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1 **PHD2 is a regulator for glycolytic reprogramming in macrophages**

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16 **Running title**

17 PHD2, metabolism and macrophages

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26 **Abstract**

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

42 **Introduction**

43 Macrophages are an essential component of innate immunity and well recognized to play a
44 critical role in inflammation, tumor progression and tissue repair for example after an
45 ischemic insult (1). In aerobic conditions the oxidative breakdown of pyruvate within the
46 mitochondria is the prevalent source of energy in most cells. Upon a decrease in oxygen
47 availability cells shift the metabolism towards anaerobic glycolysis. In line, macrophages can
48 use aerobic or anaerobic glycolysis for energy production depending on the context. There is
49 growing understanding that macrophage function can be altered by cellular metabolism (2).
50 One of the key factors at the transcriptional level in switching aerobic to anaerobic
51 metabolism is the hypoxia-inducible factor (HIF). HIF comprises two subunits, i.e. the
52 constitutively regulated HIF β subunit and one of three oxygen-regulated HIF α subunits (HIF-
53 1 α , HIF-2 α or HIF-3 α) (3). The protein stability of HIF α is regulated by the three prolyl-4-
54 hydroxylase domain (PHD) enzymes PHD1, 2 and 3, which hydroxylate HIF α in an oxygen-
55 dependent manner (for review (4,5)). The hydroxylated product is recognized by the pVHL
56 protein, which results in ubiquitination and proteasomal degradation of the α -subunit. In
57 hypoxia the hydroxylation and degradation is inhibited and thus HIF α is stabilized, which
58 finally results in HIF-dependent transcriptional activation of a repertoire of target genes.
59 Besides many others, glycolytic enzymes and the pyruvate dehydrogenase kinase 1 (PDK1)
60 belong to the target genes (6,7). Both determine the glycolytic cellular program. PHD
61 enzymes are of interest for the ongoing development of small molecule inhibitors, which
62 would allow stimulating HIF-dependent gene expression in normoxia (8).

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

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

70

71 **Materials and Methods**

72 **Chemicals**

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

78

79 **Myeloid-specific conditional knock out mice**

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

87

88 **Isolation and differentiation of BMDM**

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

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

102

103 **Generation of shPHD2 RAW cells**

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

108

109 **Preparation of MDA-MB231 conditioned medium**

110 5 flasks each with 1×10^6 MDA-MB231 cells were cultivated in 15 ml culture medium for 4
111 days. The medium was transferred to a reaction tube and centrifuged at 4000 g for 20 min at 4
112 °C. The supernatant was pooled and frozen at -80 °C.

113

114 **Hypoxic incubation**

115 For culturing cells in defined hypoxic conditions (1% O₂) an *in vivo* Hypoxia workstation was
116 used (Ruskinn Technologies).

117

118 Phagocytosis

119 Fluorescent beads (ProtonexTM Red 600-Latex Beads, AAT Bioquest, Sunnyvale, USA) were
120 administered to cells for 4 h. After washing, uptake of fluorescent beads was analyzed by
121 FACS (BD FACS Canto II, BD Biosciences).

122

123 Quantification of apoptotic cells

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

129

130 Single cell migration

131 2.5×10^4 RAW cells or 5×10^4 BMDMs were seeded in a 6-well plate. The next day the
132 medium was replaced with either normal medium or MDA-MB231 conditioned medium. The
133 migration of the cells was investigated using the T1-5M Nikon microscope inside the Sci-tive
134 work station (Ruskinn, Bridgend, South Wales) at normoxic (20% O₂) or hypoxic (1% O₂)
135 conditions. An image was taken every 10 min for in total 6 h. The migration was analyzed
136 using the chemotaxis plug-in installed in ImageJ.

137

138

139

140 **Boyden chamber assay**

141 0.7 x 10⁵ BMDM or RAW cells were seeded in 500 µL culture medium into inserts (BD
 142 Biosciences, Heidelberg, Germany) containing 3 µm pores. Inserts were placed into 24-well
 143 plates containing either 500 µl MDA-MB 231 conditioned medium or 500 µL cell culture
 144 medium as control. 18 h later inserts were placed into medium with 5 µM calcein. Cells that
 145 had not migrated were removed by scraping of the upper side of the insert after 1 h, while
 146 migrated macrophages on the lower side were analyzed by fluorescence microscopy (Axio
 147 Observer D1, Carl Zeiss, Göttingen, Germany).

148

149 **RNA isolation and qRT PCR**

150 Cells were washed once with PBS and harvested in Trizol (Invitrogen, Darmstadt, Germany).
 151 RNA was isolated according to the manufacturer's instructions and 1 µg was transcribed
 152 using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Transcript
 153 levels were analyzed by qRT PCR amplifying 1 µL of cDNA with Brilliant II SYBR Green
 154 qPCR Master Mix in an MX3005Pro light cycler (Agilent, Böblingen, Germany). Applied
 155 primer sequences were: ms12 for 5'-GAAGCTGCCAAGGCCTTAGA -3', rev 5'-
 156 AACTGCAACCAACCACCTTC-3'; phd2 for 5'-TTGCTGACATTGAACCCAAA-3',
 157 rev 5'-GGCAACTGAGAGGCTGTAGG-3'; phd3 for 5'-GGCCGCTGTATCACCTGTAT-
 158 3', rev 5'-TTCTGCCCTTTCTTCAGCAT -3'; glut1 for 5'-
 159 TGGCCTTGCTGGAACGGCTG-3', rev 5'-TCCTTGGGCTGCAGGGAGCA -3'; pfk1 for
 160 5'-ACGAGGCCATCCAGCTCCGT-3', rev 5'-
 161 TGGGGCTTGGGCAGTGTCCCT -3'; pdk1 for 5'-TTCACGTCACGCTGGGCGAG -3', rev
 162 5'-GGCTGGGCACACACCAGTCG -3'; cox4.2for 5'-
 163 CAGAGAAGGTGGCCTTGTACC -3', rev 5'-AGAAGAAGACGCAGCCCATC -3'; LonP
 164 for 5'-CATCGCCTTGAACCCTCTGT-3', rev 5'-

165 AGCCGCTTAAGGATGTTGGT-3'; BNIP3 for 5'-GTCCAGTGTCGCCTGGCCTC -3', 5'-
 166 TGGGAGCGAGGTGGGCTGTC -3', mCCR2 for 5'-CCACACCCTGTTTCGCTG-3',
 167 mCCR2 rev 5'-ACCTCTTCAGCACTTGC-3'; mCCR4 for
 168 5'-GCCTCTTGTTTCAGCACTTGC-3', mCCR4 rev 5'-ATAAGCAGCCCCAGGACG-3';
 169 mCCR5 for 5'-CCAGAGGAGGTGAGACATCCGTTC-3', mCCR5 rev 5'-
 170 GGCAGGAGCTGAGCCGCAATTT -3'; mCCR7 for 5'-
 171 ATGGACCCAGGGAAACCCAGGAA-3',
 172 mCCR7 rev 5'-GCACACCGACTCGTACAGGG-3'; mCXCR4 for 5'-
 173 GCTCCGGTAACCACCACGGC-3', mCXCR4 rev 5'-GCGAGGTACCGGTCCAGGCT-3'.
 174

175 **Microarray-Based Gene Expression**

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

177

178 **Western blots**

179 Cells were lysed with 50 mM Tris, 150 mM NaCl, 0.5 mM PMSF, 100 mM MgCl₂, 1% NP-
 180 40 supplemented with protease inhibitors (Roche). Protein samples were resolved by SDS-
 181 PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). Primary
 182 antibodies used were: anti-HIF-1 α (NB-100-479, Novus), anti-HIF-2 α (AF2997, R&D
 183 Systems), anti-PHD2 (NB-100-2219, Novus), anti-phospho pyruvate dehydrogenase
 184 (ABS204, Merck), anti-pyruvate dehydrogenase (3205, Cell Signaling), anti-PDK1 (ADI-
 185 KAP-PK112-D, Stressgene), and anti- β -actin (A5441, Sigma). For detection of
 186 immunocomplexes horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-
 187 mouse antibodies (Santa Cruz Biotechnology) were used and membranes were incubated with
 188 chemiluminescent HRP substrate (Millipore).

189 ATP and lactate measurements

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

195

196 PDH activity assay

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

198

199 Oxygen consumption rate and extracellular acidification rate

200 Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were analyzed
201 in the Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA, USA).
202 For OCR 2.5×10^4 RAW or 4.0×10^4 BMDM cells per well were seeded. The medium was
203 replaced with XF assay medium supplemented with 4.5 g/L glucose and 1 mM sodium
204 pyruvate and incubated without CO₂ for 30 min. After measuring basal respiration, the
205 oxygen consumption was analyzed after sequential addition of 1.5 μM oligomycin, 1 μM
206 FCCP, 2 μM rotenone and 1 μM antimycin A.

207 For determining the ECAR, 2.5×10^4 (RAW) or 8×10^4 (BMDM) cells per well were seeded.
208 Cells were washed with XF Glycostress-medium (DMEM D 5030, 134 mM NaCl, 3 mg
209 Phenol Red, 2 mM L-glutamine, pH 7.35). The cells were incubated for 15 min at 37°C
210 without CO₂. ECAR was analyzed after sequential addition of 10 mM glucose, 1.5-3 μM
211 oligomycin, and 100 mM 2-deoxy-glucose (2-DG).

212

213 **Statistical analyses**

214 Statistical analyses were performed using Student's two-tailed t-test. Data are shown as
215 means \pm SEM. Values of $p < 0.05$ were considered statistically significant.

216

217

218 **Results**

219 **PHD2-deficient macrophages induce a hypoxic gene expression pattern in normoxia** 220 **including PDK1, a central regulator of the pyruvate dehydrogenase**

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

239 PHD2 protein levels were decreased in PHD2 cKO and shPHD2 cells likewise (Fig.
240 1C). Whereas HIF-1 α and HIF-2 α were detectable in BMDM isolated from wt mice in
241 hypoxia only, PHD2 cKO BMDM revealed high HIF-1 α and HIF-2 α protein levels in
242 normoxia. In the RAW cells HIF-2 α was not detectable with the antibodies applied. For HIF-

243 1 α a similar pattern as in the BMDM was observed. Comparable to the HIF target RNA
244 expression HIF-1 α and HIF-2 α protein levels were further increased after exposing the PHD2
245 cKO cells and shPHD2 RAW cells to hypoxia. Taken together this demonstrates a
246 biologically relevant reduction of PHD2 with subsequent stabilization of the HIF- α proteins
247 and induction of HIF target genes like PDK1 in the cell line model and the genetically
248 modified primary macrophages partially mimicking hypoxia. The increased expression of
249 PDK1 was further analyzed at protein level. PDK1 is a major regulator in central metabolic
250 pathways including glucose consumption. It acts in part by regulating the activity of the
251 pyruvate dehydrogenase (PDH) by phosphorylation, which results in inactivation of the
252 enzyme. PDH is one part of a mitochondrial multi-enzyme complex that catalyzes the
253 oxidative decarboxylation of pyruvate and is one of the major enzymes responsible for the
254 regulation of homeostasis of carbohydrate fuels in mammals. The induction of PDK1 in the
255 PHD2-deficient cells in normoxia was also detectable at the protein level (Fig. 1D). In line,
256 PDH was found to be more phosphorylated in the shPHD2 RAW cell and the PHD2 cKO
257 BMDM compared to their wild type cells. In hypoxia both, wt and the PHD2-deficient cells,
258 showed increased PDK1 protein level and phosphorylation of PDH. In parallel we determined
259 the PDH activity in cell extracts isolated from wt cells and PHD2-deficient cells after
260 exposure to normoxia or hypoxia (Fig. 1E). shPHD2 cells exhibited a significantly lower
261 PDH activity in normoxia, which was also observed in wt cells after treatment with the PHD
262 inhibitor DMOG. In hypoxia, a decreased PDH activity was observed in wt and shPHD2
263 cells, still with significantly lower levels in the knock down cells. Since PDK1 is a critical
264 regulator of cellular metabolism, we next analyzed the glycolytic capacity in the PHD2-
265 deficient cells.

266

267

268

269 PHD2-deficient macrophages show a switch to glycolytic metabolism

270 Glycolysis utilization for energy demand can be characterized by the oxygen consumption
271 and the extracellular acidification rate after stimulation. Basal oxygen consumption rate and
272 maximal respiration after uncoupling the mitochondria with FCCP were significantly
273 decreased in the PHD2-deficient cells or after treating wt cells with DMOG, which indicates
274 that as a consequence of inhibiting PHD activity macrophages shift their source of energy to
275 anaerobic glycolysis (Fig. 2A and B). This was further supported by a significantly increased
276 glycolytic function as determined by the extracellular acidification rate after glucose or
277 oligomycin treatment, which reflects basal glycolysis and the overall glycolytic capacity,
278 respectively (Fig. 2C-F). In line we found increased lactate levels in the supernatant of
279 shPHD2 RAW cells and PHD2 cKO BMDM compared to their respective wt cells (Fig. 2G).
280 Lactate levels were likewise increased after DMOG treatment or exposure of the cells to
281 hypoxia. Incubating wt and PHD2 cKO BMDM without glucose at 20% O₂ abolished the
282 lactate production demonstrating that the glucose in the culture medium is indeed the major
283 source for lactate production (Fig. 2H).

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

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

315

316 **Impaired migratory and phagocytic capacity of PHD2 cKO BMDM and shPHD2 RAW** 317 **cells**

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

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

332 Differential migration was not due to a modulated chemokine receptor expression since
333 quantifying CCR2, CCR4, CCR5, CCR7 and CXCR4 RNA levels by RT-PCR after exposing
334 wild type and PHD2 cKO BMDM to 20% O₂ or 1% O₂ did not reveal any difference between
335 the cell types besides an upregulation of CCR1 in the PHD2 cKO cells (Fig. 4E). We however
336 observed a significant upregulation of CCR1 and CCR5 and a slight upregulation of CXCR4
337 in hypoxia, which is in line with the literature (17,18). Since the migration deficit was not due
338 to a striking differential expression of chemokine receptors we further analyzed the impact of
339 the metabolic alterations for the migratory and phagocytosis capacity.

340

341 **Regulation of PHD activity by PDK is critical for macrophage function**

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

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

359 PDK1 has been described to be a HIF target gene (7). In a previous report, we demonstrated
360 that in PHD3 cKO BMDM HIF-1 α is not stabilized in normoxia (12), which is in sharp
361 contrast to the effect seen in the PHD2 cKO BMDM described here. This was further
362 supported by the fact, that HIF target genes including PDK1 were not significantly increased
363 in PHD3 cKO BMDM other than PHD2 cKO or wt BMDMs in normoxia and hypoxia (Fig.
364 6A). The genes identified in the gene array analysis (PHD2 cKO versus wt BMDM) were
365 additionally compared to PHD3 regulated genes described earlier (12). Most interestingly just
366 13 genes were found to be regulated in the PHD2 cKO as well as in the PHD3 cKO BMDM,
367 indicating PHD isoform specific effects (Suppl. Table 3). In line ATP and lactate levels as
368 well as accumulated migration difference were not altered in PHD3 cKO versus wt BMDM
369 (Fig. 6B). To further analyze, if PHD2 mediates its effects via HIF-1 α we generated dcKO
370 mice. dcKO BMDM had a blunted response in RNA levels of the HIF target genes PHD3,
371 Glut-1, PDK1, PDK1, Cox4.2 and LonP in hypoxia (Fig. 7A). The metabolic phenotype of
372 the PHD2 cKO BMDM, i.e. decreased ATP levels, increased lactate levels, decreased OCR

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

376

377 **Discussion**

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

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

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

405 In stark contrast to the unaltered polarization, we found an influence of PHD2 on
406 reprogramming of mitochondrial metabolism. This effect was mediated via PHD2 dependent
407 regulation of PDK1 expression. This is in accordance with a recent report showing in
408 livers/hepatocytes that PHD2 can regulate PDK1 (27). In line with higher levels of PDK1 and
409 higher phosphorylation of its target PDH, the basal and stimulated oxygen consumption was
410 significantly lower in the PHD2-deficient cells accompanied by an increased glycolytic
411 capacity. PDK1 is a key regulatory enzyme in glucose metabolism. The PDH complex, which
412 is regulated by PDK1, converts pyruvate produced from the glycolytic flux to acetyl-CoA.
413 Pyruvate-derived acetyl-CoA then enters the TCA cycle that generates NADH that fuels the
414 electron transport chain for oxidative phosphorylation. In hypoxia PDH activity is inhibited
415 via PDK1 mediated phosphorylation, which induces the anaerobic glucose metabolic
416 homeostasis under limited oxygen availability. This mechanism is widely used by tumor cells
417 and is part of the so-called *Warburg effect*. Warburg described that unlike most normal
418 tissues, cancer cells tend to “ferment” glucose into lactate even in the presence of sufficient
419 oxygen to support mitochondrial oxidative phosphorylation (28). In case of immune cells the
420 metabolic adaptation however is part of their physiological response. Innate immune cells like
421 neutrophils likewise depend on anaerobic glycolysis for ATP production, which is also
422 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.

475

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479 research, analyzed data and wrote the manuscript; J.D. performed research and analyzed data;
480 P.R., C.X.C.S. and A.M.S. evaluated the data and corrected the paper; D.M.K. designed
481 research and wrote the manuscript; and all authors read and edited the manuscript.

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487

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489 **References**

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

- 522 (PHD3) is a critical terminator for cell survival of macrophages under stress
523 conditions. *J Leukoc Biol.* **96**: 365-375.
- 524 13. **Burgess, AW, Metcalf, D, Kozka, IJ, Simpson, RJ, Vairo, G, Hamilton, JA, Nice,**
525 **EC.** 1985. Purification of two forms of colony-stimulating factor from mouse L-cell-
526 conditioned medium. *J Biol Chem.* **260**: 16004-16011.
- 527 14. **Klotzsche-von Ameln, A, Muschter, A, Mamlouk, S, Kalucka, J, Prade, I,**
528 **Franke, K, Rezaei, M, Poitz, DM, Breier, G, Wielockx, B.** 2011. Inhibition of HIF
529 prolyl hydroxylase-2 blocks tumor growth in mice through the antiproliferative
530 activity of TGFbeta. *Cancer Res.* **71**: 3306-3316.
- 531 15. **Hölscher, M, Silter, M, Krull, S, von Ahlen, M, Hesse, A, Schwartz, P, Wielockx,**
532 **B, Breier, G, Katschinski, DM, Zieseniss, A.** Cardiomyocyte-specific Prolyl-4-
533 hydroxylase Domain 2 Knock Out Protects from Acute Myocardial Ischemic Injury. *J*
534 *Biol Chem.* **286**: 11185-11194.
- 535 16. **Mosser, DM, Edwards, JP.** 2008. Exploring the full spectrum of macrophage
536 activation. *Nat Rev Immunol.* **8**: 958-969.
- 537 17. **Bosco, MC, Puppo, M, Santangelo, C, Anfosso, L, Pfeffer, U, Fardin, P, Battaglia,**
538 **F, Varesio, L.** 2006. Hypoxia modifies the transcriptome of primary human
539 monocytes: modulation of novel immune-related genes and identification of CC-
540 chemokine ligand 20 as a new hypoxia-inducible gene. *J Immunol.* **177**: 1941-1955.
- 541 18. **Dong, F, Khalil, M, Kiedrowski, M, O'Connor, C, Petrovic, E, Zhou, X, Penn,**
542 **MS.** 2010. Critical role for leukocyte hypoxia inducible factor-1alpha expression in
543 post-myocardial infarction left ventricular remodeling. *Circ Res.* **106**: 601-610.
- 544 19. **Kato, M, Li, J, Chuang, JL, Chuang, DT.** 2007. Distinct structural mechanisms for
545 inhibition of pyruvate dehydrogenase kinase isoforms by AZD7545, dichloroacetate,
546 and radicicol. *Structure.* **15**: 992-1004.
- 547 20. **Shi, C, Pamer, EG.** 2011. Monocyte recruitment during infection and inflammation.
548 *Nat Rev Immunol.* **11**: 762-774.
- 549 21. **Ikeda, J, Ichiki, T, Matsuura, H, Inoue, E, Kishimoto, J, Watanabe, A, Sankoda,**
550 **C, Kitamoto, S, Tokunou, T, Takeda, K, Fong, GH, Sunagawa, K.** 2013. Deletion
551 of phd2 in myeloid lineage attenuates hypertensive cardiovascular remodeling. *J Am*
552 *Heart Assoc.* **2**: e000178.
- 553 22. **Mamlouk, S, Kalucka, J, Singh, RP, Franke, K, Muschter, A, Langer, A, Jakob,**
554 **C, Gassmann, M, Baretton, GB, Wielockx, B.** 2014. Loss of prolyl hydroxylase-2 in

- 555 myeloid cells and T-lymphocytes impairs tumor development. *Int J Cancer*. **134**: 849-
556 858.
- 557 23. **Mantovani, A, Sica, A, Sozzani, S, Allavena, P, Vecchi, A, Locati, M.** 2004. The
558 chemokine system in diverse forms of macrophage activation and polarization. *Trends*
559 *Immunol*. **25**: 677-686.
- 560 24. **Lawrence, T, Natoli, G.** 2011. Transcriptional regulation of macrophage polarization:
561 enabling diversity with identity. *Nat Rev Immunol*. **11**: 750-761.
- 562 25. **Takeda, N, O'Dea, EL, Doedens, A, Kim, JW, Weidemann, A, Stockmann, C,**
563 **Asagiri, M, Simon, MC, Hoffmann, A, Johnson, RS.** 2010. Differential activation
564 and antagonistic function of HIF- α isoforms in macrophages are essential for
565 NO homeostasis. *Genes Dev*. **24**: 491-501.
- 566 26. **Takeda, Y, Costa, S, Delamarre, E, Roncal, C, Leite de Oliveira, R, Squadrito,**
567 **ML, Finisguerra, V, Deschoemaeker, S, Bruyere, F, Wenes, M, Hamm, A,**
568 **Serneels, J, Magat, J, Bhattacharyya, T, Anisimov, A, Jordan, BF, Alitalo, K,**
569 **Maxwell, P, Gallez, B, Zhuang, ZW, Saito, Y, Simons, M, De Palma, M, Mazzone,**
570 **M.** 2011. Macrophage skewing by Phd2 haploinsufficiency prevents ischaemia by
571 inducing arteriogenesis. *Nature*. **479**: 122-126.
- 572 27. **Suhara, T, Hishiki, T, Kasahara, M, Hayakawa, N, Oyaizu, T, Nakanishi, T,**
573 **Kubo, A, Morisaki, H, Kaelin, WG, Jr., Suematsu, M, Minamishima, YA.** 2015.
574 Inhibition of the oxygen sensor PHD2 in the liver improves survival in lactic acidosis
575 by activating the Cori cycle. *Proc Natl Acad Sci U.S.A.* **112**: 11642-11647.
- 576 28. **Warburg, O.** 1956. On respiratory impairment in cancer cells. *Science*. **124**: 269-270.
- 577 29. **Kelly, B, O'Neill, LA.** 2015. Metabolic reprogramming in macrophages and dendritic
578 cells in innate immunity. *Cell Res*. **25**: 771-784.
- 579 30. **Pavlova, NN, Thompson, CB.** 2016. The Emerging Hallmarks of Cancer
580 Metabolism. *Cell Metab*. **23**: 27-47.
- 581 31. **Mills, EL, O'Neill, LA.** 2016. Reprogramming mitochondrial metabolism in
582 macrophages as an anti-inflammatory signal. *Eur J Immunol*. **46**: 13-21.
- 583 32. **Myllyharju, J.** 2009. HIF prolyl 4-hydroxylases and their potential as drug targets.
584 *Curr Pharm Des*. **15**: 3878-3885.
- 585 33. **Tan, Z, Xie, N, Cui, H, Moellering, DR, Abraham, E, Thannickal, VJ, Liu, G.**
586 2015. Pyruvate dehydrogenase kinase 1 participates in macrophage polarization via
587 regulating glucose metabolism. *J Immunol*. **194**: 6082-6089.
- 588

- 589 34. **Haas, R, Smith, J, Rocher-Ros, V, Nadkarni, S, Montero-Melendez, T,**
590 **D'Acquisto, F, Bland, EJ, Bombardieri, M, Pitzalis, C, Perretti, M, Marelli-Berg,**
591 **FM, Mauro, C.** 2015. Lactate Regulates Metabolic and Pro-inflammatory Circuits in
592 Control of T Cell Migration and Effector Functions. *PLoS Biol.* **13:** e1002202.
- 593 35. **Gottfried, E, Kunz-Schughart, LA, Ebner, S, Mueller-Klieser, W, Hoves, S,**
594 **Andreesen, R, Mackensen, A, Kreutz, M.** 2006. Tumor-derived lactic acid
595 modulates dendritic cell activation and antigen expression. *Blood.* **107:** 2013-2021.
- 596 36. **Barile, E, De, SK, Pellecchia, M.** 2012. PDK1 inhibitors. *Pharm Pat Anal.* **1:** 145-
597 163.
- 598 37. **Chan, MC, Holt-Martyn, JP, Schofield, CJ, Ratcliffe, PJ.** 2016. Pharmacological
599 targeting of the HIF hydroxylases - A new field in medicine development. *Mol*
600 *Aspects Med.* **47-48:** 54-75.

601

602

603

604 **Figure legends**

605 **Fig. 1:** *PHD2 knock down RAW cells and PHD2 knock out (PHD2 cKO) BMDMs display*
606 *increased PDK1 expression and activity. (A)* wt RAW and shPHD2 knock down cells as well
607 as wt BMDM and PHD2 cKO macrophages were incubated for 24 hrs at 20% or 1% O₂. RNA
608 levels of the indicated genes were analyzed by qRT-PCR. RNA levels of wt RAW and wt
609 BMDM cells were set to one. Fold change of the RNA levels of shPHD2 or the PHD2 cKO
610 BMDM or wt cells in hypoxia of the indicated genes compared to the wt cells in normoxia
611 was analyzed. n = 3-6 independent samples per condition. **(B)** Annexin V (AV) single-
612 positive cells were analyzed in wt BMDM and PHD2 cKO macrophages with and without
613 treatment with 1 mM DMOG for 24 hrs. **(C)** HIF-1 α , HIF-2 α , PHD2 and β -actin protein
614 levels of wt RAW and shPHD2 as well as wt BMDM and PHD2 cKO macrophages in
615 normoxia (20% O₂) or hypoxia (1% O₂ for 24 hrs). **(D)** phospho-PDH, total PDH, PDK and
616 β -actin protein levels of wt RAW and shPHD2 cells as well as wt BMDM and PHD2 cKO
617 macrophages in normoxia (20% O₂) or hypoxia (1% O₂ for 24 hrs). **(E)** PDH activity in wt
618 RAW, shPHD2 RAW cells and wt RAW cells treated with 1 mM DMOG for 24 hrs in
619 normoxia or hypoxia (1% O₂ for 24 hrs). n = 6 independent samples per condition. mean \pm
620 SEM, * p<0.05.

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

629 FCCP to obtain basal respiration and maximum respiration values, respectively. (C)
630 Extracellular acidification rate (ECAR) was determined in wt RAW and shPHD2 RAW as
631 well as (D) wt BMDM and PHD2 cKO cells or wt RAW cells treated with 1 mM DMOG for
632 24 hrs after addition of glucose, oligomycin and 2-deoxy glucose (2-DG). n = 7 independent
633 samples per condition. (E, F) Glycolysis and anaerobic glycolytic capacity were analyzed
634 based on the experiments shown in C and D. The ECAR after addition of 2-DG was
635 subtracted from the ECAR after addition of glucose or oligomycin to obtain glycolysis and
636 glycolytic capacity values, respectively. (G) Lactate levels were determined in the supernatant
637 of wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells after incubation of the cells at
638 the indicated conditions. n = 4 independent samples per condition. mean \pm SEM, * p<0.05.
639 (H) Lactate levels were determined in the supernatant of wt BMDM and cKO cells after
640 incubation of the cells at 20% O₂, 1% O₂ with or without addition of glucose in the cell
641 culture medium. Cells were incubated for 24 hrs in the respective cell culture medium, n = 4
642 independent samples per condition.

643 **Fig. 3:** *Decreased ATP levels and unaltered polarization in PHD2 deficient macrophages.*

644 (A) wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells were incubated at 20% O₂ or
645 1% O₂ for 24 hrs. Subsequently intracellular ATP levels were determined. n = 6 independent
646 samples per condition. Right panel: wt RAW cells were incubated for the indicated times with
647 1 mM DMOG and ATP levels were determined. n = 6-7 independent samples per condition.
648 (B) RNA levels of M1- and M2-markers in resting wt BMDM and cKO cells or (C) after
649 stimulation with IL-4 (20 nM) or LPS (100 ng/ml) and IFN γ (20 nM) for 24 hrs. (D) RNA
650 levels of M1 and M2-markers in resting wt BMDM and PHD2 cKO cells were analyzed after
651 treatment of the cells with 1mM DMOG for 24 hrs. Fold change of the RNA levels of DMOG
652 treated wt or cKO BMDM of the indicated genes compared to the non-treated cells was
653 analyzed. n = 3-6 independent samples per condition. mean \pm SEM, * p<0.05.

654 **Fig. 4:** *A reduction of PHD2 expression in RAW cells or BMDMs results in a defect in*
655 *macrophage migration and phagocytosis. (A)* wt RAW, shPHD2 RAW, wt BMDM and
656 PHD2 cKO cells were tested for their migration capacity in Boyden chambers using FCS or
657 conditioned medium of MDA-MB231 cells as stimulants. n = 4 (RAW cells), n = 4 (BMDM)
658 independent samples. **(B)** The accumulated migration distance over 6 hrs of wt RAW,
659 shPHD2 RAW, wt BMDM and PHD2 cKO cells was tested in single cell migration
660 experiments using FCS or conditioned medium of MDA-MB231 cells as stimulants. n = 59-
661 64 cells per condition (RAW cells), n = 51-71 cells per conditions (BMDM cells). **(C)** wt
662 RAW cells were incubated at 20% O₂ ± 1 mM DMOG or 1% O₂ for 6 hrs. The accumulated
663 migration distance was tested in single cell migration experiments using FCS or conditioned
664 medium of MDA-MB231 cells as stimulants. For cells analyzed at 1% O₂ the hypoxic
665 conditions were kept during the single cell migration experiments without reoxygenation. n =
666 50-53 cells per condition. **(D)** wt RAW, shPHD2 RAW, wt BMDM and PHD2 cKO cells
667 were incubated at 20% O₂ ± 1 mM DMOG or 1% O₂ in total for 20 hrs. Subsequently, the
668 capacity of the cells to phagocytose labeled beads was analyzed. Fluorescence beads were
669 added to the cells for 4 hrs without reoxygenation. n = 5 independent samples per condition.
670 mean ± SEM, * p<0.05. **(E)** RNA levels of chemokine receptors in resting wt BMDM and
671 PHD2 cKO cells after incubation in normoxia or hypoxia (1% O₂) for 24 hrs. Fold change of
672 the RNA levels in the PHD2 cKO BMDM of the indicated genes compared to the wt cells in
673 normoxia was analyzed. n = 3 independent samples per condition. mean ± SEM. *p<0.05.

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

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

689 **Fig. 6:** *No metabolic phenotype in PHD3-deficient macrophages.* (A) wt BMDM and PHD3
690 cKO macrophages were incubated for 24 hrs at 20% or 1% O₂. RNA levels of the indicated
691 genes were analyzed by qRT-PCR. RNA levels of wt BMDM cells were set to one. Fold
692 change of the RNA levels of PHD3 cKO BMDM or wt cells in hypoxia of the indicated genes
693 compared to the wt cells in normoxia was analyzed. n = 3 independent samples per condition.
694 (B) ATP (n = 5 independent samples) and lactate levels (n = 4 independent samples) as well
695 as accumulated migration distance were determined in wt BMDM and PHD3 cKO
696 macrophages.

697 **Fig. 7:** *HIF-1 α mediates the metabolic alterations in PHD2-deficient macrophages.* (A) wt
698 BMDM and dcKO macrophages were incubated for 24 hrs at 20% or 1% O₂. RNA levels of
699 the indicated genes were analyzed by qRT-PCR. RNA levels of wt BMDM cells were set to
700 one. Fold change of the RNA levels of dcKO BMDM or wt cells in hypoxia of the indicated
701 genes compared to the wt cells in normoxia was analyzed. n = 3 independent samples per
702 condition. mean \pm SEM, * p<0.05 compared to wt 20% O₂, # p<0.05 compared to wt 1% O₂.
703 (B) Intracellular ATP levels were determined in wt BMDM, PHD2 cKO and dcKO cells after
704 incubation for 24 hrs in 20% O₂ or 1% O₂. (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 \pm SEM, * p<0.05.

709

Figure 1

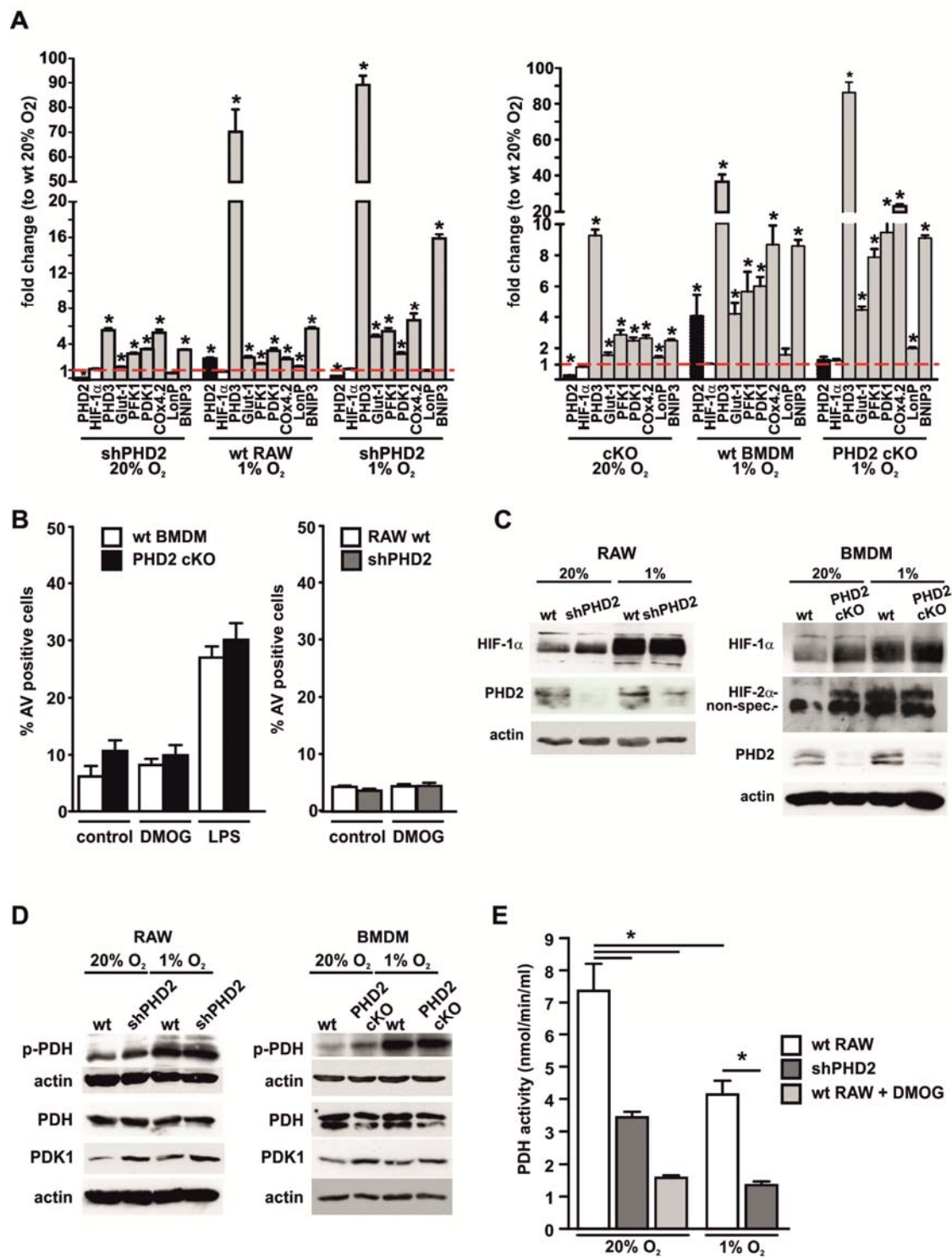


Figure 2

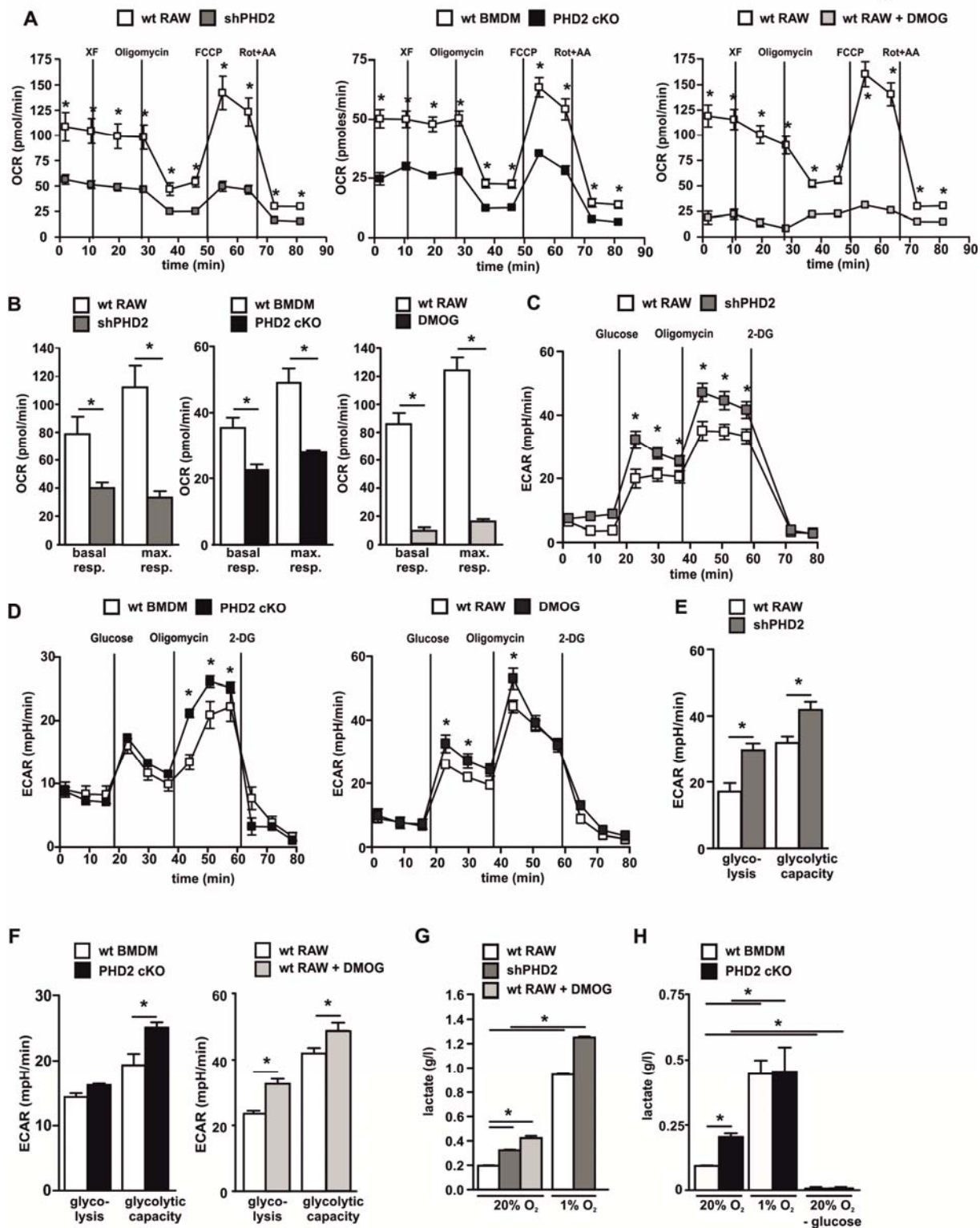


Figure 3

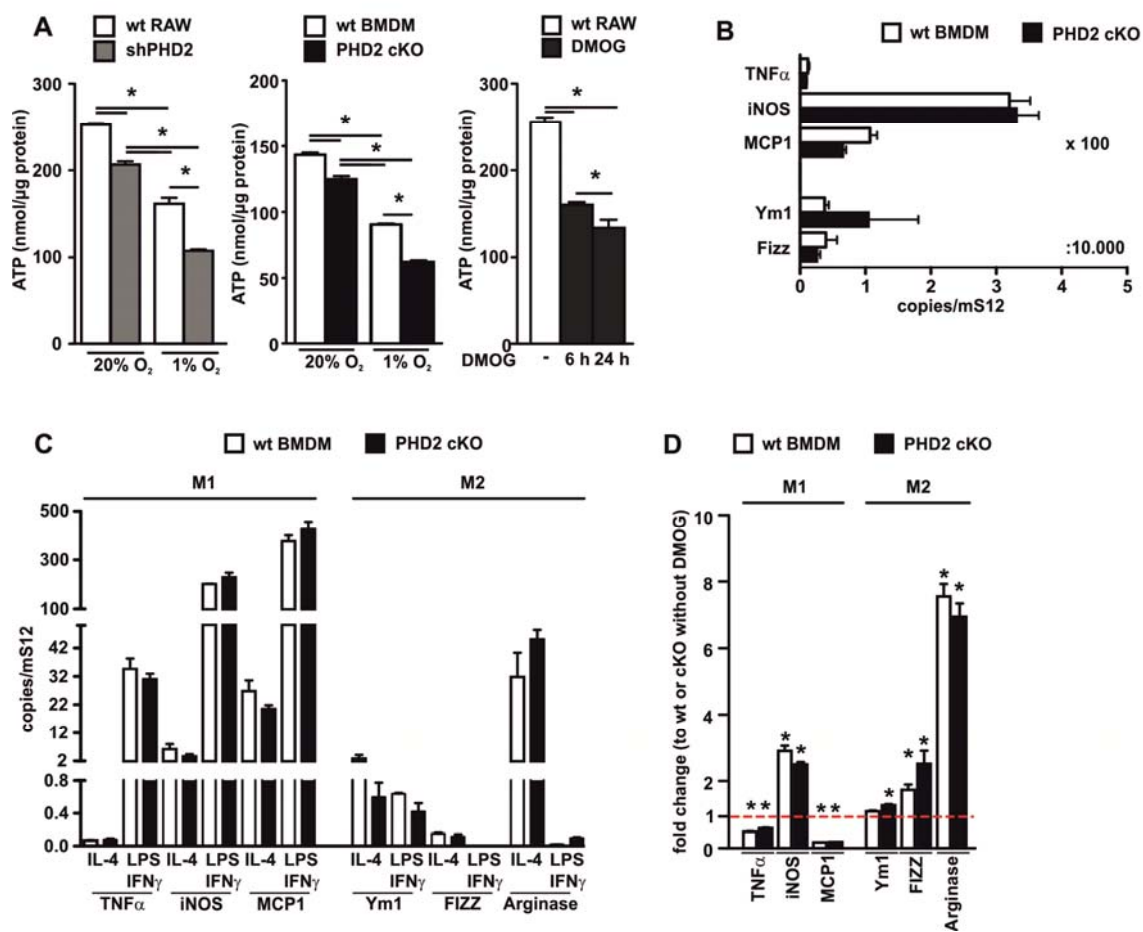


Figure 4

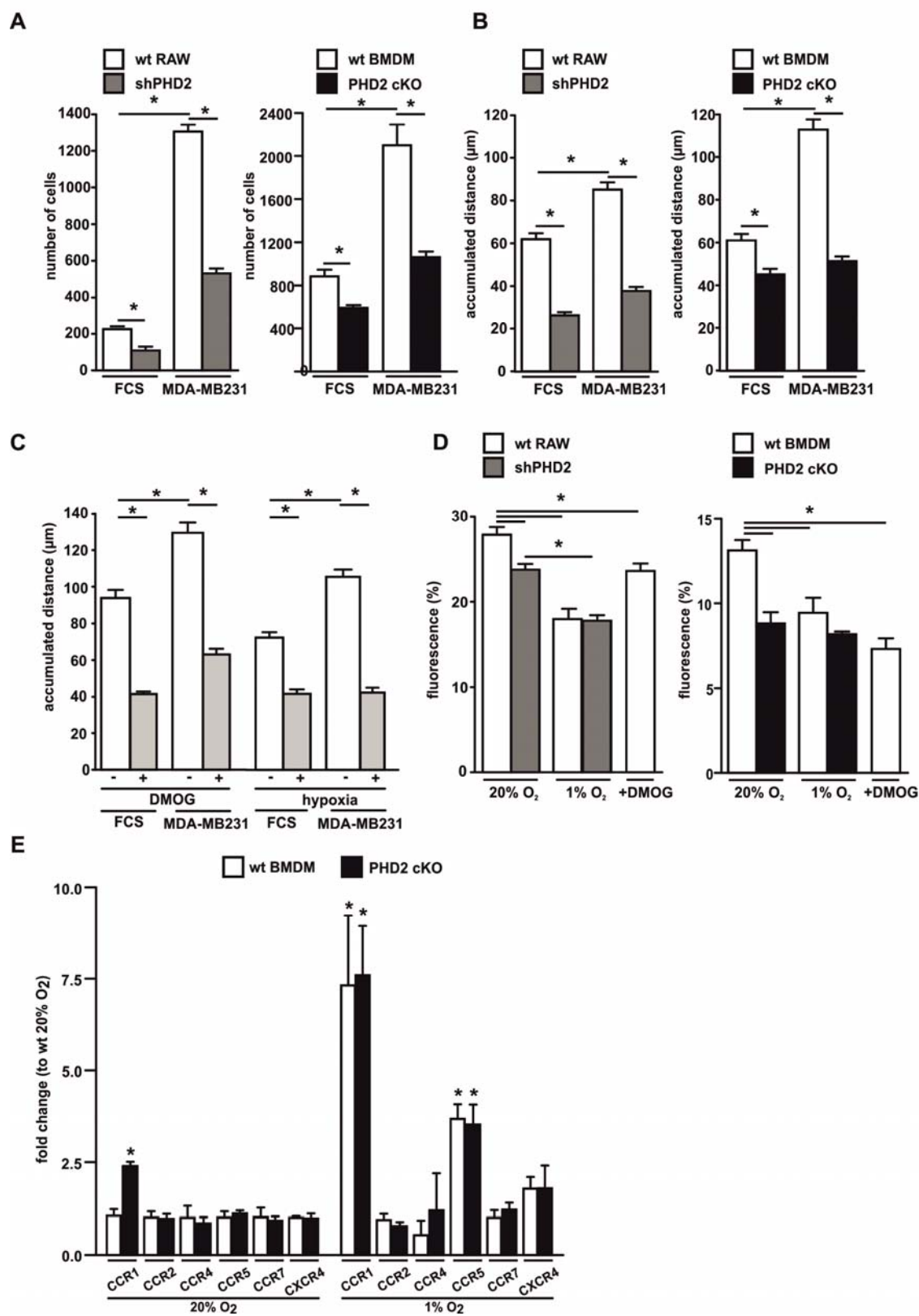


Figure 5

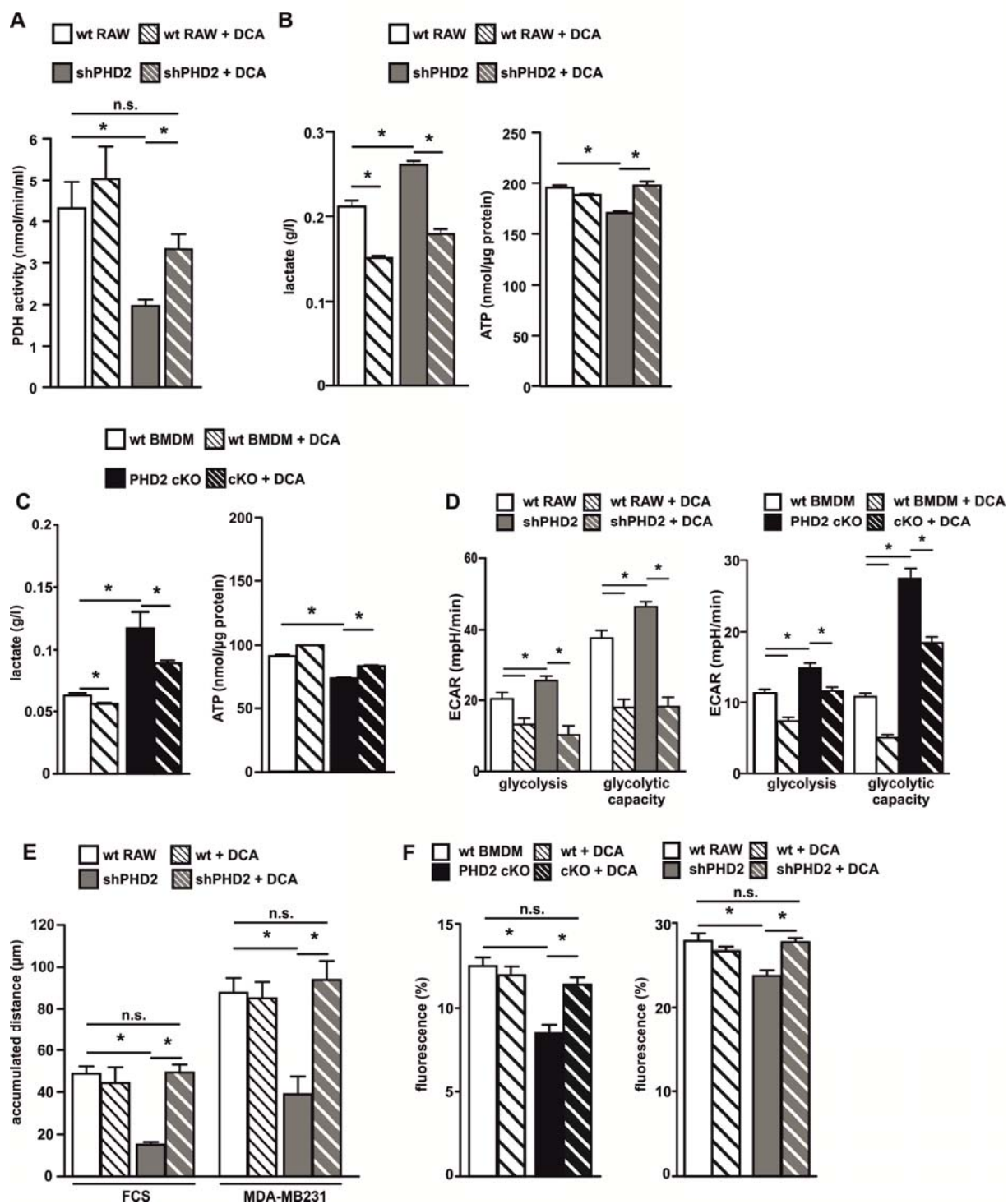
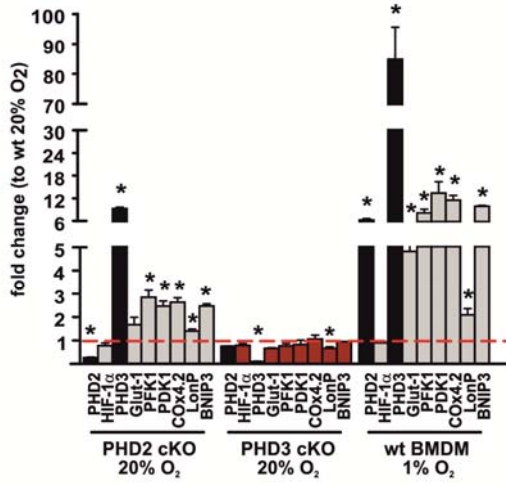
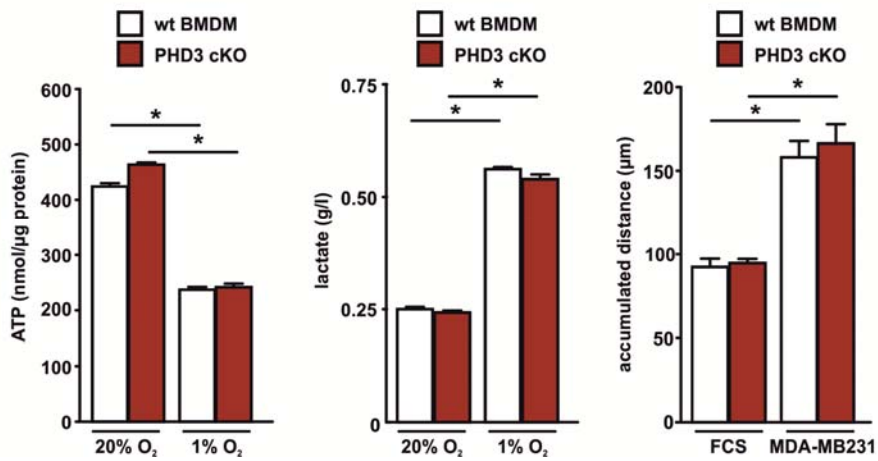


Figure 6

A

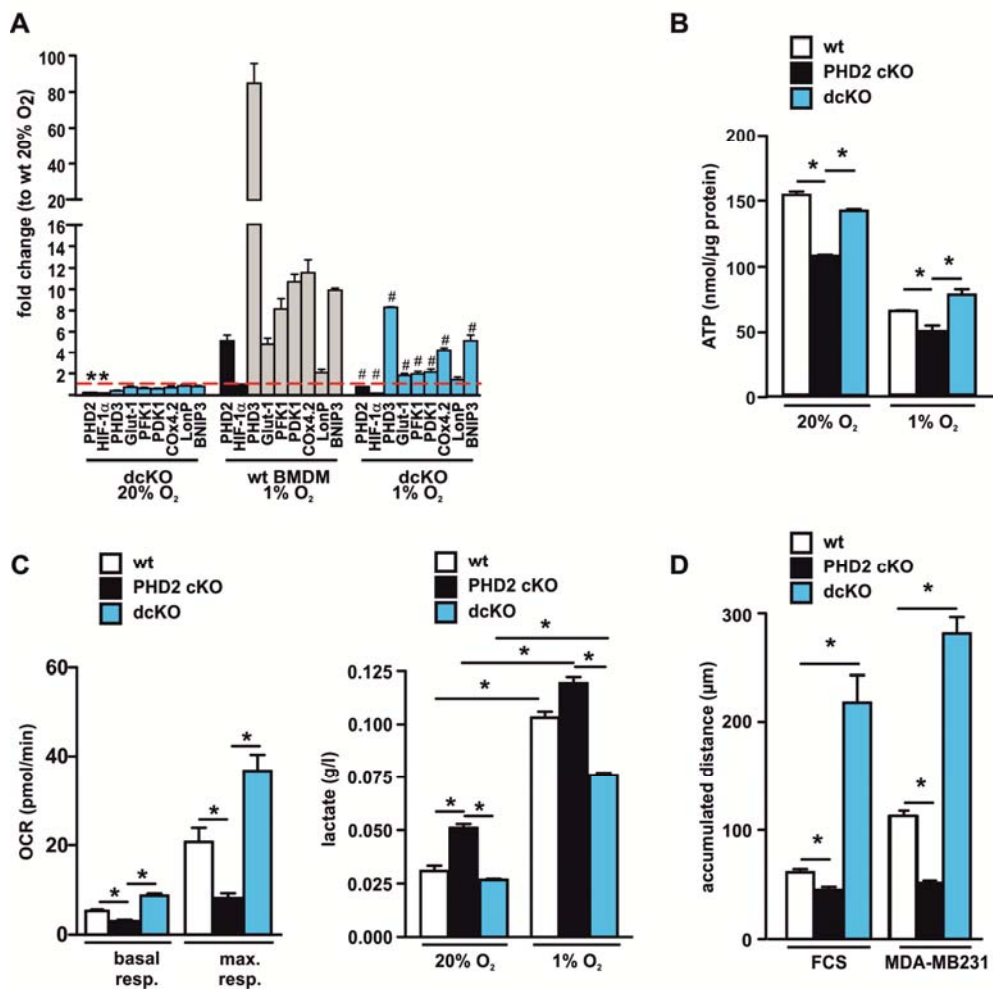


B



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Figure 7



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