical role in inflammation, tumor progression and tissue repair for example after an ischemic insult (1). In aerobic conditions the oxidative breakdown of pyruvate within the mitochondria is the prevalent source of energy in most cells. Upon a decrease in oxygen availability cells shift the metabolism towards anaerobic glycolysis. In line, macrophages can use aerobic or anaerobic glycolysis for energy production depending on the context. There is growing understanding that macrophage function can be altered by cellular metabolism (2). One of the key factors at the transcriptional level in switching aerobic to anaerobic metabolism is the hypoxia-inducible factor (HIF). HIF comprises two subunits, i.e. the constitutively regulated HIFβ subunit and one of three oxygen-regulated HIFα subunits (HIF-1α, HIF-2α or HIF-3α) (3). The protein stability of HIFα is regulated by the three prolyl-4-hydroxylase domain (PHD) enzymes PHD1, 2 and 3, which hydroxylate HIFα in an oxygen-dependent manner (for review (4,5)). The hydroxylated product is recognized by the pVHL protein, which results in ubiquitination and proteasomal degradation of the α-subunit. In hypoxia the hydroxylation and degradation is inhibited and thus HIFα is stabilized, which finally results in HIF-dependent transcriptional activation of a repertoire of target genes. Besides many others, glycolytic enzymes and the pyruvate dehydrogenase kinase 1 (PDK1) belong to the target genes (6,7). Both determine the glycolytic cellular program. PHD enzymes are of interest for the ongoing development of small molecule inhibitors, which would allow stimulating HIF-dependent gene expression in normoxia (8).

PHD1-3 have common but also non redundant functions (9). In case of innate immunity the role of PHD3 has been analyzed in detail (10-12). The role of PHD2 however is less understood. Especially, if and how PHD2 affects macrophage metabolism has not been described before. We therefore analyzed the consequences of a knock out of PHD2 in bone marrow derived macrophages (BMDM) isolated from LysM $Cre^{+/-}$ x $Phd2^{fl/fl}$ (named

conditional knock out, PHD2 cKO in the following text) mice and the monocyte/macrophage cell line RAW264 for their cell metabolism and function.

Materials and Methods

Chemicals

The oxoglutarate analogue dimethyloxalylglycine (DMOG, Enzo) and the PDK inhibitor dichloroacetate (DCA, Sigma) were used in final concentrations of 1 mM and 5 mM, respectively. LPS (Enzo Life Sciences, Lörrach, Germany), IFNγ and IL-4 (Peprotech, Hamburg, Germany) were applied in a concentration of 100 ng/mL, 20 nM and 20 nM, respectively or as indicated.

Myeloid-specific conditional knock out mice

All animals in this study were backcrossed to C57BL/6 mice at least five times. *Phd2*$^{flox/flox}$ x *LysMcre*$^{+/-}$ mice were crossed with *Phd2*$^{flox/flox}$ mice to obtain PHD2 cKO (*Phd2*$^{flox/flox}$ x *LysMcre*$^{+/-}$) mice and littermate control wild type mice (*Phd2*$^{flox/flox}$). Generation of *Phd3*$^{flox/flox}$ x *LysMcre*$^{+/-}$ (PHD3 cko) mice is described in (12). To obtain PHD2/HIF-1α double knock out macrophages *Phd2*$^{flox/flox}$ x *LysMcre*$^{+/-}$ were crossed with *Hif-1α*$^{flox/flox}$ mice (B6.129-Hif1atm3Rsjo/J, Jackson Laboratories) to obtain *PHD2*$^{flox/flox}$ x *HIF-1α*$^{flox/flox}$ x *LysMcre*$^{+/-}$ mice (dcKO).

Isolation and differentiation of BMDM

Bone marrow cells were isolated from the femur. After 24 h of culturing the cells non adherent monocytes were harvested and seeded in Pluznik´s medium (DMEM supplemented with 0.2 mM L-glutamine, 0.1 mM sodium pyruvate, 50 U/mL penicillin G, 50 µg/mL streptomycin, 10% heat-inactivated fetal calf serum, 5% heat-inactivated horse serum (Pan Biotech, Aidenbach, Germany), 0.05% 1:1000 diluted ß-mercaptoethanol (Carl Roth GmbH, Karlsruhe, Germany), and 15% L929 cell-conditioned medium (13)) and differentiated for 7

days. Adherent bone marrow-derived macrophages (BMDM) were detached with 3.5 mL accutase (PAA Laboratories, Cölbe, Germany) and resuspended in culture medium (DMEM supplemented with, 0.2 mM L-glutamine, 0.1 mM sodium pyruvate, 1 mM HEPES, 50 U/mL penicillin G, 50 µg/mL streptomycin and 10% heat-inactivated fetal calf serum). For analyzing lactate production without addition of glucose, glucose-free medium (Pan Biotech) was used as indicated. Successful differentiation of the BMDM was controlled by FACS-staining for the macrophage marker F4/80.

Generation of shPHD2 RAW cells

The mouse macrophage cell line RAW 264.7 was infected with Lentivirus encoding shRNA targeting mPHD2 (5'-ATTCGAAGAATACCTCCAC-3') and cotransfected with EGFP as described earlier (14). Cells were used as a pool of sorted $EGFP^+$ cells and knock-down efficiency was tested via qPCR.

Preparation of MDA-MB231 conditioned medium

5 flasks each with $1x10^6$ MDA-MB231 cells were cultivated in 15 ml culture medium for 4 days. The medium was transferred to a reaction tube and centrifuged at 4000 g for 20 min at 4 ºC. The supernatant was pooled and frozen at -80 ºC.

Hypoxic incubation

For culturing cells in defined hypoxic conditions (1% O_2) an *invivo* Hypoxia workstation was used (Ruskinn Technologies).

Phagocytosis

Fluorescent beads (Protonex^{TM} Red 600-Latex Beads, AAT Bioquest, Sunnyvale, USA) were administered to cells for 4 h. After washing, uptake of fluorescent beads was analyzed by FACS (BD FACS Canto II, BD Biosciences).

Quantification of apoptotic cells

Supernatant and detached cells were collected. Samples were centrifuged and washed in PBS. Subsequently, cells were stained for 30 min at 4°C with Pacific Blue Annexin V (70 µg/ml) per sample (640918, Biolegend, San Diego, USA) diluted 1:150 in the dark. Samples were washed in Annexin V binding buffer (422201, Biolegend) and analyzed by FACS (BD FACS Canto II, BD Biosciences).

Single cell migration

2.5 x 10^4 RAW cells or 5 x 10^4 BMDMs were seeded in a 6-well plate. The next day the medium was replaced with either normal medium or MDA-MB231 conditioned medium. The migration of the cells was investigated using the T1-5M Nikon microscope inside the Sci-tive work station (Ruskinn, Bridgend, South Wales) at normoxic (20% O_2) or hypoxic (1% O_2) conditions. An image was taken every 10 min for in total 6 h. The migration was analyzed using the chemotaxis plug-in installed in ImageJ.

Boyden chamber assay

0.7 x 10^5 BMDM or RAW cells were seeded in 500 µL culture medium into inserts (BD Biosciences, Heidelberg, Germany) containing 3 µm pores. Inserts were placed into 24-well plates containing either 500 µl MDA-MB 231 conditioned medium or 500 µL cell culture medium as control. 18 h later inserts were placed into medium with 5 µM calcein. Cells that had not migrated were removed by scraping of the upper side of the insert after 1 h, while migrated macrophages on the lower side were analyzed by fluorescence microscopy (Axio Observer D1, Carl Zeiss, Göttingen, Germany).

RNA isolation and qRT PCR

Cells were washed once with PBS and harvested in Trizol (Invitrogen, Darmstadt, Germany). RNA was isolated according to the manufacturer's instructions and 1 µg was transcribed using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Transcript levels were analyzed by qRT PCR amplifying 1 µL of cDNA with Brilliant II SYBR Green qPCR Master Mix in an MX3005Pro light cycler (Agilent, Böblingen, Germany). Applied primer sequences were: ms12 for 5′-GAAGCTGCCAAGGCCTTAGA -3′, rev 5′-AACTGCAACCAACCACCTTC-3′; phd2 for 5′-TTGCTGACATTGAACCCAAA-3′, rev 5′-GGCAACTGAGAGGCTGTAGG-3′; phd3 for 5′-GGCCGCTGTATCACCTGTAT-3′, rev 5′-TTCTGCCCTTTCTTCAGCAT -3′; glut1 for 5′-TGGCCTTGCTGGAACGGCTG-3′, rev 5′-TCCTTGGGCTGCAGGGAGCA -3′; pfk1 for 5′-ACGAGGCCATCCAGCTCCGT-3′, rev 5′-TGGGGCTTGGGCAGTGTCCT -3′; pdk1 for 5′-TTCACGTCACGCTGGGCGAG -3′, rev 5′-GGCTGGGCACACACCAGTCG -3′; cox4.2for 5′-CAGAGAAGGTGGCCTTGTACC -3′, rev 5′-AGAAGAAGACGCAGCCCATC -3′; LonP for 5′-CATCGCCTTGAACCCTCTGT-3′, rev 5′-

AGCCGCTTAAGGATGTTGGT-3′; BNIP3 for 5′-GTCCAGTGTCGCCTGGCCTC -3′, 5′-TGGGAGCGAGGTGGGCTGTC -3′, mCCR2 for 5′-CCACACCCTGTTTCGCTG-3′, mCCR2 rev 5′-ACCTCTTCAGCACTTGC-3′; mCCR4 for 5′-GCCTCTTGTTCAGCACTTGC-3′, mCCR4 rev 5′-ATAAGCAGCCCCAGGACG-3′; mCCR5 for 5′-CCAGAGGAGGTGAGACATCCGTTC-3′, mCCR5 rev 5′-GGCAGGAGCTGAGCCGCAATTT -3′; mCCR7 for 5′-ATGGACCCAGGGAAACCCAGGAA-3′, mCCR7 rev 5′-GCACACCGACTCGTACAGGG-3′; mCXCR4 for 5′-GCTCCGGTAACCACCACGGC-3′, mCXCR4 rev 5′-GCGAGGTACCGGTCCAGGCT-3′.

Microarray-Based Gene Expression

Microarray-based gene expression was analysed as described before (12).

Western blots

Cells were lysed with 50 mM Tris, 150 mM NaCl, 0.5 mM PMSF, 100 mM $MgCl_2$, 1% NP-40 supplemented with protease inhibitors (Roche). Protein samples were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). Primary antibodies used were: anti-HIF-1α (NB-100-479, Novus), anti-HIF-2α (AF2997, R&D Systems), anti-PHD2 (NB-100-2219, Novus), anti-phospho pyruvate dehydrogenase (ABS204, Merck), anti-pyruvate dehydrogenase (3205, Cell Signaling), anti-PDK1 (ADI-KAP-PK112-D, Stressgene), and anti-β-actin (A5441, Sigma). For detection of immunocomplexes horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (Santa Cruz Biotechnology) were used and membranes were incubated with chemilumiescent HRP substrate (Millipore).

ATP and lactate measurements

Supernatant of 0.75 x 10^5 BMDM or RAW cells cultivated for 24 h in 24-well plates was analyzed for lactate production using a l-lactate kit according to the manufacturer's instructions (R-Biopharm, Darmstadt, Germany). For determination of ATP levels 0.2 x 10^5 BMDM or RAW cells were seeded. ATP levels were determined using the Cell titer-Glo ATP kit (Promega, Madison, USA).

PDH activity assay

For determining PDH activity, the MAK183 kit (Sigma, St. Louis, USA) was used.

Oxygen consumption rate and extracellular acidification rate

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were analyzed in the Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA, USA). For OCR 2.5 x 10^4 RAW or 4.0 x 10^4 BMDM cells per well were seeded. The medium was replaced with XF assay medium supplemented with 4.5 g/L glucose and 1 mM sodium pyruvate and incubated without CO_2 for 30 min. After measuring basal respiration, the oxygen consumption was analyzed after sequential addition of 1.5 µM oligomycin, 1 µM FCCP, 2 µM rotenone and 1 µM antimycin A.

For determining the ECAR, 2.5 x 10^4 (RAW) or 8 x 10^4 (BMDM) cells per well were seeded. Cells were washed with XF Glycostress-medium (DMEM D 5030, 134 mM NaCl, 3 mg Phenol Red, 2 mM L-glutamine, pH 7.35). The cells were incubated for 15 min at 37ºC without CO_2. ECAR was analyzed after sequential addition of 10 mM glucose, 1.5-3 µM oligomycin, and 100 mM 2-deoxy-glucose (2-DG).

213 **Statistical analyses**

214 Statistical analyses were performed using Student`s two-tailed t-test. Data are shown as
215 means ± SEM. Values of $p < 0.05$ were considered statistically significant.

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Results

PHD2-deficient macrophages induce a hypoxic gene expression pattern in normoxia including PDK1, a central regulator of the pyruvate dehydrogenase

BMDM isolated from PHD2$^{fl/fl}$ x LysMCre mice (PHD2 cKO) and RAW cells, which were transfected with a constitutively active shRNA targeting PHD2 (shPHD2) showed an 80% reduction of PHD2 RNA with a consequential increase of PHD3 RNA expression compared to wt BMDM and wt RAW cells (Fig. 1A). The compensatory increase of the HIF-1 target gene PHD3 is in line with other cell/tissue-specific PHD2 knock out mouse models (15). Besides PHD3 other metabolism-related HIF target genes like Glut-1, PFK1, PDK1, COX4-2, LonP and BNIP3 were upregulated. The gene expression pattern of the PHD2 cKO and shPHD2 cells resembled the pattern of HIF target genes in wt BMDMs and wt RAW cells after incubation in hypoxia. In quantitative means, however the levels of the HIF target genes were lower in the shPHD2 and PHD2 cKO cells in normoxia compared to the respective wt cells in hypoxia, which indicates that the reduction of PHD2 induced a partial HIF-response possibly due to the fact that the other PHDs, i.e. PHD1 and PHD3 are still active. This assumption was further supported by the fact that after hypoxic incubation of shPHD2 and PHD2 cKO cells the RNA levels of the HIF target genes were further increased to a similar extent as the respective wt cells in hypoxia. Cell viability/cell death, as determined by the number of AV single-positive cells, were not different in untreated wt BMDM and wt RAW cells compared to PHD2 cKO and shPHD2, respectively or after treatment with 1 mM DMOG (Fig. 1B).

PHD2 protein levels were decreased in PHD2 cKO and shPHD2 cells likewise (Fig. 1C). Whereas HIF-1α and HIF-2α were detectable in BMDM isolated from wt mice in hypoxia only, PHD2 cKO BMDM revealed high HIF-1α and HIF-2α protein levels in normoxia. In the RAW cells HIF-2α was not detectable with the antibodies applied. For HIF-

1α a similar pattern as in the BMDM was observed. Comparable to the HIF target RNA expression HIF-1α and HIF-2α protein levels were further increased after exposing the PHD2 cKO cells and shPHD2 RAW cells to hypoxia. Taken together this demonstrates a biologically relevant reduction of PHD2 with subsequent stabilization of the HIF-α proteins and induction of HIF target genes like PDK1 in the cell line model and the genetically modified primary macrophages partially mimicking hypoxia. The increased expression of PDK1 was further analyzed at protein level. PDK1 is a major regulator in central metabolic pathways including glucose consumption. It acts in part by regulating the activity of the pyruvate dehydrogenase (PDH) by phosphorylation, which results in inactivation of the enzyme. PDH is one part of a mitochondrial multi-enzyme complex that catalyzes the oxidative decarboxylation of pyruvate and is one of the major enzymes responsible for the regulation of homeostasis of carbohydrate fuels in mammals. The induction of PDK1 in the PHD2-deficient cells in normoxia was also detectable at the protein level (Fig. 1D). In line, PDH was found to be more phosphorylated in the shPHD2 RAW cell and the PHD2 cKO BMDM compared to their wild type cells. In hypoxia both, wt and the PHD2-deficient cells, showed increased PDK1 protein level and phosphorylation of PDH. In parallel we determined the PDH activity in cell extracts isolated from wt cells and PHD2-deficient cells after exposure to normoxia or hypoxia (Fig. 1E). shPHD2 cells exhibited a significantly lower PDH activity in normoxia, which was also observed in wt cells after treatment with the PHD inhibitor DMOG. In hypoxia, a decreased PDH activity was observed in wt and shPHD2 cells, still with significantly lower levels in the knock down cells. Since PDK1 is a critical regulator of cellular metabolism, we next analyzed the glycolytic capacity in the PHD2-deficient cells.

PHD2-deficient macrophages show a switch to glycolytic metabolism

Glycolysis utilization for energy demand can be characterized by the oxygen consumption and the extracellular acidification rate after stimulation. Basal oxygen consumption rate and maximal respiration after uncoupling the mitochondria with FCCP were significantly decreased in the PHD2-deficient cells or after treating wt cells with DMOG, which indicates that as a consequence of inhibiting PHD activity macrophages shift their source of energy to anaerobic glycolysis (Fig. 2A and B). This was further supported by a significantly increased glycolytic function as determined by the extracellular acidification rate after glucose or oligomycin treatment, which reflects basal glycolysis and the overall glycolytic capacity, respectively (Fig. 2C-F). In line we found increased lactate levels in the supernatant of shPHD2 RAW cells and PHD2 cKO BMDM compared to their respective wt cells (Fig. 2G). Lactate levels were likewise increased after DMOG treatment or exposure of the cells to hypoxia. Incubating wt and PHD2 cKO BMDM without glucose at 20% O_2 abolished the lactate production demonstrating that the glucose in the culture medium is indeed the major source for lactate production (Fig. 2H).

A fully operative cellular metabolism of macrophages is important to provide the necessary amount of ATP. Aerobic versus anaerobic glycolysis are differing in the net production of ATP. In line with the less efficient anaerobic glycolysis, levels of ATP were significantly diminished in the PHD2 cKO BMDM, shPHD2 RAW cells as well as after treating RAW wt cells with DMOG or exposing them to hypoxia (Fig. 3A), which might impact their functionality namely polarization, migration and phagocytosis. Macrophages display remarkable plasticity and can change their phenotype upon stimulation (16). The most prominent macrophage populations are the M1- and M2-polarized macrophages. To analyze if the metabolic alterations in the PHD2-deficient cells affects macrophage polarization we analysed RNA levels of the M1 markers TNFα, iNOS, and MCP-1 as well as the the M2 markers Ym1 and Fizz in wt and PHD2 cKO BMDM. Arginase, which is also a M2 marker,

was not detectable in non-stimulated wt BMDM. None of the M1 or M2 markers were changed in the PHD2 cKO BMDMs (Fig. 3B). Subsequently, we stimulated the cells with LPS and the Th1 cytokine IFNγ or the Th2 cytokine IL-4 to functionally characterize M1- and M2-polarization, respectively (Fig. 3C). Successful M1- and M2-polarization after stimulation was verified by increased RNA levels of TNFα, iNOS and MCP-1 after treatment with LPS as well as the Ym1, arginase and Fizz after treatment with IL-4. Expression levels of the M1 and M2-marker RNAs were not different comparing wild type and PHD2 cKO BMDM. A lack in macrophage polarization in the PHD2 cKO BMDM was additionally confirmed by a non-biased RNA microarray gene expression assay (Suppl. Table 1 and Table 2). In total 42 genes were found to be significantly upregulated in PHD2 cKO BMDM including PHD3, BNIP3, PFK1, and PDK1 and 56 were found to be significantly downregulated. Pathway analysis of the RNAs identified to be up- or downregulated in the PHD2 cKO BMDM versus wt BMDM did not reveal any indications for a macrophage polarization. Likewise treatment of wt or PHD2 cKO BMDM with DMOG resulted in altered RNA expression but no clear pattern of M1 or M2 associated genes (Fig. 3D). Whereas DMOG resulted in significantly decreased RNA levels of TNFα and MCP1, iNOS, FIZZ and arginase were significantly increased in wt as well as cKO BMDM. Taken collectively, the metabolic alterations in consequence if DMOG treatment results in the downregulation of RNAs of some M1 associated genes, however no stringent impact on the polarization of macrophages was observed in cKO BMDM.

Impaired migratory and phagocytic capacity of PHD2 cKO BMDM and shPHD2 RAW cells

Whereas most cells in the body are fixed in place, macrophages are motile and able to migrate into surrounding tissues, where one of their major tasks is phagocytosis of invading pathogens or cell debris. We analyzed the migration capacity of the PHD2 cKO BMDM and shPHD2

RAW cells by confronting the macrophages with conditioned supernatant of the MDA-MB231 breast carcinoma cells in a Boyden chamber and in single cell migration experiments. Significantly less PHD2 cKO BMDM and shPHD2 RAW cells migrated compared to their respective wt cells in the Boyden chamber (Fig. 4A). Additionally the accumulated distance in the single cell migration experiments was found to be reduced (Fig. 4B). The impaired migration capacity of the PHD2-deficient RAW cells could be mimicked by treating wt RAW cells with the PHD inhibitor DMOG or exposure to hypoxia (Fig. 4C). In line with the migration deficit, phagocytosis capacity was disturbed in the shPHD2 RAW cells and the PHD2 cKO BMDM (Fig. 4D). Comparable to the migration capacity the decreased phagocytosis could be mimicked in wt RAW cells by incubating the cells in hypoxia or by treatment with DMOG.

Differential migration was not due to a modulated chemokine receptor expression since quantifying CCR2, CCR4, CCR5, CCR7 and CXCR4 RNA levels by RT-PCR after exposing wild type and PHD2 cKO BMDM to 20% O_2 or 1% O_2 did not reveal any difference between the cell types besides an upregulation of CCR1 in the PHD2 cKO cells (Fig. 4E). We however observed a significant upregulation of CCR1 and CCR5 and a slight upregulation of CXCR4 in hypoxia, which is in line with the literature (17,18). Since the migration deficit was not due to a striking differential expression of chemokine receptors we further analyzed the impact of the metabolic alterations for the migratory and phagocytosis capacity.

Regulation of PHD activity by PDK is critical for macrophage function

To obtain insight into the importance of the altered PDK1 levels and PDH activity for the functional impairment of the PHD2-deficient macrophages, we treated cells with the PDK inhibitor DCA. DCA binds to PDK1 near the helix bundle in the N-terminal part. Bound DCA promotes local conformational changes that are communicated to both nucleotide-binding and lipoyl-binding pockets of PDK1, leading to the inactivation of kinase activity (19). DCA

treatment reestablished the decreased PDH activity in the shPHD2 RAW cells (Fig. 5A). In line with this shPHD2 and PHD2 cKO BMDM produced less lactate and demonstrated significantly increased ATP levels after DCA treatment (Fig. 5B and C). Reversal of the metabolic reprogramming after DCA treatment was also observed when analyzing the ECAR. Whereas the non-treated shPHD2 cells and PHD2 cKO BMDM had a significantly increased glycolysis and glycolytic capacity, this was significantly decreased in the DCA treated cells (Fig. 5D). Finally, we tested if DCA treatment is able to normalize the migration and phagocytosis deficit (Fig. 5E and F). Most interestingly, DCA treatment indeed was able to increase the impaired migration and phagocytosis capacity in the shPHD2 RAW and PHD2 cKO cells to comparable levels of the wild type cells, strongly indicating that the metabolic programming via PDK1 *per se* is responsible for the functional alterations in consequence of downregulating PHD2 expression.

PDK1 has been described to be a HIF target gene (7). In a previous report, we demonstrated that in PHD3 cKO BMDM HIF-1α is not stabilized in normoxia (12), which is in sharp contrast to the effect seen in the PHD2 cKO BMDM described here. This was further supported by the fact, that HIF target genes including PDK1 were not significantly increased in PHD3 cKO BMDM other than PHD2 cKO or wt BMDMs in normoxia and hypoxia (Fig. 6A). The genes identified in the gene array analysis (PHD2 cKO versus wt BMDM) were additionally compared to PHD3 regulated genes described earlier (12). Most interestingly just 13 genes were found to be regulated in the PHD2 cKO as well as in the PHD3 cKO BMDM, indicating PHD isoform specific effects (Suppl. Table 3). In line ATP and lactate levels as well as accumulated migration difference were not altered in PHD3 cKO versus wt BMDM (Fig. 6B). To further analyze, if PHD2 mediates its effects via HIF-1α we generated dcKO mice. dcKO BMDM had a blunted response in RNA levels of the HIF target genes PHD3, Glut-1, PDK1, PDK1, Cox4.2 and LonP in hypoxia (Fig. 7A). The metabolic phenotype of the PHD2 cKO BMDM, i.e. decreased ATP levels, increased lactate levels, decreased OCR

and decreased migration was rescued in consequence of the HIF-1α knock out demonstrating that HIF-1 and PDK1 are the two main mediators for the PHD2-induced metabolic alterations (Fig. 7B-D).

Discussion

Macrophages are critical effector cells for innate immunity but also adaptive immune function by recruiting further cells to the inflamed tissue (20). To fulfill their functions macrophages need to efficiently migrate into the affected tissue and phagocytose invading pathogens or cell debris. Both characteristic features were severely impaired in the PHD2-deficient RAW cell line model as well as in the primary BMDM in our study. The impaired migration is in line with two previous studies analyzing the migratory capacity of PHD2-deficient peritoneal macrophages as well as shPHD2 RAW cells towards MCP-1 as a stimulus (21,22). Infiltrating inflammatory cells including macrophages play an important role in tissue remodeling after an insult. In line with this, LysM Cre PHD2$^{fl/fl}$ animals showed less macrophage infiltration in the aorta during hypertensive cardiovascular remodeling (21). This was associated with a protection from hypertension-induced left ventricular hypertrophy and reduced ejection fraction. The basis for the impaired macrophage migration, however, has not been analyzed in further depth so far.

Functionality of macrophages is significantly affected by their polarization as well as their metabolic phenotype. Macrophages can be generally classified into two major groups, i.e. the M1- (classically activated) and M2- (alternatively activated) macrophages (23). Both subgroups have specific functions for the inflammatory clearance of pathogens and tissue repair, respectively (24). HIF-1α and HIF-2α are known to affect macrophage polarization with a predominant role of HIF-1α for M1-macrophages and HIF-2α for M2-macrophages

(25). Since the PHD enzymes regulate HIFα stability it is tempting to speculate that inhibition of their enzymatic activity would also affect macrophage polarization. Characterization of resting as well as stimulated wt and PHD2 cKO macrophages, however, did not reveal any striking pattern, which would indicate a clear polarization. It is important to note that this observation is not in contrast with the study by Takeda et al, in which an M2-polarization has been described as a consequence of a PHD2 knock out (26). M2-polarization was observed in heterozygous but not homozygous PHD2-deficient macrophages by Takeda et al., which matches our observations with the homozygous PHD2-deficient macrophages.

In stark contrast to the unaltered polarization, we found an influence of PHD2 on reprogramming of mitochondrial metabolism. This effect was mediated via PHD2 dependent regulation of PDK1 expression. This is in accordance with a recent report showing in livers/hepatocytes that PHD2 can regulate PDK1 (27). In line with higher levels of PDK1 and higher phosphorylation of its target PDH, the basal and stimulated oxygen consumption was significantly lower in the PHD2-deficient cells accompanied by an increased glycolytic capacity. PDK1 is a key regulatory enzyme in glucose metabolism. The PDH complex, which is regulated by PDK1, converts pyruvate produced from the glycolytic flux to acetyl-CoA. Pyruvate-derived acetyl-CoA then enters the TCA cycle that generates NADH that fuels the electron transport chain for oxidative phosphorylation. In hypoxia PDH activity is inhibited via PDK1 mediated phosphorylation, which induces the anaerobic glucose metabolic homeostasis under limited oxygen availability. This mechanism is widely used by tumor cells and is part of the so-called *Warburg effect*. Warburg described that unlike most normal tissues, cancer cells tend to “ferment” glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation (28). In case of immune cells the metabolic adaptation however is part of their physiological response. Innate immune cells like neutrophils likewise depend on anaerobic glycolysis for ATP production, which is also resembled by the fact, that they harbor only few mitochondria (29). In contrast macrophages

423 have comparable numbers of mitochondria like other body cells and thus a higher metabolic
424 flexibility, which allows a quick metabolic switch from aerobic to anaerobic glycolysis.
425 Hallmarks of anaerobic glycolysis are a reduced ATP production, increased lactate levels and
426 decreased oxygen consumption (30). All three features were significantly altered in the
427 PHD2-deficient macrophages indicating an anaerobic metabolic shift. Neutrophils and
428 monocytes/macrophages fulfill their physiological function in severely hypoxic areas like
429 inflammation or ischemia. Unlike short-lived neutrophils, macrophages survive longer in the
430 body up to a maximum of several months. Compared to the oxygenated blood the pO2 in
431 most tissues is significantly lower. Thus, compared to monocytes, macrophages need to be
432 able to adapt to the hypoxic conditions, which reflect their physiological environment. In
433 contrast, short lived neutrophils enter inflamed hypoxic tissue where they die quickly to fulfill
434 their function. This is also reflected by the fact that in contrast to other cells, they die upon
435 exposure to hypoxia. Most interestingly the metabolic phenotype observed in the shPHD2
436 RAW cells and cKO BMDM mimicked the effects seen in wt cells in hypoxia. The metabolic
437 adaptation thus might ensure function and viability of the cells as long as possible in the
438 hypoxic conditions. A lower migration rate as a consequence helps to keep the cells in place
439 until they are stimulated during the course of an acute inflammation.

440 Recent evidence suggests an intricate link between metabolism and macrophage activation
441 (31). To this end it becomes important to know what kind of metabolic changes occur after
442 immune cell activation and if the altered metabolism *per se* can serve as a controller of the
443 immunomodulatory functionality. Analyzing metabolic aspects as a consequence of blocking
444 PHD2 thus might answer the question about what changes in the regulation of energy
445 metabolism are necessary for macrophages and if these can be targeted to control innate
446 immune function. In this regard our data clearly indicate that deleting PHD2 in macrophages
447 is sufficient to drive an anaerobic glycolytic phenotype in normoxia and interferes with
448 migration and phagocytosis. PHD enzyme activity can be inhibited with competitive

449 oxoglutarate analogues. Respective inhibitors are being developed to specifically interfere
450 with the PHD/HIF signaling pathway (32). Short term treatment of wt macrophages with the
451 PHD inhibitor DMOG or exposing the cells to hypoxia was indeed sufficient to mimic the
452 metabolic features of the genetically modified macrophages. Moreover, the metabolic switch
453 in the PHD2-deficient macrophages was readily reversible by inhibition of PDK1
454 demonstrating that PHD2-mediated metabolic changes are not decretory. DCA treatment
455 rescued all hallmarks of anaerobic glycolysis in the PHD2-deficient cells including the
456 impaired functionality. A critical role of PDK1 for macrophage metabolism and function has
457 been described earlier and is in line with our findings (33). Via altering PDK1 activity the
458 cellular ATP levels as well as extracellular lactate levels are modulated. ATP as well as a
459 lactate-enriched environment has been demonstrated to add to immunomodulatory functions
460 by altering the migratory activity of defense cells (34,35). PDK1 as a molecule to target
461 deregulated energy metabolism is an emerging strategy for cancer therapy (36). Redirection of
462 glucose metabolism from glycolysis to oxidation, which reverses the Warburg effect, leads to
463 inhibition of proliferation and induction of caspase-mediated apoptosis in tumor cells. Thus
464 far, DCA is the most extensively studied PDK1 inhibitor; however, it has limited use for
465 therapeutic purposes because of its low potency and high toxicity. PHD inhibitors on the other
466 side have entered pre-clinical models and clinical trials and thus interfering with PHD2
467 activity might serve as better strategy to influence immune functions via metabolic
468 reprogramming (37).

469 Taken collectively our study shows that while PHD2 is not required for macrophage
470 polarization it controls macrophage metabolism and function. Mechanistically the balance
471 between aerobic and anaerobic glycolysis is affected by PHD2 via the expression and activity
472 of PDK1. This adds to our understanding of the functionality of macrophages in normoxia
473 and hypoxia. In addition our finding might point to a possibility to specifically interfere with
474 the inflammatory function of macrophages by inhibiting PHD2 activity.

Acknowledgements

We thank Annette Hillemann for expert technical support.

Contribution: A.G., A.B., L.S., K.F., S.N., B.W., A.Z., A.J. and M.C. designed and performed research, analyzed data and wrote the manuscript; J.D. performed research and analyzed data; P.R., C.X.C.S. and A.M.S. evaluated the data and corrected the paper; D.M.K. designed research and wrote the manuscript; and all authors read and edited the manuscript.

Conflict-of-interest disclosure The authors declare no competing financial interests.

Funding information

This study was supported by research funding from the Deutsche Forschungsgemeinschaft (IRTG1816) to A.G. and A.B.; P.R. is supported by the SFB1002; M.C., C.X.C.S. and A.M.S. are supported by the British Heart Foundation.

References

1. **Wynn, TA, Vannella, KM**. 2016. Macrophages in Tissue Repair, Regeneration, and Fibrosis. Immunity. **44:** 450-462.
2. **Goda, N, Kanai, M**. 2012. Hypoxia-inducible factors and their roles in energy metabolism. Int J Hematol. **95:** 457-463.
3. **Bishop, T, Ratcliffe, PJ**. 2015. HIF hydroxylase pathways in cardiovascular physiology and medicine. Circ Res. **117:** 65-79.
4. **Kaelin Jr, WG**. 2004. Proline Hydroxylation and Gene Expression. Annu Rev Biochem. **19:** 19.
5. **Schofield, CJ, Ratcliffe, PJ**. 2004. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol. **5:** 343-354.
6. **Papandreou, I, Cairns, RA, Fontana, L, Lim, AL, Denko, NC**. 2006. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab. **3:** 187-197.
7. **Kim, JW, Tchernyshyov, I, Semenza, GL, Dang, CV**. 2006. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. **3:** 177-185.
8. **Katschinski, DM**. 2009. In vivo functions of the prolyl-4-hydroxylase domain oxygen sensors: direct route to the treatment of anaemia and the protection of ischaemic tissues. Acta Physiol (Oxf). **195:** 407-414.
9. **Myllyharju, J**. 2013. Prolyl 4-hydroxylases, master regulators of the hypoxia response. Acta Physiol (Oxf). **208:** 148-165.
10. **Walmsley, SR, Chilvers, ER, Thompson, AA, Vaughan, K, Marriott, HM, Parker, LC, Shaw, G, Parmar, S, Schneider, M, Sabroe, I, Dockrell, DH, Milo, M, Taylor, CT, Johnson, RS, Pugh, CW, Ratcliffe, PJ, Maxwell, PH, Carmeliet, P, Whyte, MK**. 2011. Prolyl hydroxylase 3 (PHD3) is essential for hypoxic regulation of neutrophilic inflammation in humans and mice. J Clin Invest. **121:** 1053-1063.
11. **Kiss, J, Mollenhauer, M, Walmsley, SR, Kirchberg, J, Radhakrishnan, P, Niemietz, T, Dudda, J, Steinert, G, Whyte, MK, Carmeliet, P, Mazzone, M, Weitz, J, Schneider, M**. 2012. Loss of the oxygen sensor PHD3 enhances the innate immune response to abdominal sepsis. J Immunol. **189:** 1955-1965.
12. **Swain, L, Wottawa, M, Hillemann, A, Beneke, A, Odagiri, H, Terada, K, Endo, M, Oike, Y, Farhat, K, Katschinski, DM**. 2014. Prolyl-4-hydroxylase domain 3

(PHD3) is a critical terminator for cell survival of macrophages under stress conditions. J Leukoc Biol. **96:** 365-375.

13. **Burgess, AW, Metcalf, D, Kozka, IJ, Simpson, RJ, Vairo, G, Hamilton, JA, Nice, EC**. 1985. Purification of two forms of colony-stimulating factor from mouse L-cell-conditioned medium. J Biol Chem. **260:** 16004-16011.
14. **Klotzsche-von Ameln, A, Muschter, A, Mamlouk, S, Kalucka, J, Prade, I, Franke, K, Rezaei, M, Poitz, DM, Breier, G, Wielockx, B**. 2011. Inhibition of HIF prolyl hydroxylase-2 blocks tumor growth in mice through the antiproliferative activity of TGFbeta. Cancer Res. **71:** 3306-3316.
15. **Hölscher, M, Silter, M, Krull, S, von Ahlen, M, Hesse, A, Schwartz, P, Wielockx, B, Breier, G, Katschinski, DM, Zieseniss, A**. Cardiomyocyte-specific Prolyl-4-hydroxylase Domain 2 Knock Out Protects from Acute Myocardial Ischemic Injury. J Biol Chem. **286:** 11185-11194.
16. **Mosser, DM, Edwards, JP**. 2008. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. **8:** 958-969.
17. **Bosco, MC, Puppo, M, Santangelo, C, Anfosso, L, Pfeffer, U, Fardin, P, Battaglia, F, Varesio, L**. 2006. Hypoxia modifies the transcriptome of primary human monocytes: modulation of novel immune-related genes and identification of CC-chemokine ligand 20 as a new hypoxia-inducible gene. J Immunol. **177:** 1941-1955.
18. **Dong, F, Khalil, M, Kiedrowski, M, O'Connor, C, Petrovic, E, Zhou, X, Penn, MS**. 2010. Critical role for leukocyte hypoxia inducible factor-1alpha expression in post-myocardial infarction left ventricular remodeling. Circ Res. **106:** 601-610.
19. **Kato, M, Li, J, Chuang, JL, Chuang, DT**. 2007. Distinct structural mechanisms for inhibition of pyruvate dehydrogenase kinase isoforms by AZD7545, dichloroacetate, and radicicol. Structure. **15:** 992-1004.
20. **Shi, C, Pamer, EG**. 2011. Monocyte recruitment during infection and inflammation. Nat Rev Immunol. **11:** 762-774.
21. **Ikeda, J, Ichiki, T, Matsuura, H, Inoue, E, Kishimoto, J, Watanabe, A, Sankoda, C, Kitamoto, S, Tokunou, T, Takeda, K, Fong, GH, Sunagawa, K**. 2013. Deletion of phd2 in myeloid lineage attenuates hypertensive cardiovascular remodeling. J Am Heart Assoc. **2:** e000178.
22. **Mamlouk, S, Kalucka, J, Singh, RP, Franke, K, Muschter, A, Langer, A, Jakob, C, Gassmann, M, Baretton, GB, Wielockx, B**. 2014. Loss of prolyl hydroxylase-2 in

myeloid cells and T-lymphocytes impairs tumor development. Int J Cancer. **134:** 849-858.

23. **Mantovani, A, Sica, A, Sozzani, S, Allavena, P, Vecchi, A, Locati, M**. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. **25:** 677-686.

24. **Lawrence, T, Natoli, G**. 2011. Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat Rev Immunol. **11:** 750-761.

25. **Takeda, N, O'Dea, EL, Doedens, A, Kim, JW, Weidemann, A, Stockmann, C, Asagiri, M, Simon, MC, Hoffmann, A, Johnson, RS**. 2010. Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. Genes Dev. **24:** 491-501.

26. **Takeda, Y, Costa, S, Delamarre, E, Roncal, C, Leite de Oliveira, R, Squadrito, ML, Finisguerra, V, Deschoemaeker, S, Bruyere, F, Wenes, M, Hamm, A, Serneels, J, Magat, J, Bhattacharyya, T, Anisimov, A, Jordan, BF, Alitalo, K, Maxwell, P, Gallez, B, Zhuang, ZW, Saito, Y, Simons, M, De Palma, M, Mazzone, M**. 2011. Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. Nature. **479:** 122-126.

27. **Suhara, T, Hishiki, T, Kasahara, M, Hayakawa, N, Oyaizu, T, Nakanishi, T, Kubo, A, Morisaki, H, Kaelin, WG, Jr., Suematsu, M, Minamishima, YA**. 2015. Inhibition of the oxygen sensor PHD2 in the liver improves survival in lactic acidosis by activating the Cori cycle. Proc Natl Acad Sci U.S.A. **112:** 11642-11647.

28. **Warburg, O**. 1956. On respiratory impairment in cancer cells. Science. **124:** 269-270.

29. **Kelly, B, O'Neill, LA**. 2015. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell Res. **25:** 771-784.

30. **Pavlova, NN, Thompson, CB**. 2016. The Emerging Hallmarks of Cancer Metabolism. Cell Metab. **23:** 27-47.

31. **Mills, EL, O'Neill, LA**. 2016. Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. Eur J Immunol. **46:** 13-21.

32. **Myllyharju, J**. 2009. HIF prolyl 4-hydroxylases and their potential as drug targets. Curr Pharm Des. **15:** 3878-3885.

33. **Tan, Z, Xie, N, Cui, H, Moellering, DR, Abraham, E, Thannickal, VJ, Liu, G**. 2015. Pyruvate dehydrogenase kinase 1 participates in macrophage polarization via regulating glucose metabolism. J Immunol. **194:** 6082-6089.

34. **Haas, R, Smith, J, Rocher-Ros, V, Nadkarni, S, Montero-Melendez, T, D'Acquisto, F, Bland, EJ, Bombardieri, M, Pitzalis, C, Perretti, M, Marelli-Berg, FM, Mauro, C**. 2015. Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration and Effector Functions. PLoS Biol. **13:** e1002202.

35. **Gottfried, E, Kunz-Schughart, LA, Ebner, S, Mueller-Klieser, W, Hoves, S, Andreesen, R, Mackensen, A, Kreutz, M**. 2006. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. Blood. **107:** 2013-2021.

36. **Barile, E, De, SK, Pellecchia, M**. 2012. PDK1 inhibitors. Pharm Pat Anal. **1:** 145-163.

37. **Chan, MC, Holt-Martyn, JP, Schofield, CJ, Ratcliffe, PJ**. 2016. Pharmacological targeting of the HIF hydroxylases - A new field in medicine development. Mol Aspects Med. **47-48:** 54-75.

Figure legends

Fig. 1: *PHD2 knock down RAW cells and PHD2 knock out (PHD2 cKO) BMDMs display increased PDK1 expression and activity.* (**A**) wt RAW and shPHD2 knock down cells as well as wt BMDM and PHD2 cKO macrophages were incubated for 24 hrs at 20% or 1% O_2. RNA levels of the indicated genes were analyzed by qRT-PCR. RNA levels of wt RAW and wt BMDM cells were set to one. Fold change of the RNA levels of shPHD2 or the PHD2 cKO BMDM or wt cells in hypoxia of the indicated genes compared to the wt cells in normoxia was analyzed. n = 3-6 independent samples per condition. (**B**) Annexin V (AV) single-positive cells were analyzed in wt BMDM and PHD2 cKO macrophages with and without treatment with 1 mM DMOG for 24 hrs. (**C**) HIF-1α, HIF-2α, PHD2 and β-actin protein levels of wt RAW and shPHD2 as well as wt BMDM and PHD2 cKO macrophages in normoxia (20% O_2) or hypoxia (1% O_2 for 24 hrs). (**D**) phospho-PDH, total PDH, PDK and β-actin protein levels of wt RAW and shPHD2 cells as well as wt BMDM and PHD2 cKO macrophages in normoxia (20% O_2) or hypoxia (1% O_2 for 24 hrs). (**E**) PDH activity in wt RAW, shPHD2 RAW cells and wt RAW cells treated with 1 mM DMOG for 24 hrs in normoxia or hypoxia (1% O_2 for 24 hrs). n = 6 independent samples per condition. mean ± SEM, * $p<0.05$.

Fig. 2: *As a consequence of a reduction of PHD2 expression macrophages shift their metabolism towards anaerobic glycolysis.* (**A**) wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells or wt RAW cells treated with 1 mM DMOG for 24 hrs were tested for their oxygen consumption rate (OCR) after addition of oligomycin, FCCP as well as rotenone and antimycin A (Rot + AA). n = 6 (RAW) and n = 10 (BMDM) independent samples per condition. (**B**) Basal respiration and maximum respiration were analyzed in wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells based on the experiments shown in A. OCR after addition of Rot + AA was subtracted from the OCR after addition of oligomycin and

FCCP to obtain basal respiration and maximum respiration values, respectively. (**C**) Extracellular acidification rate (ECAR) was determined in wt RAW and shPHD2 RAW as well as (**D**) wt BMDM and PHD2 cKO cells or wt RAW cells treated with 1 mM DMOG for 24 hrs after addition of glucose, oligomycine and 2-desoxy glucose (2-DG). n = 7 independent samples per condition. (**E, F**) Glycolysis and anaerobic glycolytic capacity were analyzed based on the experiments shown in C and D. The ECAR after addition of 2-DG was subtracted from the ECAR after addition of glucose or oligomycine to obtain glycolysis and glycolytic capacity values, respectively. (**G**) Lactate levels were determined in the supernatant of wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells after incubation of the cells at the indicated conditions. n = 4 independent samples per condition. mean ± SEM, * $p<0.05$. (**H**) Lactate levels were determined in the supernatant of wt BMDM and cKO cells after incubation of the cells at 20% O_2, 1% O_2 with or without addition of glucose in the cell culture medium. Cells were incubated for 24 hrs in the respective cell culture medium, n = 4 independent samples per condition.

Fig. 3: *Decreased ATP levels and unaltered polarization in PHD2 deficient macrophages.* (**A**) wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells were incubated at 20% O_2 or 1% O_2 for 24 hrs. Subsequently intracellular ATP levels were determined. n = 6 independent samples per condition. Right panel: wt RAW cells were incubated for the indicated times with 1 mM DMOG and ATP levels were determined. n = 6-7 independent samples per condition. (**B**) RNA levels of M1- and M2-markers in resting wt BMDM and cKO cells or (**C**) after stimulation with IL-4 (20 nM) or LPS (100 ng/ml) and IFNγ (20 nM) for 24 hrs. (**D**) RNA levels of M1 and M2-markers in resting wt BMDM and PHD2 cKO cells were analyzed after treatment of the cells with 1mM DMOG for 24 hrs. Fold change of the RNA levels of DMOG treated wt or cKO BMDM of the indicated genes compared to the non-treated cells was analyzed. n = 3-6 independent samples per condition. mean ± SEM, * $p<0.05$.

Fig. 4: *A reduction of PHD2 expression in RAW cells or BMDMs results in a defect in macrophage migration and phagocytosis.* (**A**) wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells were tested for their migration capacity in Boyden chambers using FCS or conditioned medium of MDA-MB231 cells as stimulants. n = 4 (RAW cells), n = 4 (BMDM) independent samples. (**B**) The accumulated migration distance over 6 hrs of wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells was tested in single cell migration experiments using FCS or conditioned medium of MDA-MB231 cells as stimulants. n = 59-64 cells per condition (RAW cells), n = 51-71 cells per conditions (BMDM cells). (**C**) wt RAW cells were incubated at 20% O_2 ± 1 mM DMOG or 1% O_2 for 6 hrs. The accumulated migration distance was tested in single cell migration experiments using FCS or conditioned medium of MDA-MB231 cells as stimulants. For cells analyzed at 1% O_2 the hypoxic conditions were kept during the single cell migration experiments without reoxygenation. n = 50-53 cells per condition. (**D**) wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells were incubated at 20% O_2 ± 1 mM DMOG or 1% O_2 in total for 20 hrs. Subsequently, the capacity of the cells to phagocytose labeled beads was analyzed. Fluorescence beads were added to the cells for 4 hrs without reoxygenation. n = 5 independent samples per condition. mean ± SEM, * $p<0.05$. (**E**) RNA levels of chemokine receptors in resting wt BMDM and PHD2 cKO cells after incubation in normoxia or hypoxia (1% O_2) for 24 hrs. Fold change of the RNA levels in the PHD2 cKO BMDM of the indicated genes compared to the wt cells in normoxia was analyzed. n = 3 independent samples per condition. mean ± SEM. *$p<0.05$.

Fig. 5: *Inhibition of PDK1 by dichloroacetate (DCA) reverses the metabolic phenotype and the migration defect in PHD2-deficient macrophages.* (**A**) PDH activity was determined in lysates of wt RAW, shPHD2 RAW cells after incubation of the cells ± 5 mM DCA for 24 hrs. n = 6 independent samples per condition. Lactate levels in the supernatants and intracellular ATP levels of wt RAW and shPHD2 RAW (**B**) as well as wt BMDM and cKO macrophages (**C**) after incubation of the cells ± 5 mM DCA for 24 hrs. (**D**) Glycolysis and glycolytic

capacity were analyzed in wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO after incubating the cells ± 5 mM DCA for 24 hrs. n = 6-10 independent samples per condition. (**E**) The accumulated migration distance over 6 hrs of wt RAW and shPHD2 RAW cells after incubation of the cells ± 5 mM DCA for 24 hrs was determined in single cell migration experiments using FCS or conditioned medium of MDA-MB231 cells as stimulants. n = at least 20 cells per condition. (**F**) wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells were incubated ± 5 mM DCA for 24 hrs. Subsequently, the capacity of the cells to phagocytose labeled beads was analyzed. n = 5-8 independent samples per condition. mean ± SEM, * $p<0.05$.

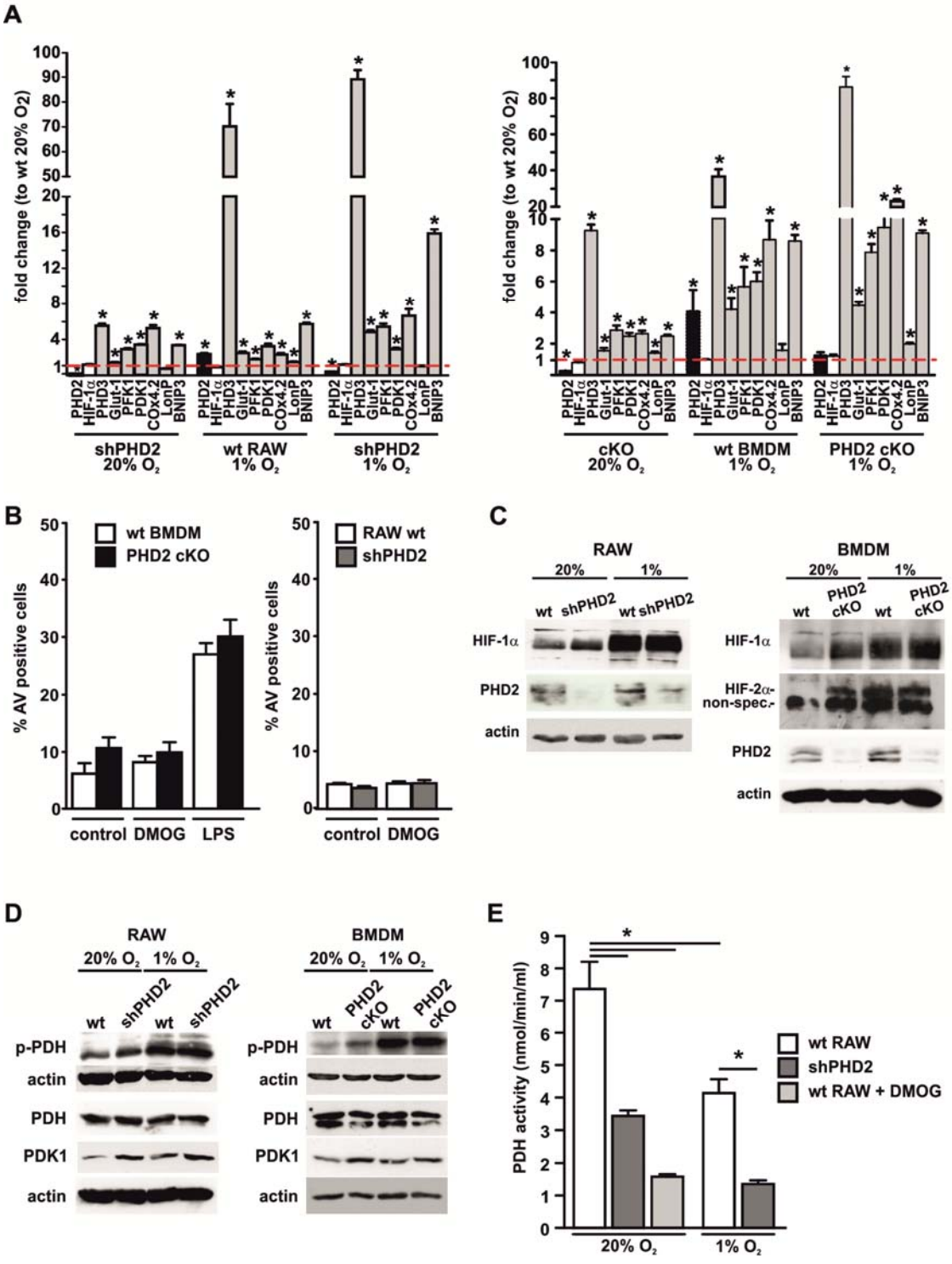
Fig. 6: *No metabolic phenotype in PHD3-deficient macrophages*. (**A**) wt BMDM and PHD3 cKO macrophages were incubated for 24 hrs at 20% or 1% O_2. RNA levels of the indicated genes were analyzed by qRT-PCR. RNA levels of wt BMDM cells were set to one. Fold change of the RNA levels of PHD3 cKO BMDM or wt cells in hypoxia of the indicated genes compared to the wt cells in normoxia was analyzed. n = 3 independent samples per condition. (**B**) ATP (n = 5 independent samples) and lactate levels (n = 4 independent samples) as well as accumulated migration distance were determined in wt BMDM and PHD3 cKO macrophages.

Fig. 7: *HIF-1α mediates the metabolic alterations in PHD2-deficient macrophages.* (**A**) wt BMDM and dcKO macrophages were incubated for 24 hrs at 20% or 1% O_2. RNA levels of the indicated genes were analyzed by qRT-PCR. RNA levels of wt BMDM cells were set to one. Fold change of the RNA levels of dcKO BMDM or wt cells in hypoxia of the indicated genes compared to the wt cells in normoxia was analyzed. n = 3 independent samples per condition. mean ± SEM, * $p<0.05$ compared to wt 20% O_2, # $p<0.05$ compared to wt 1% O_2. (**B**) Intracellular ATP levels were determined in wt BMDM, PHD2 cKO and dcKO cells after incubation for 24 hrs in 20% O_2 or 1% O_2. (**C**) Oxygen consumption rate (OCR) and lactate in

705 the supernatants of wt BMDM PHD2 cKO and dcKO cells were analyzed. (**D**) The
706 accumulated migration distance over 6 hrs of wt BMDM, PHD2 cKO and dcKO cells was
707 determined in single cell migration experiments using FCS or conditioned medium of MDA-
708 MB231 cells as stimulants. n = at least 20 cells per condition. mean ± SEM, * $p<0.05$.

709

Figure 1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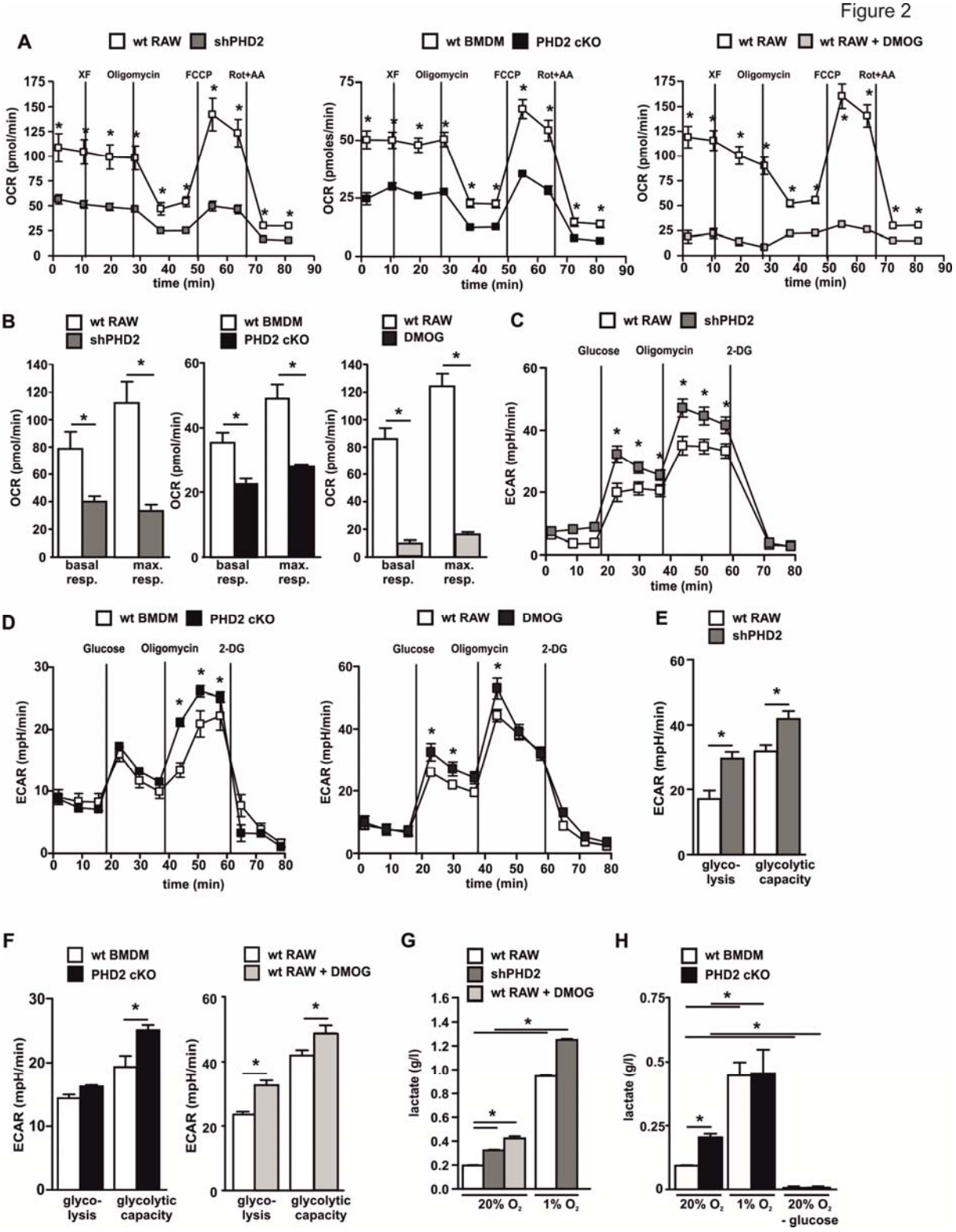
fold change (to wt 20% O2)
PHD2
HIF-1α
PHD3
Glut-1
PFK1
PDK1
COx4.2
LonP
BNIP3
shPHD2 20% O2
wt RAW 1% O2
shPHD2 1% O2
cKO 20% O2
wt BMDM 1% O2
PHD2 cKO 1% O2
B
wt BMDM
PHD2 cKO
RAW wt
shPHD2
% AV positive cells
control
DMOG
LPS
C
RAW
BMDM
20%
1%
wt
shPHD2
PHD2 cKO
HIF-1α
HIF-2α-non-spec.-
PHD2
actin
D
RAW
BMDM
20% O2
1% O2
p-PDH
actin
PDH
PDK1
E
PDH activity (nmol/min/ml)
wt RAW
shPHD2
wt RAW + DMOG
20% O2
1% O2

Figure 2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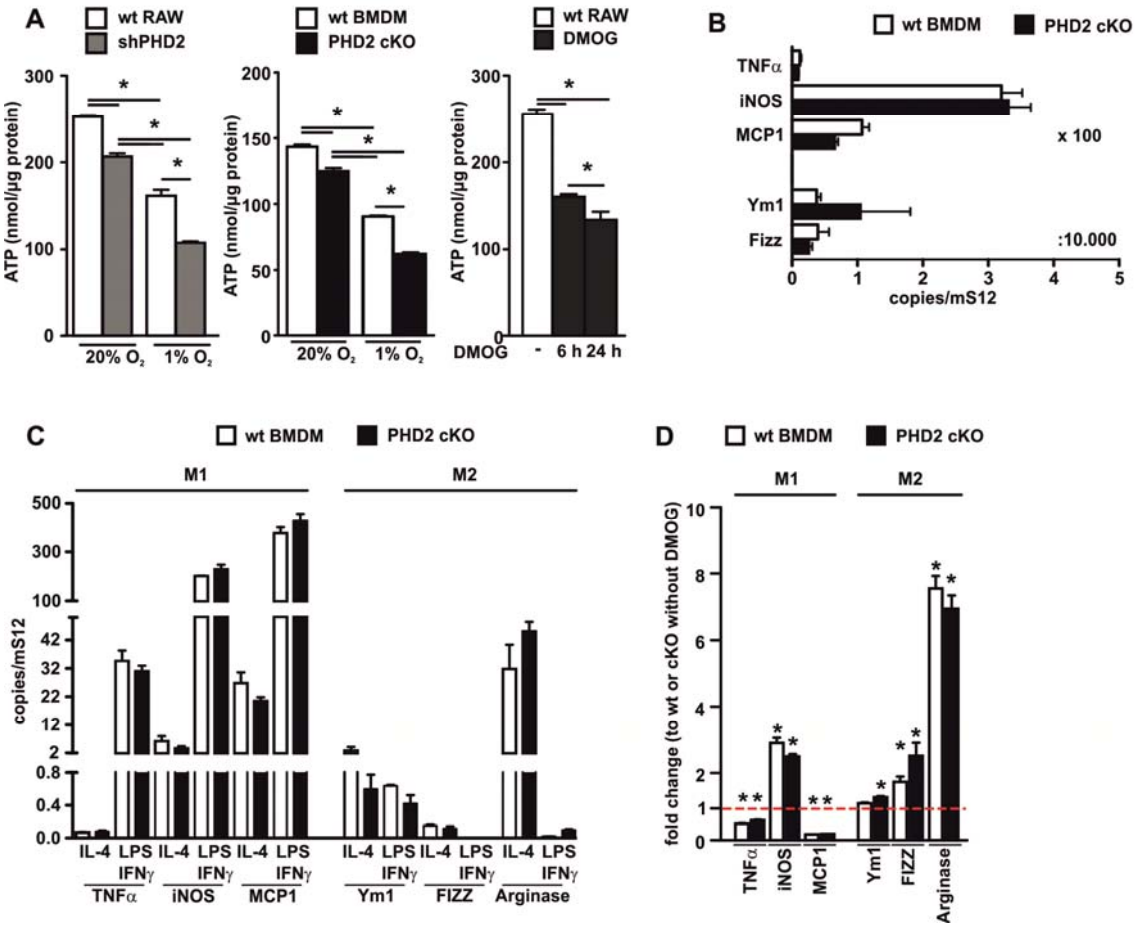
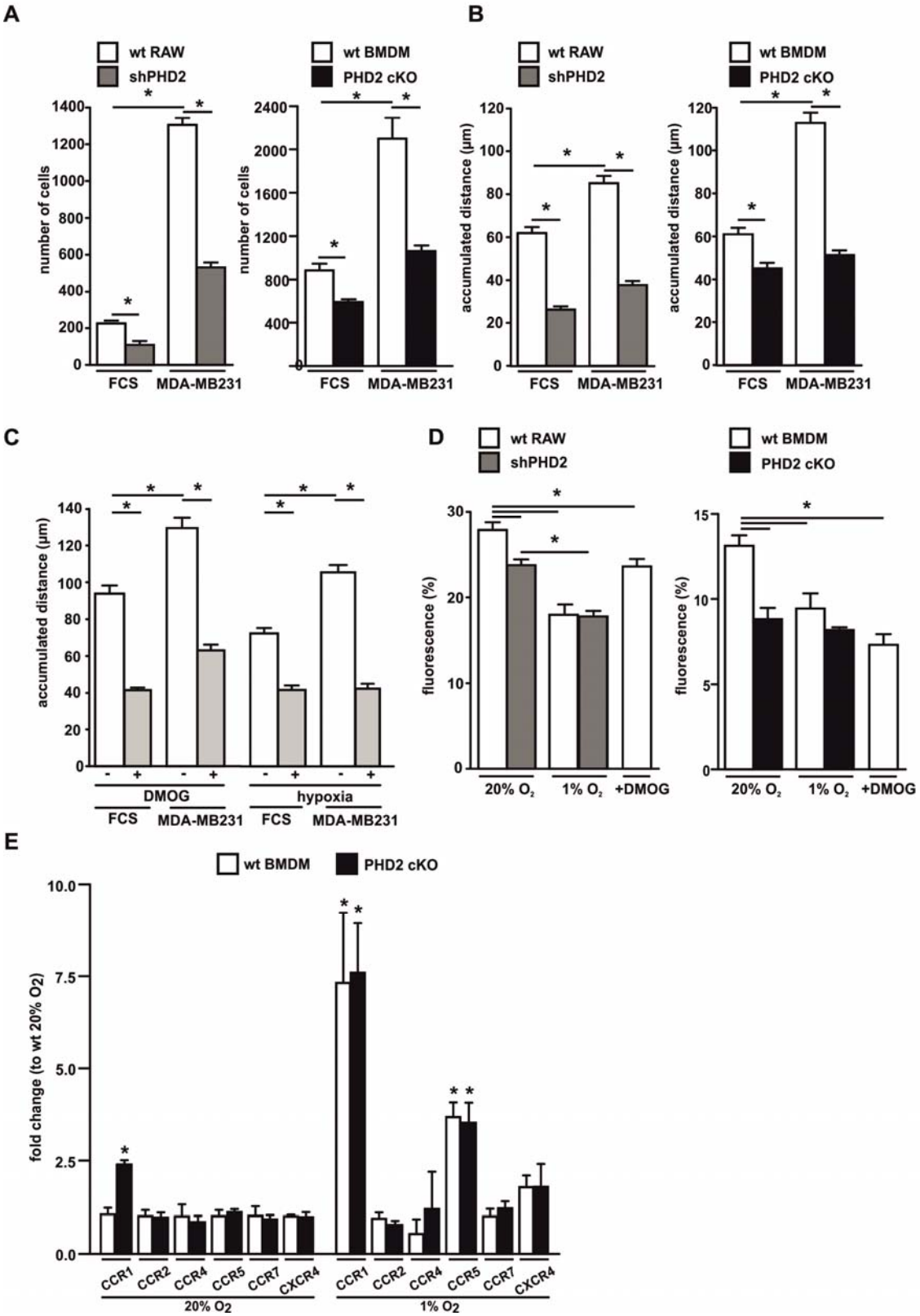
wt RAW
shPHD2
wt BMDM
PHD2 cKO
wt RAW + DMOG
XF
Oligomycin
FCCP
Rot+AA
OCR (pmol/min)
OCR (pmoles/min)
time (min)
B
wt RAW
shPHD2
wt BMDM
PHD2 cKO
DMOG
OCR (pmol/min)
basal resp.
max. resp.
C
wt RAW
shPHD2
Glucose
Oligomycin
2-DG
ECAR (mpH/min)
time (min)
D
wt BMDM
PHD2 cKO
wt RAW
DMOG
Glucose
Oligomycin
2-DG
ECAR (mpH/min)
time (min)
E
wt RAW
shPHD2
ECAR (mpH/min)
glyco-lysis
glycolytic capacity
F
wt BMDM
PHD2 cKO
wt RAW
wt RAW + DMOG
ECAR (mpH/min)
glyco-lysis
glycolytic capacity
G
wt RAW
shPHD2
wt RAW + DMOG
lactate (g/l)
20% O2
1% O2
H
wt BMDM
PHD2 cKO
lactate (g/l)
20% O2
1% O2
20% O2 - glucose

Figure 3


A
wt RAW
shPHD2
wt BMDM
PHD2 cKO
wt RAW
DMOG
ATP (nmol/µg protein)
20% O2
1% O2
DMOG
- 6 h 24 h
B
wt BMDM
PHD2 cKO
TNFα
iNOS
MCP1
Ym1
Fizz
x 100
:10.000
copies/mS12
C
wt BMDM
PHD2 cKO
M1
M2
copies/mS12
IL-4
LPS
IFNγ
TNFα
iNOS
MCP1
Ym1
FIZZ
Arginase
D
wt BMDM
PHD2 cKO
M1
M2
fold change (to wt or cKO without DMOG)
TNFα
iNOS
MCP1
Ym1
FIZZ
Arginase

712

Figure 4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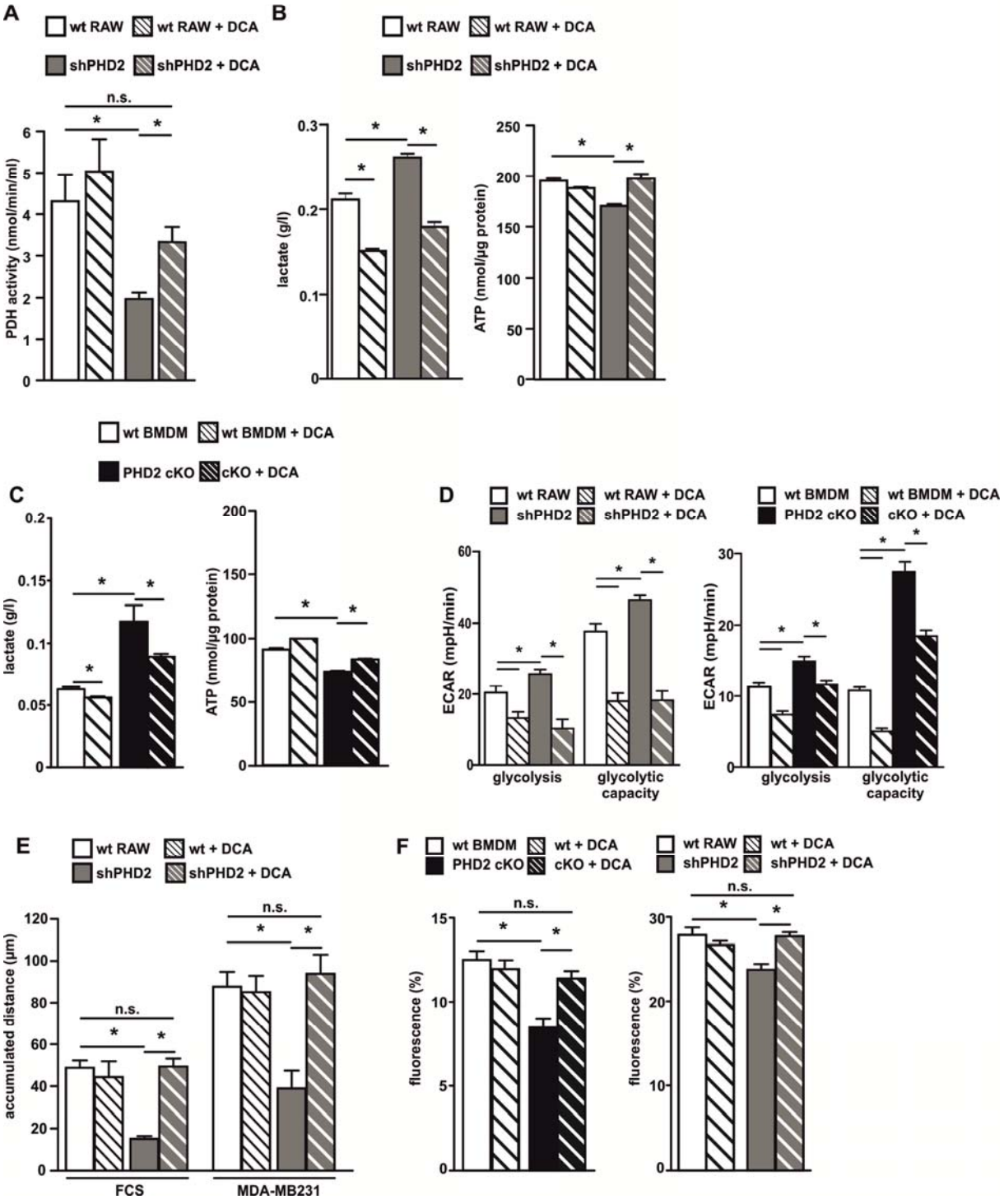
wt RAW
shPHD2
number of cells
FCS
MDA-MB231
wt BMDM
PHD2 cKO
B
accumulated distance (µm)
C
DMOG
hypoxia
D
fluorescence (%)
20% O2
1% O2
+DMOG
E
fold change (to wt 20% O2)
CCR1
CCR2
CCR4
CCR5
CCR7
CXCR4

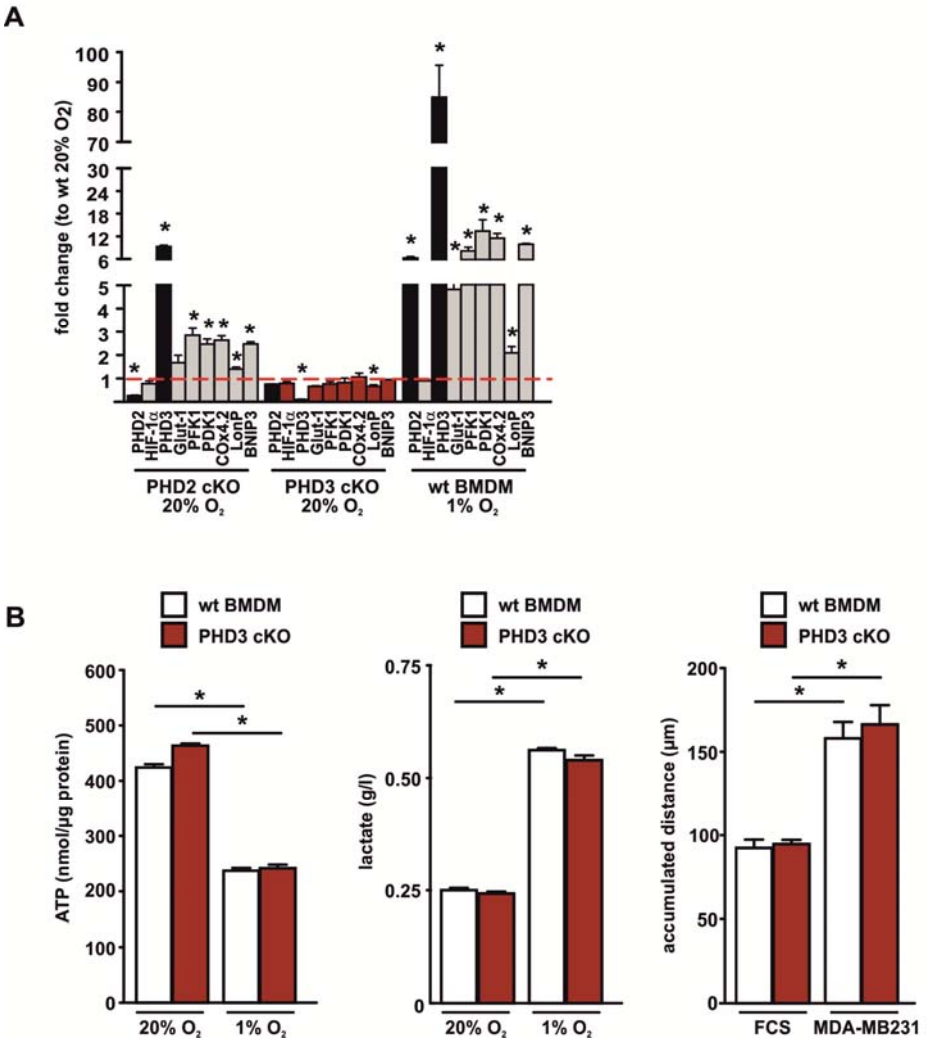
713

Figure 5


A
wt RAW
wt RAW + DCA
shPHD2
shPHD2 + DCA
n.s.
PDH activity (nmol/min/ml)
B
lactate (g/l)
ATP (nmol/µg protein)
wt BMDM
wt BMDM + DCA
PHD2 cKO
cKO + DCA
C
D
ECAR (mpH/min)
glycolysis
glycolytic capacity
E
accumulated distance (µm)
wt + DCA
FCS
MDA-MB231
F
fluorescence (%)

714

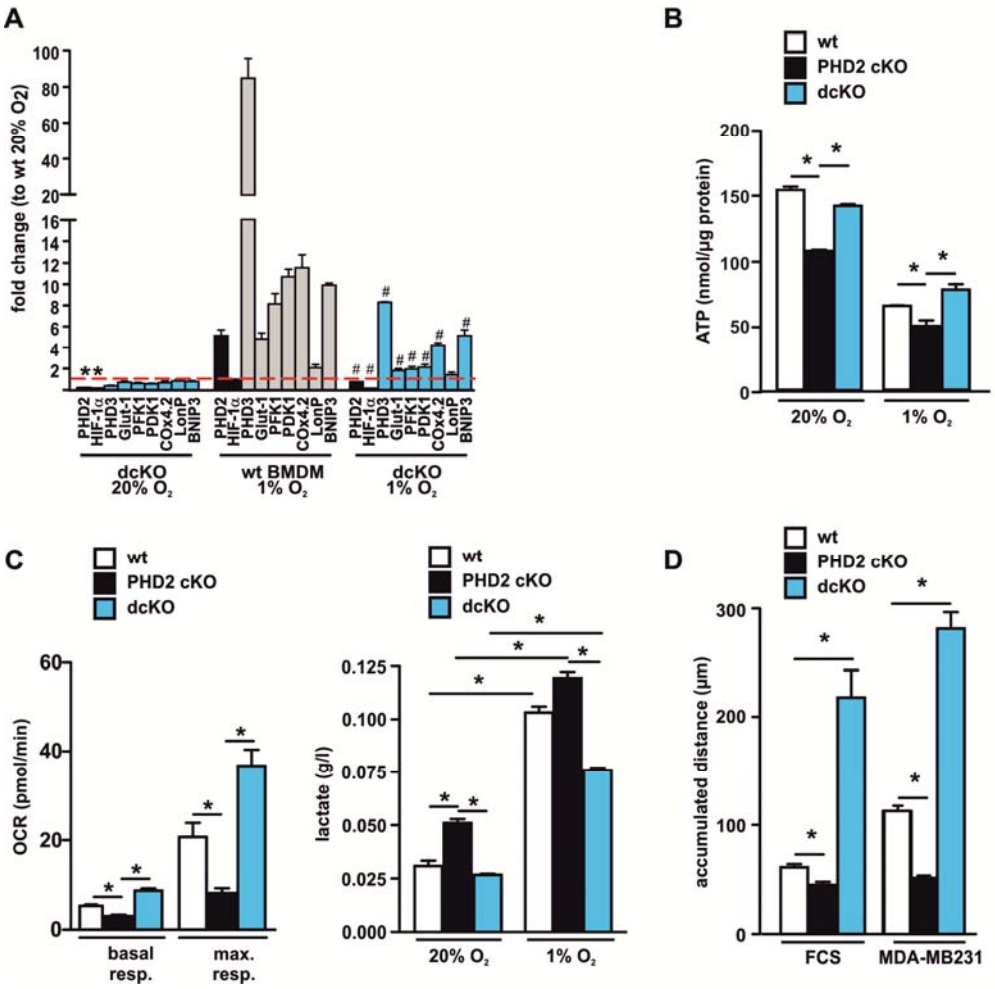
Figure 6


A
fold change (to wt 20% O2)
PHD2
HIF-1α
PHD3
Glut-1
PFK1
PDK1
COx4.2
LonP
BNIP3
PHD2 cKO 20% O2
PHD3 cKO 20% O2
wt BMDM 1% O2
B
wt BMDM
PHD3 cKO
ATP (nmol/µg protein)
20% O2
1% O2
lactate (g/l)
accumulated distance (µm)
FCS
MDA-MB231

715

716

Figure 7



717