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Prolyl Hydroxylase 3 Attenuates MCL-1–Mediated ATP Production to Suppress the Metastatic Potential of Colorectal Cancer Cells

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Abstract

Hypoxia is a common feature of solid tumors. Prolyl hydroxylase enzymes (PHD1–3) are molecular oxygen sensors that regulate hypoxia-inducible factor activity, but their functions in metastatic disease remain unclear. Here, we assessed the significance of PHD enzymes during the metastatic spread of colorectal cancer. PHD expression analysis in 124 colorectal cancer patients revealed that reduced tumoral expression of PHD3 correlated with increased frequency of distant metastases and poor outcome. Tumorigenicity and metastatic potential of colorectal tumor cells over and underexpressing PHD3 were investigated in orthotopic and heterotopic tumor models. PHD3 overexpression in a syngeneic tumor model resulted in fewer liver metastases, whereas PHD3 knockdown induced tumor spread. The migration of PHD3-overexpressing tumor cells was also attenuated *in vitro*. Conversely, migratory potential and colony formation were enhanced in PHD3-deficient cells, and this phenotype was associated with enhanced mitochondrial ATP production. Furthermore, the effects of PHD3 deficiency were accompanied by increased mitochondrial expression of the BCL-2 family member, member myeloid cell leukemia sequence 1 (MCL-1), and could be reversed by simultaneous inhibition of MCL-1. MCL-1 protein expression was likewise enhanced in human colorectal tumors expressing low levels of PHD3. Therefore, we demonstrate that downregulation of PHD3 augments metastatic spread in human colorectal cancer and identify MCL-1 as a novel downstream effector of oxygen sensing. Importantly, these findings offer new insight into the possible, context-specific deleterious effects of pharmacologic PHD inhibition. *Cancer Res; 76(8); 2219–30. ©2016 AACR.*

Introduction

Metastatic colorectal cancer represents a leading cause for cancer-related deaths worldwide (1). When tumor growth exceeds the formation of nourishing blood vessels, hypoxia occurs and leads to the stabilization of hypoxia-inducible transcription factors (HIF; ref. 2). In hypoxia, transcriptionally active HIF complexes bind to the promoter region of downstream target genes, which collectively mount an adaptive response aiming at securing cellular survival and restoring oxygen supply (3). Three HIF prolyl hydroxylases (PHD1, PHD2, and PHD3) regulate the stability of HIFs in an oxygen-dependent manner (4, 5). Their capacity to abrogate the hypoxic response depending on the availability of oxygen predestines these PHD enzymes as molecular oxygen sensors and makes them interesting targets for pharmacologic intervention (6).

HIFs are frequently overexpressed in human tumors (7), and several studies have revealed that the PHD enzymes are implicated in cancer growth. Albeit conflicting evidence has likewise been reported (8, 9), a majority of these studies suggest that PHD enzymes exert tumor-suppressive effects. For instance, forced overexpression of PHD1 in tumor cells suppresses HIF-1α activation and inhibits tumor growth in mice (10). Loss of PHD2 increases the growth of tumors derived from human colorectal and pancreatic cancer cells (11, 12), and silencing of PHD3 expression enhances the growth of heterotopically implanted colorectal tumors in mice (13). However, although PHD3 has been assigned a tumor-suppressive role in colorectal cancer, its function in metastatic tumor spread has not been studied.

Here, we assessed the significance of PHD enzymes in metastatic colorectal cancer. We report that underexpression of PHD3 in human colorectal tumors is associated with the occurrence of distant metastases and impaired patient survival. Furthermore, we identify the BCL-2 family member myeloid cell leukemia sequence 1 (MCL-1) as a downstream effector, causing improved mitochondrial efficacy and enhanced metastatic spread of PHD3-deficient tumor cells. These studies confirm the significance of PHD3 as a tumor suppressor in human metastatic colorectal cancer and identify MCL-1 as a novel link between the hypoxia-sensing pathway and energy homeostasis in tumor cells.

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Materials and Methods

Mouse models

Female C57BL/6 mice (10–12 weeks, 18–22 g) were used. All animal experiments were approved by the ethical commission of the local government (no. 35-9185.81/G-60/11) and carried out at Interfacultary Biomedical Faculty, University of Heidelberg (Heidelberg, Germany). All animal experiments were conducted according to FELASA and GV-SOLAS guidelines. Syngeneic tumors were heterotopically grown by subcutaneous injection of murine MC38 colon cancer cells (1×10^6, C57BL/6 background). Subcutaneous tumors grown for 3 weeks were cut into small pieces (1 mm^3) and implanted into the cecal wall to generate orthotopic tumors. Liver metastases were induced by splenic injection of tumor cells (1×10^6). Tumor volumes were calculated using the formula: $V = \pi \times (\text{width}^2 \times \text{length})/6$.

Human samples

Primary colorectal cancers and corresponding healthy mucosa were obtained from 124 patients undergoing surgical resection at the University Hospital Heidelberg (Heidelberg, Germany). Tissue collection and correlation analyses were approved by the Medical Ethics Committee of the University of Heidelberg (Heidelberg, Germany; no. 323/2004). Informed consent was obtained from each subject or subject's guardian. For enumeration of circulating tumor cells (CTC), 7.5 mL of whole blood was collected in CellSave Tubes (Janssen Diagnostics and analyzed applying the CELLSEARCH Circulating Tumor Cell Kit (Janssen Diagnostics) according to the manufacturer's instructions.

Cell culture experiments

MC38 colon cancer cells were kindly provided by H. Abken (University Hospital Cologne, Cologne, Germany) and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2. Testing for mycoplasma was performed routinely. All cell lines were free of contaminants.

For knockdown of PHD3, a knockdown vector was constructed by transferring a fragment containing an shRNA specifically targeting murine PHD3 from a donor vector (kindly provided by T. Acker, Justus Liebig University, Giessen, Germany; ref. 14) into the pLenti6/V5-GW/lacZ vector (Invitrogen) via KpnI/EcoRI lacZ removal. Conversely, for overexpression of PHD3, a vector was constructed by transferring a fragment containing the open reading frame of human PHD3 from a donor vector (kindly provided by T. Acker, Giessen, Germany) into the pLenti6/V5-GW/lacZ vector. Viral supernatants were prepared by cotransfection of the appropriate plasmid vector and the packaging vectors from the ViraPower Lentiviral Expression Systems (Invitrogen) into 293FT cells using Lipofectamine 2000 (Invitrogen). Subsequently, transfection of MC38 cells was performed according to the manufacturer's instructions, and stably transfected cells were selected with blasticidin (Invitrogen). Control cells were transduced with the plasmid vector encoding lacZ.

Proliferation was determined using the cell proliferation reagent WST1 (Roche Diagnostics GmbH) according to the manufacturer's instructions. Migration and invasion were assayed using a modified Boyden chamber assay (Greiner Bio-one) and FBS as a chemoattractant. For migration assays, filters were coated with Matrigel (250 μg/mL; BD Biosciences). Migrated cells were quantified by dissolving cell-bound crystal violet in 10% acetic acid, and absorbance was measured at 540 nm. For colony formation assays, 10,000 cells were seeded in petri dishes. After 3 weeks, colonies were fixed, stained with crystal violet, and quantified by two independent observers. Cellular ATP was quantified using a luciferase-based assay (Promega Corporation) according to the manufacturer's protocol. siRNA-mediated knockdown of MCL-1 in MC38 cells was carried out by applying FlexiTube Gene Solution (Qiagen; target sequences: AAAGGTTTGATTTCCTATACTA, catalog number SI03851407 and CGGGACTGGCTTGTCAAACAA, catalog number SI03851400) with Lipofectamine RNAiMAX (Thermo Fisher Scientific Inc).

Mouse embryonic fibroblasts (MEF) were isolated from $PHD3^{-/-}$ mice (13 days postcoitum) and MEFs lacking MCL-1 protein ($Mcl\text{-}1^{\Delta/null}$) as described previously (15, 16). Carcasses were minced and suspended in trypsin-EDTA with DNase. Single-cell suspensions were resuspended and maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mmol/L L-glutamine.

Histology and immunostaining

Paraffin-embedded tissues were sectioned at 6 μm thickness. PHD3 and MCL-1 IHC in human samples were performed using a rabbit anti-PHD3 antibody (Proteintech Group Inc, 1:500), and a rabbit anti-MCL-1 antibody (Santa Cruz Biotechnology, 1:200). Blood vessels were analyzed by double labeling with Anti-Actin, α-Smooth Muscle-Cy3 (Sigma-Aldrich, 1:2000) and anti-CD31 antibodies (Abcam, 1:200). Images were captured using a Nikon A1R Confocal Microscope. Histomorphometric quantification was performed by two independent investigators. Metastatic areas in murine livers were detected with an anti-V5 tag antibody (Abcam, 1:100).

Western blot analysis

Whole-cell lysates were prepared in RIPA lysis buffer (Merck Millipore). Nuclear proteins were isolated using NE-PER Nuclear and Cytosolic Extraction Reagents (Thermo Fisher). Primary antibodies against HIF-1α, MTCO1 (Abcam), MTCO2 (Proteintech), HIF-2α, PHD3 (Novus Biologicals), MCL-1, tubulin, histone H3 (Cell Signaling Technology), Tom20, and MCL-1 (Santa Cruz Biotechnology) were used. After development with an HRP-conjugated secondary antibody, semiquantitative analysis was performed applying ImageJ software (NIH, Bethesda, MD).

qRT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA synthesis and real-time PCR were performed with a first strand cDNA Synthesis Kit (Thermo Fisher) and LightCycler 480 SYBR Green I Master (Roche) using specific primers (sequences available on request). Transcript levels in human tumors were normalized to corresponding healthy colon mucosa. GAPDH was used as housekeeping gene.

Mitochondrial assays

Mitochondria were isolated applying the Mitochondrial Isolation Kit for Cultured Cells (Thermo Fisher). Complex I activity was measured using the Complex I Enzyme Activity Microplate Assay Kit (Abcam MitoSciences). Citrate synthase activity was measured using Citrate Synthase Assay Kit (Sigma-Aldrich).

Statistical analysis

All values are represented as mean ± SEM. The significance of experimental differences was evaluated by the Student *t* test or

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ANOVA, where applicable. $P \leq 0.05$ was considered significant. Survival data were analyzed by the construction of Kaplan–Meier plots and application of the log-rank test.

Results

Significance of PHD3 in human colorectal cancer

To investigate the significance of the HIF PHDs in human colorectal cancer, we analyzed the expression of PHD1, PHD2, and PHD3 mRNA transcripts in surgically resected specimens from 124 patients. PHD1 and PHD2 were differentially expressed in primary colorectal tumors (Fig. 1A, top and middle). However, a majority of tumors displayed downregulated PHD3 mRNA expression (Fig. 1A, bottom). Immunohistochemical analyses revealed spatial expression of PHD3 protein in colonic adenocarcinoma cells (Fig. 1B, bottom), albeit at decreased frequency when compared with PHD3 expression in healthy colon mucosal cells (Fig. 1B, top). Transcript expression levels of PHD1 or PHD2 did not correlate with local tumor invasiveness (histopathologic T-stage), lymphatic spread (N-stage), or the occurrence of distant metastases (M-stage). Likewise, PHD3 transcript expression in human colorectal tumors did not correlate with local or lymphatic tumor progression (Table 1). However, decreased expression of PHD3 correlated significantly with the occurrence of distant metastases (M1-stage; Table 1). Consistently, quantitative expression of PHD3 mRNA transcripts was significantly higher in non-metastatic (M0-stage) compared with metastatic (M1-stage) tumors (Fig. 1C). Likely as an effect of increased metastatic frequency, the overall survival of patients with tumors expressing low levels of PHD3 was significantly impaired compared with that of patients with tumors expressing high levels of PHD3 (Fig. 1D). CTCs were counted in a subset of 64 patients, revealing that CTCs occurred more frequently in central venous blood from patients with decreased tumoral PHD3 expression than in patients with increased tumoral PHD3 expression, albeit this difference was not quite significant (Table 1).

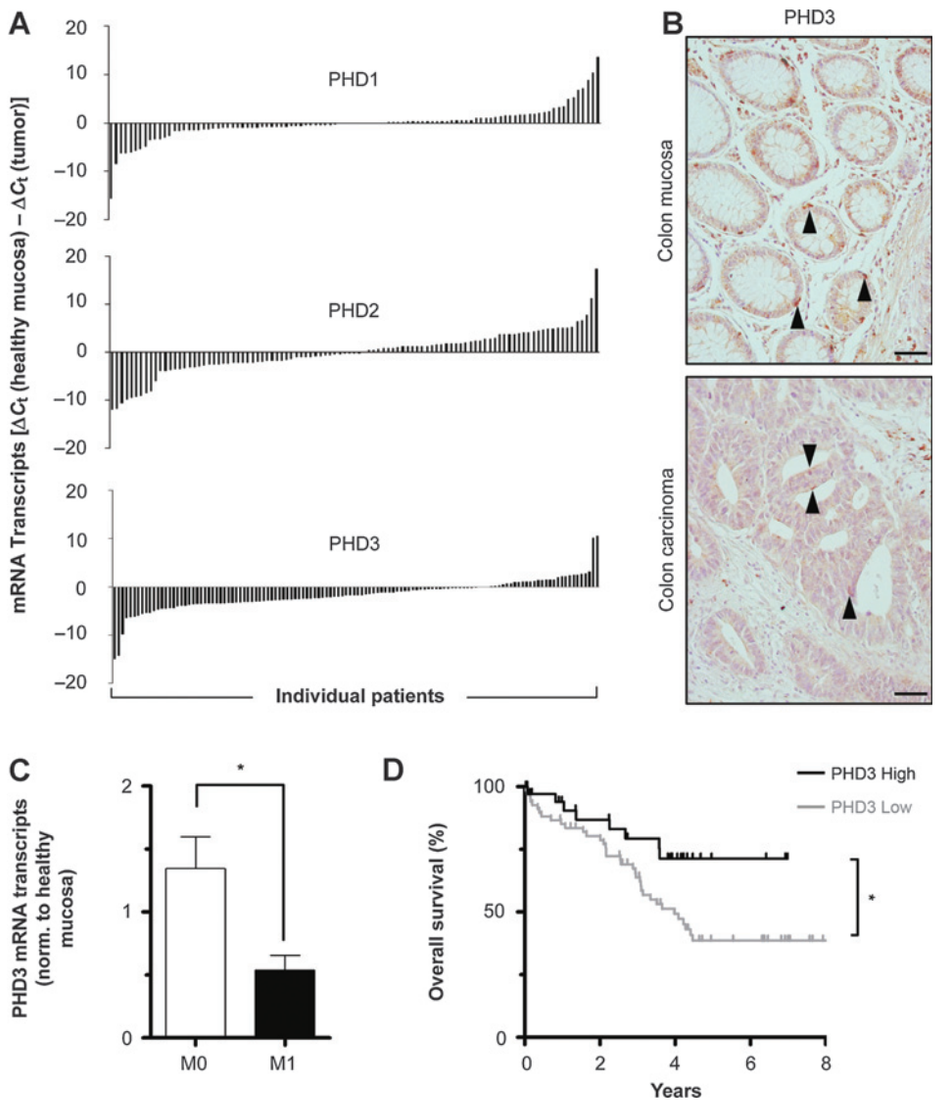

Figure 1.
Significance of PHD enzymes in human colorectal cancer. A, real-time PCR analysis revealing underexpression of PHD3 (but not PHD1 or PHD2) mRNA in primary colorectal tumors. Each bar represents an individual patient; bar values [ΔC_t (mucosa) − ΔC_t (tumor)] represent the difference of PHD mRNA between healthy mucosa and corresponding tumor. PHD1 and PHD2 mRNA expression was analyzed in 101 patients. PHD3 mRNA expression analysis was expanded to 124 patients for clinical correlation analyses. B, immunostaining revealing expression of PHD3 protein (arrowheads) in colorectal cancer cells (bottom) and healthy mucosa cells (top). Scale bars, 20 μm. C, real-time PCR revealing lower PHD3 transcript levels in primary tumors from patients suffering distant metastases (M1) compared with those without (M0). *, $P \leq 0.05$; $n = 61/63$. D, Kaplan–Meier curves revealing the survival of patients with low versus high intratumoral expression of PHD3. *, $P \leq 0.05$; $n = 124$.

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Table 1. Correlation of PHD3 expression levels in primary human colorectal tumors with clinical patient characteristics

Clinical characteristics	PHD3 Decreased ($n = 94$)	PHD3 Increased ($n = 30$)	Test of significance
Sex			
Male	61	17	$P = 0.5155$[a]
Female	33	13	
Age, y			
≤50	10	2	$P = 0.7288$[a]
>50	84	28	
Tumor site			
Colon	50	20	$P = 0.2906$[a]
Rectum	44	10	
TNM Stage			
T1	3	1	$P = 0.6476$[b]
T2	14	5	
T3	57	19	
T4	20	5	
N0	39	13	$P = 0.9841$[b]
N1	26	8	
N2	29	9	
M0	40	21	$P = 0.0116$[a,c]
M1	54	9	
UICC Stage			
I	10	2	$P = 0.0107$[b,c]
II	11	10	
III	19	9	
IV	54	9	
CTC	**PHD3 Decreased ($n = 38$)**	**PHD3 Increased ($n = 26$)**	
CTC ≥ 1	19	7	$P = 0.0759$[a]
CTC = 0	19	19	

Abbreviations: TNM, tumor/lymph node/metastasis; UICC, Union for International Cancer Control.
[a]Fisher exact test.
[b]χ^2 test.
[c]$P < 0.05$, $n = 124$ (CTC analysis: $n = 64$).

Immunohistochemical analyses were applied in an initial attempt to determine the effects of PHD3 expression on pathologic tumor features. Immunolabeling and histomorphometric quantification of $CD31^+$ vascular endothelial cells revealed comparable densities of tumor blood vessels in human colorectal tumors expressing low and high levels of PHD3 (Supplementary Fig. S1A and S1B). Furthermore, immunohistochemical double labeling of CD31 and alpha-smooth muscle actin (αSMA) revealed comparable blood vessel maturation in tumors with low and high PHD3 expression levels (Supplementary Fig. S1A and S1B). Immunohistochemical staining for the Ki67 antigen revealed similar fractions of proliferating tumor cells in tumors under or overexpressing PHD3 (% Ki67-positive cells: 5.78 ± 1.53 in PHD3 low vs. 5.62 ± 1.10 in PHD3 high tumor samples; $n = 12$, $P =$ n.s.). Thus, the expression status of PHD3 in human colorectal tumor samples was not associated with tumor angiogenesis, blood vessel maturation, or cell proliferation.

PHD3 expression affects tumor spread *in vivo*

As underexpression of PHD3 in human colorectal cancer specimens was associated with the occurrence of distant metastases, we employed functional genetic analyses to delineate specific functions of PHD3 in colorectal cancer spread. For this purpose, murine MC38 colon cancer cells were stably transduced to either knockdown or to overexpress PHD3 (referred to as PHD3kd and PHD3oe, respectively). As a control, MC38 cells were transduced with the plasmid vector encoding lacZ (PHD3lacZ). RNA expression analyses and immunoblotting confirmed successful silencing of PHD3 expression in PHD3kd cells and robust induction of PHD3 in PHD3oe cells (Fig. 2A).

Various syngeneic tumor models were applied to delineate effects of altered PHD3 expression on tumor cell expansion in mice. First, MC38 cells were subcutaneously implanted into the flank of syngeneic animals. Continuous monitoring of tumor size revealed that overexpression of PHD3 significantly delayed the growth of heterotopic tumors (Fig. 2B). Second, tumor pieces were implanted into the cecal wall. Orthotopic growth of PHD3oe tumors was likewise significantly attenuated compared with that of corresponding PHD3lacZ tumors. Conversely, implantation of PHD3kd tumors accelerated tumor growth (Fig. 2C). Third, MC38 cells were injected into the spleen to induce hepatic tumor spread. Consistent with the results outlined above, histomorphometric quantification revealed significantly attenuated metastatic tumor growth in livers of mice injected with PHD3oe cells and strikingly enhanced tumor spread in those injected with PHD3kd cells (Fig. 2D). Of note, although mice undergoing intrasplenic injection of PHD3lacZ cells succumbed to progressive tumor burden and liver failure within four weeks, no fatalities were observed in mice injected with PHD3oe cells (Fig. 2E).

Collectively, these *in vivo* analyses are in accordance with the enhanced metastatic potential observed in human colorectal tumors underexpressing PHD3 and support the hypothesis that PHD3 acts as a suppressor of colorectal tumor spread.

Inhibition of PHD3 enhances tumorigenicity *in vitro*

To elucidate cellular mechanisms underlying enhanced spread of PHD3-deficient tumor cells, we performed *in vitro* analyses, applying PHD3 knockdown or overexpression in MC38 cells.

Cellular proliferation studies revealed that knockdown of PHD3 expression was associated with slightly but significantly enhanced cellular outgrowth, whereas an opposite effect was observed in PHD3-overexpressing cells (Fig. 3A). These subtle effects of altered PHD3 expression on *in vitro* tumor cell proliferation were mirrored by slightly (albeit not quite significantly) enhanced proliferation of PHD3kd tumor cells *in vivo* (Supplementary Fig. S1C). More strikingly, tumor cell migration through Matrigel was significantly enhanced upon knockdown of PHD3 expression but markedly decreased upon upregulation of PHD3 expression (Fig. 3B). Further RT-PCR analyses revealed that the expression of matrix metalloproteinases MMP10, MMP11, and MMP14, which reportedly facilitate cancer cell invasion (17–19), was enhanced in PHD3kd tumor cells (Fig. 3C). We likewise assessed the effects of PHD3 expression on colony formation, which is a hallmark feature of malignant cells (20). Indeed, colony formation was strikingly enhanced upon knockdown of PHD3 expression (Fig. 3D).

Taken together, these *in vitro* data confirm the notion that PHD3 expression in tumor cells affects their migratory and invasive potential. These findings are consistent with our observations concerning the effects of PHD3 expression on tumor expansion *in vivo* and on metastatic spread in human patients.

PHD3 expression affects mitochondrial ATP production of tumor cells

Intriguingly, expressional analyses of the epithelial cell adhesion molecule E-cadherin and the mesenchymal marker vimentin suggested that the increased migratory potential of PHD3kd cells

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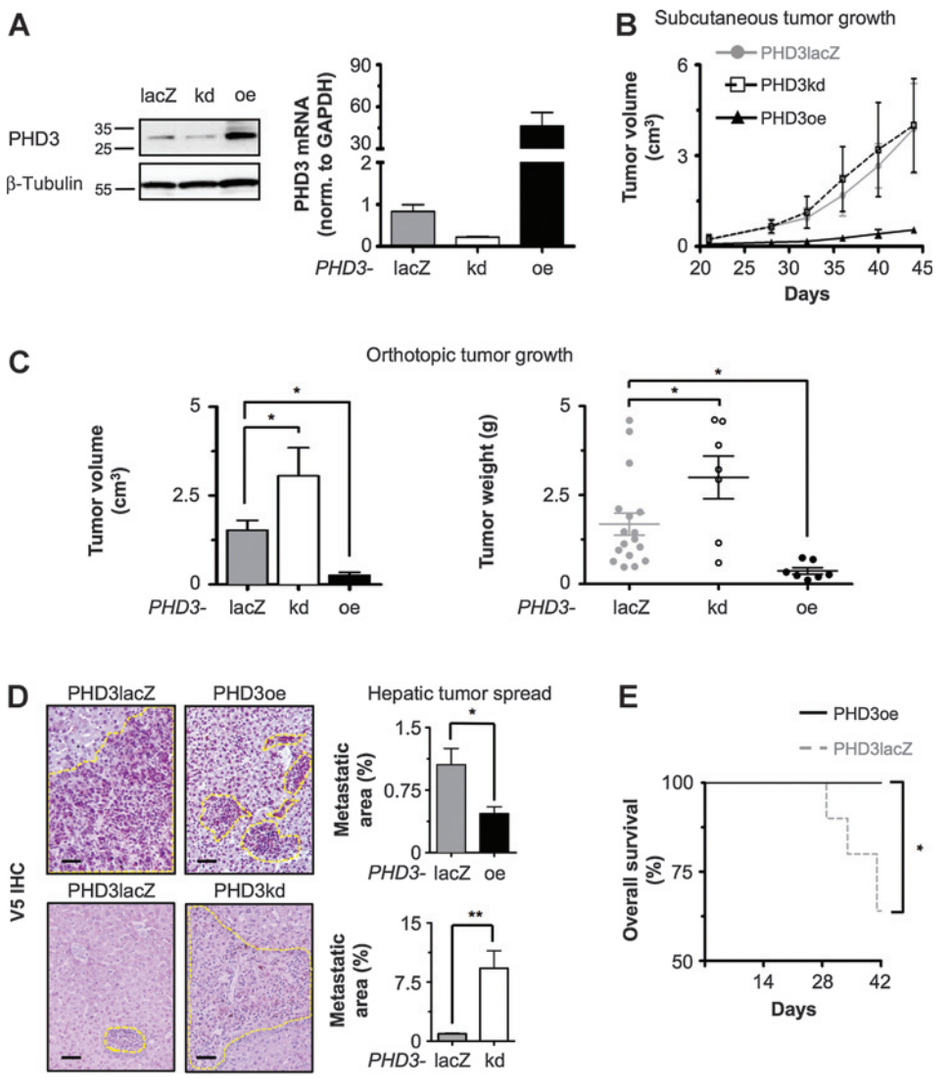

Figure 2.
PHD3 expression affects tumor spread *in vivo*. A, immunoblot (left) and real-time PCR (right) revealing reduced PHD3 expression in MC38 colon carcinoma cells upon transfection with a PHD3-silencing construct (PHD3kd) and enhanced PHD3 expression upon transfection with a PHD3 overexpression construct (PHD3oe) compared with control-transfected cells (lacZ). β-Tubulin was used to assure equal loading. B, subcutaneous growth of tumors derived from PHD3kd-, PHD3oe-, and lacZ- MC38 cells in syngeneic mice. *, $P \leq 0.05$; $n = 6$. C, volume (left) and weight (right) of tumors derived from orthotopic implantation of PHD3ue-, PHD3oe-, and lacZ- MC38 cells into the colon of syngeneic mice and harvested after 4 weeks. *, $P \leq 0.05$; $n = 10$. D, representative V5 immunolabeling (left) and histomorphometric quantification (right), revealing reduced occurrence of metastatic liver nodules (dotted lines) in mouse livers following intrasplenic injection of PHD3oe cells and enhanced metastatic spread of intrasplenically injected PHD3kd cells. *, $P \leq 0.05$; **, $P \leq 0.01$; $n = 7/5$. Scale bars, 50 μm. E, survival curves revealing enhanced survival of mice following intrasplenic injection of PHD3oe cells compared with mice injected with control-transfected cells (lacZ). *, $P \leq 0.05$; $n = 11$; kd, knockdown; oe, overexpressed.

was not linked to enhanced epithelial–mesenchymal transition (Supplementary Fig. S1D).

Among other requirements, the ability of tumor cells to migrate and invade adjacent structures depends on cellular energy production (21, 22). To test whether altered PHD3 expression affected ATP production in tumor cells, we performed ATP assays under varying conditions. Strikingly, expressional silencing of PHD3 caused elevated ATP levels in MC38 cells under normal culture conditions. Conversely, ATP production of MC38 cells could be attenuated by overexpression of PHD3 (Fig. 4A).

Cellular ATP production can be accomplished by anaerobic glycolysis and, more effectively, by mitochondrial respiration (23). To assess the relative contribution of these energy sources on enhanced ATP generation in PHD3kd cells, we assessed the ATP production of MC38 cells upon treatment with anaerobic glycolysis inhibitors (sodium iodoacetate plus 2-deoxy-D-glucose) or with mitochondrial respiration inhibitors (rotenone plus antimycin A). Inhibition of anaerobic glycolysis similarly attenuated ATP levels in PHD3lacZ, PHD3kd, and PHD3oe cells. However, even upon addition of glycolysis inhibitors, PHD3kd tumor cells remained significantly more efficient in ATP production than PHD3lacZ or PHD3oe cells (Fig. 4B, middle bars). Inhibition of mitochondrial respiration not only caused a more robust decline in cellular ATP levels than inhibition of anaerobic glycolysis, but likewise abrogated enhanced ATP levels in PHD3kd cells (Fig. 4B, right bars).

As impairment of mitochondrial energy production, but not interference with anaerobic glycolysis, abrogated enhanced ATP levels in PHD3kd cells, we hypothesized that altered PHD3 expression affects mitochondrial respiration in tumor cells. Indeed, protein expression of the mitochondrial encoded cytochrome c oxidase subunit I, a key enzyme in mitochondrial respiration, was increased upon knockdown of PHD3 (Fig. 4C). Furthermore, the activities of citrate synthase (the pacemaking enzyme of mitochondrial respiration; Fig. 4D, top) and of NADH dehydrogenase (mitochondrial complex I) were increased in PHD3kd cells (Fig. 4D, bottom).

Effects of PHD3 on ATP production and migration are mediated by MCL-1

Next, we aimed to determine putative molecular mechanisms downstream PHD3, which could facilitate mitochondrial energy

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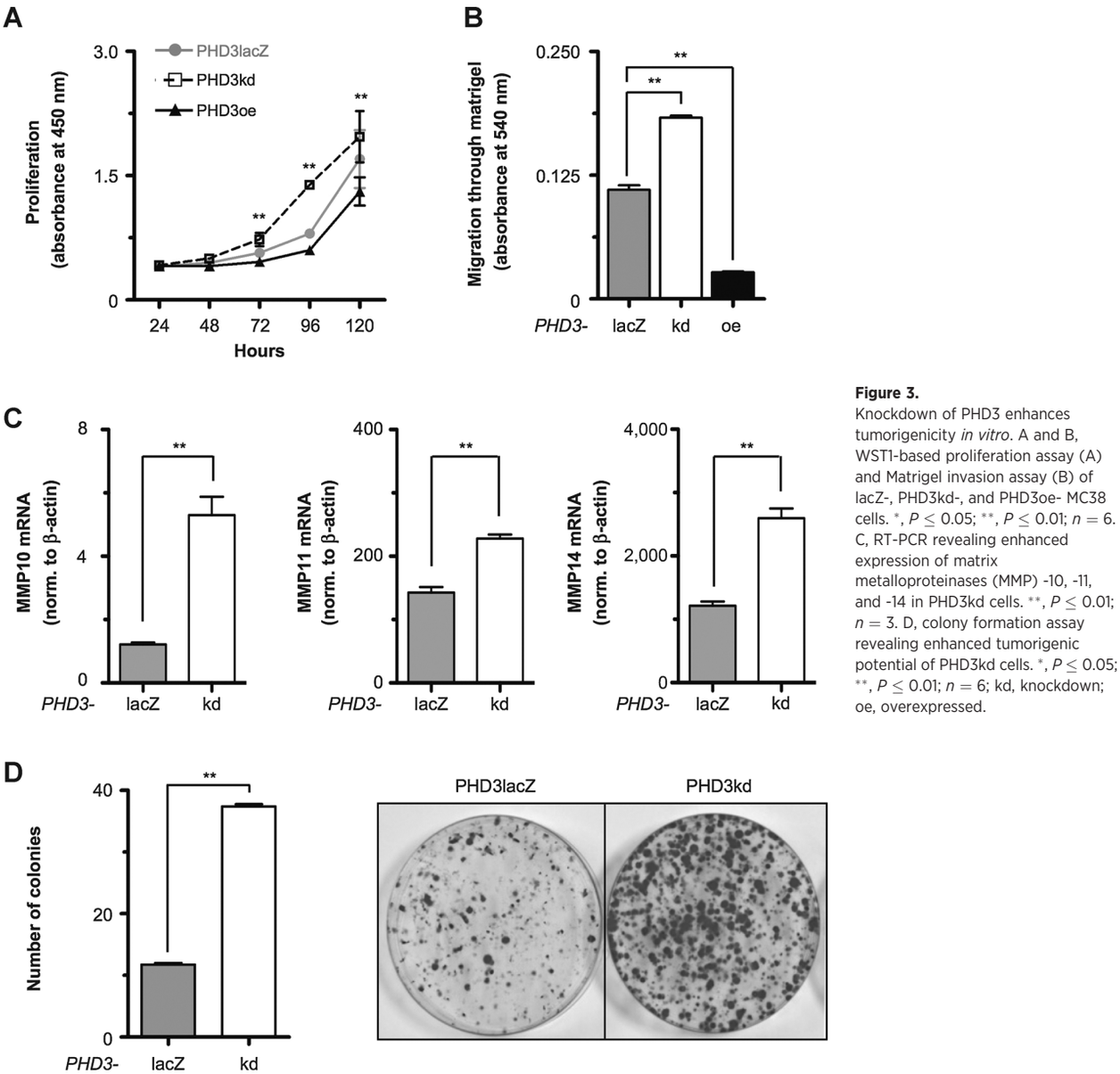

Figure 3.
Knockdown of PHD3 enhances tumorigenicity *in vitro*. A and B, WST1-based proliferation assay (A) and Matrigel invasion assay (B) of lacZ-, PHD3kd-, and PHD3oe- MC38 cells. *, $P \leq 0.05$; **, $P \leq 0.01$; $n = 6$. C, RT-PCR revealing enhanced expression of matrix metalloproteinases (MMP) -10, -11, and -14 in PHD3kd cells. **, $P \leq 0.01$; $n = 3$. D, colony formation assay revealing enhanced tumorigenic potential of PHD3kd cells. *, $P \leq 0.05$; **, $P \leq 0.01$; $n = 6$; kd, knockdown; oe, overexpressed.

production on the one hand, while on the other hand promoting cellular migration and invasion.

Among other molecules (and HIF in particular, see below), we focused on MCL-1, a BCL-2 family member, which is expressed in colorectal cancer (24, 25). MCL-1 protein undergoes proteolytic cleavage. A truncated isoform localizes to the mitochondrial matrix and has recently been linked to facilitated mitochondrial ATP production and mitochondrial dynamics (26, 27).

We initially investigated whether knockdown of PHD3 affected MCL-1 protein expression in MC38 tumor cells. Immunoblotting revealed that the abundance of MCL-1 was significantly enhanced in the mitochondrial protein fraction of PHD3kd cells (Fig. 5A; Supplementary Fig. S2A). Additional Western blotting was performed following proteinase K digestion of purified mitochondria (which reportedly degrades MCL-1 located at the outer mitochondrial membrane, while leaving the matrix-localized MCL-1 isoform intact; ref. 28). These analyses confirmed that the expression of mitochondrial matrix-localized MCL-1 was enhanced in PHD3kd tumor cells (Supplementary Fig. S2B).

The following experiments were carried out to assess the significance of altered mitochondrial MCL-1 protein expression concerning ATP production in PHD3kd cells. First, we applied RNA interference to selectively target MCL-1 protein expression in PHD3lacZ, PHD3kd, and PHD3oe cells. qRT-PCR analysis revealed that this approach reduced the expression of MCL-1 transcripts to comparable extent in lacZ- and PHD3kd MC38 cells (residual expression of MCL-1 transcripts, percentage of levels in control transfected cells: 40.9 ± 5.6 in lacZ cells vs. 42.1 ± 4.1 in PHD3kd cells; $n = 10$), and Western blotting revealed that siRNA treatment likewise reduced the abundance of MCL-1 protein in lacZ- and PHD3kd MC38 cells (Supplementary Fig. S2C, top). siRNA-mediated knockdown of MCL-1 completely abrogated

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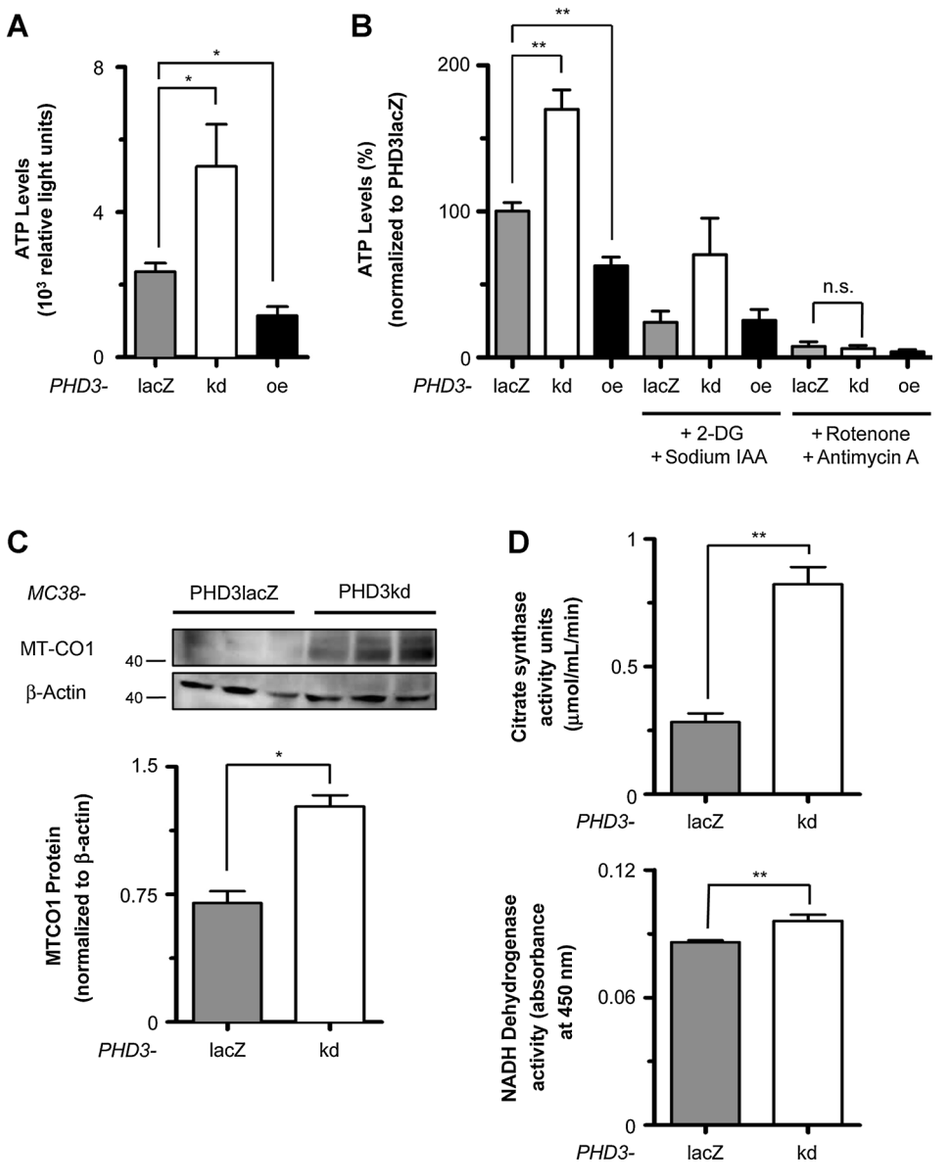

Figure 4.
Loss of PHD3 enhances mitochondrial ATP production. A, luciferase-based ATP assay revealing enhanced ATP generation in PHD3kd cells compared with lacZ or PHD3oe cells. *, $P \leq 0.05$; $n = 4$. B, ATP assay revealing that enhanced ATP production of PHD3kd cells is abrogated upon inhibition of mitochondrial respiration (rotenone + antimycin A), but not anaerobic glycolysis [2-deoxy-D-glucose (2-DG) + sodium iodoacetate (sodium IAA)]. **, $P \leq 0.01$; $n = 10$. C, immunoblot (top) and densitometric quantification (bottom) revealing enhanced expression of cytochrome c oxidase subunit I (MT-CO1) in PHD3kd cells. *, $P \leq 0.05$; $n = 3$. D, enzyme activity assays of citrate synthase (top) and NADH dehydrogenase (bottom) revealing enhanced activity in PHD3kd cells. **, $P \leq 0.01$; $n = 6$; n.s., not significant; kd, knockdown; oe, overexpressed.

enhanced ATP production in PHD3-deficient cells (Fig. 5B, left). In PHD3oe cells, knockdown of MCL-1 caused a further repression of cellular ATP levels, which were already at baseline conditions lower than those in lacZ cells (Fig. 5B, right). Second, PHD3lacZ and PHD3kd cells were treated with GX15-070 (obatoclax), a small-molecule inhibitor of BCL-2 family proteins, including MCL-1 (29). Enhanced ATP levels in PHD3kd cells could be fully reverted upon treatment with GX15-070, but not upon treatment with ABT-737, an inhibitor of BCL-2 family proteins, excluding MCL-1 (Supplementary Fig. S2D; ref. 30). Cell viability in these experimental settings was monitored in parallel, but was only slightly reduced by targeting of MCL-1. Readouts from ATP assays were corrected for these subtle differences in cell numbers.

As an alternative experimental readout to investigate whether PHD3 deficiency affects energy production in an MCL-1–dependent manner, we assessed ATP levels in MEFs lacking PHD3 ($PHD3^{-/-}$). $PHD3^{-/-}$ MEFs displayed enhanced ATP levels compared with corresponding wild-type (WT) MEFs (Fig. 5C). Consistent with our findings in PHD3-silenced MC38 tumor cells, siRNA-mediated knockdown of MCL-1 (Supplementary Fig. S2C, bottom) reduced ATP levels in $PHD3^{-/-}$ cells (Fig. 5C). In WT MEFs, knockdown of MCL-1 likewise caused a slight but significant decline in ATP levels.

Finally, to unambiguously validate the effects of MCL-1 on cellular energy production, we assessed ATP levels in MCL-1–deficient MEFs. Indeed, siRNA-induced knockdown or genetic deficiency of MCL-1 ($Mcl\text{-}1^{\Delta/null}$) significantly attenuated the ATP production in these cells (Supplementary Fig. S2E). Strikingly, reinsertion of MCL-1 via transfection with a vector expressing MCL-1 (31) fully restored ATP levels of $Mcl\text{-}1^{\Delta/null}$ cells to those observed in corresponding WT control cells (Supplementary Fig. S2E).

We likewise aimed to determine whether MCL-1–mediated effects were sufficient to induce the increased migratory properties of PHD3-deficient tumor cells. Indeed, recent reports have highlighted the significance of mitochondrial efficacy and mitochondrial dynamics for persistent migration of epithelial

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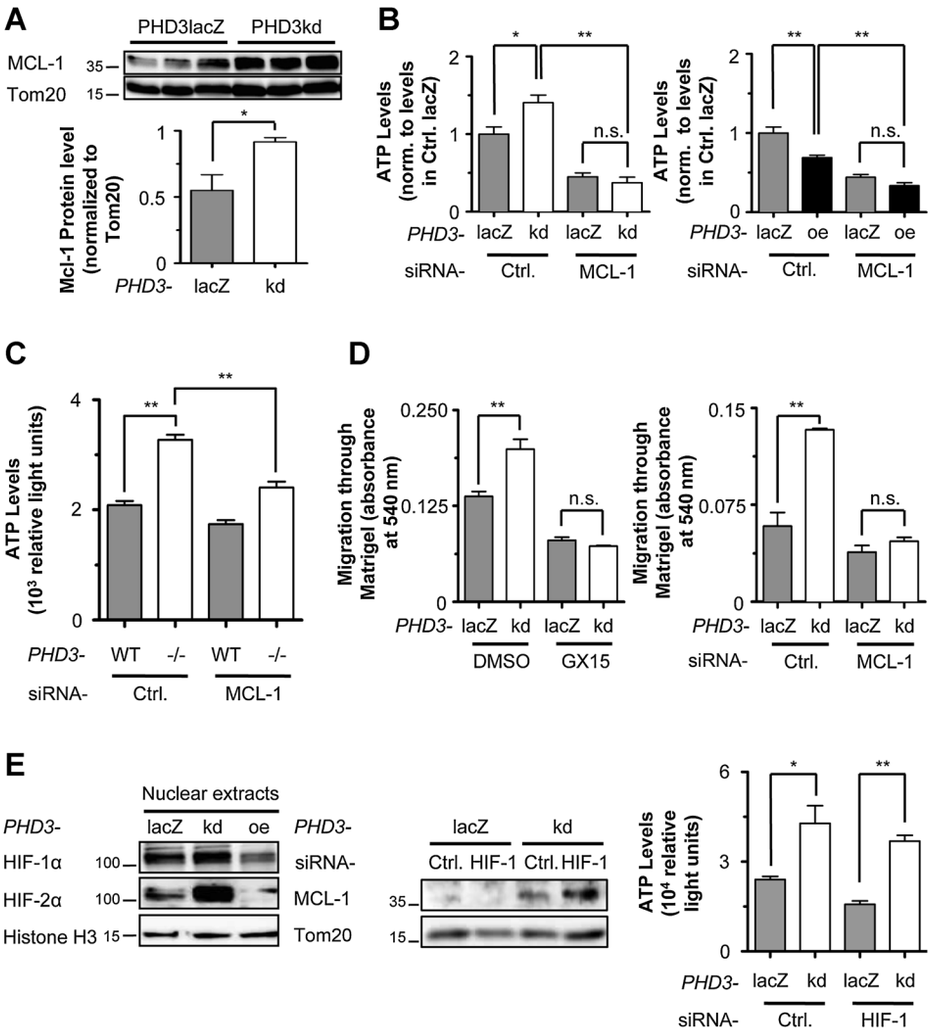

Figure 5.

Enhanced ATP production and migration of PHD3-deficient tumor cells are mediated by MCL-1. A, immunoblot of mitochondrial fractions (top) and semiquantitative analysis (bottom) revealing enhanced mitochondrial MCL-1 protein expression in PHD3kd cells. Tom20 was used as a loading control. B, ATP assays of PHD3kd, PHD3oe, and control-transfected (lacZ) MC38 cells. Enhanced ATP levels in PHD3kd cells are abrogated upon siRNA-mediated knockdown of MCL-1 (left). Reduced ATP levels in PHD3oe cells are further repressed upon knockdown of MCL-1 (Ctrl, control-siRNA–treated). C, ATP assay of PHD3 knockout ($PHD3^{-/-}$) MEFs and corresponding WT cells, following transfection with siRNA targeting MCL-1 or control siRNA (Ctrl). D, Matrigel assays revealing that enhanced migration of PHD3kd cells is abolished upon treatment with the MCL-1 inhibitor GX15-70 (GX15, left) or siRNA-mediated knockdown of MCL-1 (right). E, immunoblot of nuclear extracts (left) revealing enhanced stabilization of HIF-1α and HIF-2α in PHD3kd MC38 cells and decreased HIF stabilization in PHD3oe cells (histone H3: loading control); middle, immunoblot of mitochondrial fractions revealing that enhanced MCL-1 expression in PHD3kd MC38 cells is not affected by siRNA-mediated knockdown of HIF-1α (Tom20, loading control); right, ATP assay revealing persistently elevated ATP levels in PHD3kd cells upon silencing of HIF-1α. All ATP and migration data were normalized to cell viability as monitored by WST1 assay. $n = 3$, *, $P \leq 0.05$; **, $P \leq 0.01$; n.s., not significant; kd, knockdown; oe, overexpressed.

tumor cells (32). Strikingly, increased migration of PHD3kd cells through Matrigel could be fully abolished by pharmacologic treatment with the inhibitor of BCL-2 family proteins, GX15-070 (Fig. 5D, left). Consistently, siRNA-mediated interference with MCL-1 expression reverted the migratory potential of PHD3kd cells to the extent that was observed in corresponding PHD3lacZ cells (Fig. 5D, right).

As PHD3 is a crucial regulator of the HIF pathway, we likewise assessed the involvement of HIF-1α. Indeed, immunoblotting revealed significantly increased protein levels of HIF-1α and HIF-2α in nuclear extracts from PHD3kd MC38 tumor cells, but decreased expression of HIF-1α and HIF-2α in cells overexpressing PHD3 (Fig. 5E, left). However, enhanced mitochondrial MCL-1 protein levels in PHD3kd tumor cells persisted even upon siRNA-mediated repression of HIF-1α (Fig. 5E, middle). Moreover, enhanced ATP production of PHD3kd cells was not affected by siRNA-mediated knockdown of HIF-1α (Fig. 5E, right) and likewise persisted upon pharmacologic HIF inhibition with chetomin (Supplementary Fig. S2F), altogether suggesting that HIF-1α is not an exclusive mediator of improved ATP production in PHD3-deficient cells. Furthermore, we applied the PHD inhibitor, dimethyloxalylglycine (DMOG), to test whether the hydroxylase activity of PHD3 is required to provoke the observed effect of enhanced

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ATP production in PHD3-deficient tumor cells. Indeed, treatment with DMOG abolished the phenotype of enhanced ATP levels in MC38 tumor cells (Supplementary Fig. S2G), suggesting that the hydroxylase function of PHD3 is indeed required to mediate this effect.

Collectively, these results indicate that MCL-1 is an important downstream effector, facilitating mitochondrial ATP production and invasive potential in PHD3-deficient tumor cells.

MCL-1 expression is enhanced in colorectal tumors with low PHD3 expression

Finally, we aimed to delineate whether the link between downregulation of PHD3 and enhanced expression of MCL-1, which could be documented in tumor cells *in vitro*, could likewise be recapitulated *in vivo*. For this purpose, we determined the expression of MCL-1 protein in murine colon tumors, which were derived by orthotopic injection of PHD3kd or PHD3lacZ cells in syngeneic mice. Indeed, Western blotting analysis revealed significantly enhanced abundance of MCL-1 protein in PHD3kd tumors compared with PHD3lacZ tumors (Fig. 6A).

To assess whether downregulation of PHD3 is likewise linked to enhanced MCL-1 expression in primary human colorectal tumors, further analyses were applied on a representative collective of human tumors, either lowly or highly expressing PHD3 (as compared with PHD3 expression in healthy mucosa from each respective patient). IHC and morphometric quantification revealed that MCL-1 protein was significantly more abundant in human colorectal tumors with low PHD3 expression than in those with high PHD3 expression (Fig. 6B). Consistently, Western blotting analysis revealed enhanced expression levels of MCL-1 protein in both whole tissue lysates (Fig. 6C, left and middle), as well as in isolated mitochondrial fractions (Fig. 6C, right, and Supplementary Fig. S2A) from human colorectal tumors expressing low PHD3 levels compared with those expressing high PHD3 levels.

Collectively, these data confirm that downregulation of PHD3 causes enhanced expression of MCL-1 protein in

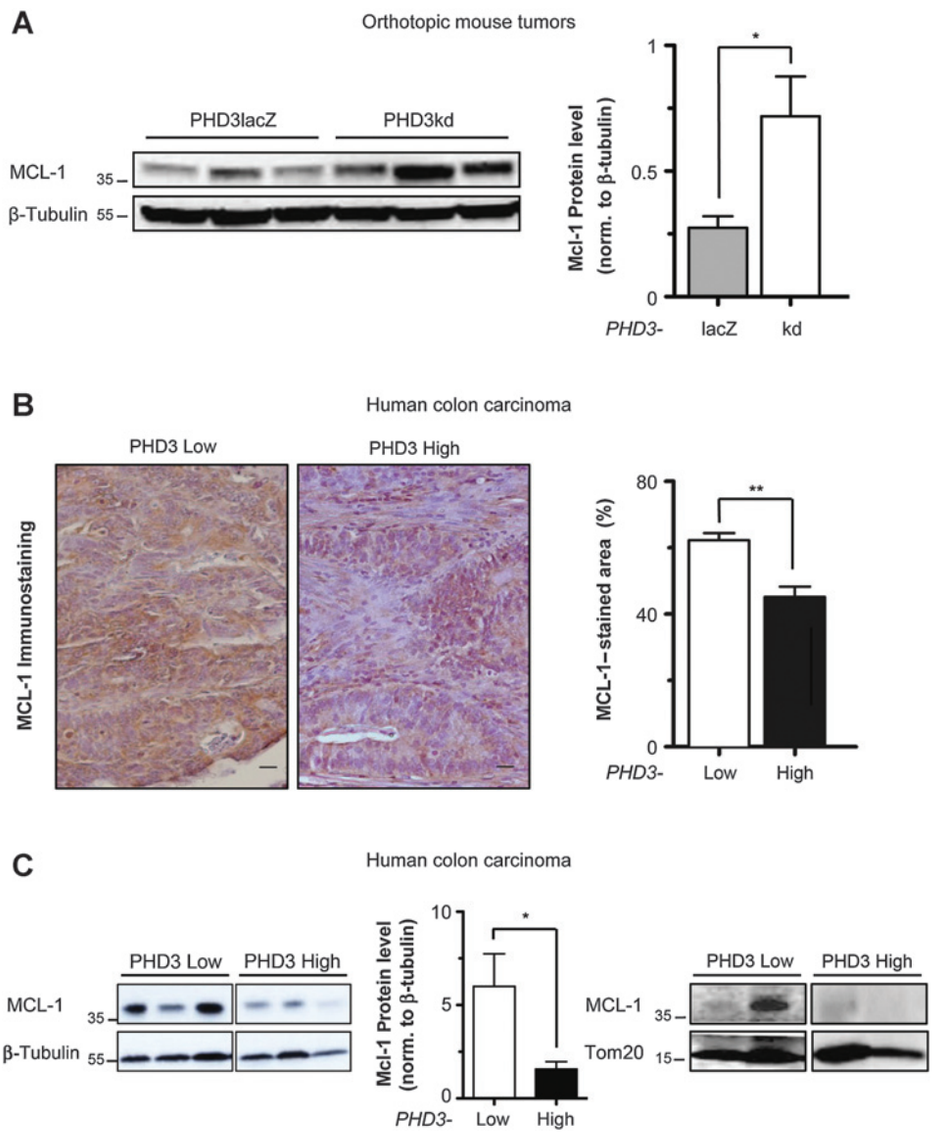

Figure 6.
MCL-1 expression in murine and human tumors underexpressing PHD3. A, immunoblot of tumor lysates (left) and semiquantitative analysis (right) revealing enhanced MCL-1 protein levels in tumors derived from orthotopic implantation of PHD3kd MC38 cells in syngeneic mice (lacZ, control-transfected tumors). $n = 6$; *, $P \leq 0.05$. B, representative IHC of human colon tumors (left) and histomorphometric quantification (right) revealing higher MCL-1 expression in tumors with decreased PHD3 expression (PHD3 low) than in those with enhanced PHD3 expression (PHD3 high). $n = 9$; **, $P \leq 0.01$. Scale bars, 50 μm. C, immunoblot of human colorectal tumor lysates (left) and semiquantitative analysis (middle) revealing enhanced MCL-1 expression in PHD3 low tumors (β-tubulin, loading control). $n = 9$; *, $P \leq 0.05$. Immunoblot of mitochondrial fractions isolated from human colon tumors revealing higher mitochondrial MCL-1 protein levels in PHD3 low tumors than in PHD3 high tumors (right). kd, knockdown.

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colorectal cancer cells *in vivo* and that this likewise occurs in human colorectal cancer.

Discussion

Here, we applied expressional studies in human colorectal tumor samples to demonstrate that underexpression of the oxygen sensor PHD3 in cancer cells is associated with enhanced metastatic frequency and impaired patient survival. This effect was specific for PHD3, as neither PHD1 nor PHD2 expression correlated with tumor invasiveness or metastatic spread, and further corroborated by functional genetic approaches, revealing that underexpression of PHD3 in tumor cells enhanced their migratory and invasive capacity.

The concept that PHD3 can suppress the growth of visceral tumors has been documented in previous studies (33, 34). In human colorectal cancer, in particular, downregulated PHD3 expression has been correlated with higher tumor grade and lymph node metastases (13). Our results extend and specify these insights by linking downregulated PHD3 expression in primary human colorectal cancer to increased detection of disseminated tumor cells in the bloodstream and to more frequent occurrence of distant metastases. Remarkably, we did not detect significant effects of tumoral PHD3 expression on local or lymphatic tumor spread. In this regard, our findings differ from those reported by Xue and colleagues, who found that underexpression of PHD3 was correlated with advanced primary tumor size (T-stage) and lymph node involvement (N-stage) in a collective of 60 Chinese colorectal cancer patients (13). This discrepancy might be explained by peculiarities of the investigated patient collectives: a significant proportion of patients analyzed in our study suffered cancer of the low rectum, which is associated with higher local recurrence rates due to its challenging anatomy and surgical treatment (35). Other differences might relate to distinct tumor biologic features within both collectives of patients, which may affect local tumor spread independently from tumoral expression of PHD3 and could be influenced by ethnic disparities (36, 37). Regardless of its impact on local and lymphatic tumor spread, our findings corroborate the notion that PHD3 acts as a tumor suppressor in human colorectal cancer. Furthermore, we were able to document, for the first time, that tumoral downregulation of PHD3 is associated with impaired survival in colorectal cancer patients.

Further analyses in tumor cells over and underexpressing PHD3 identified facilitated ATP production as a major mechanism, contributing to enhanced migration and infiltration of PHD3-deficient colorectal cancer cells. Indeed, the metastatic potential of tumor cells depends on cellular energy production (38, 39). Surprisingly, however, our results link improved energy efficiency in PHD3kd cells to facilitated mitochondrial energy production, rather than anaerobic glycolysis (Warburg effect), which can be initiated in tumor cells as a major effect mediated by HIF (a primary PHD3 target; ref. 23). Indeed, we found that enhanced ATP-production in PHD3-underexpressing cells could be reverted by the inhibition of mitochondrial respiration, but not anaerobic glycolysis. Albeit unexpected, this finding does not appear inconceivable; it has been recognized that cancer cells depend on functional mitochondria to maintain their biosynthetic capacity (39), and, even more, that epithelial cancer cells actively exploit oxidative mitochondrial metabolism to enhance their potential to migrate and to form distant metastases (38). It thus appears justified to conclude that downregulation of PHD3 facilitates the metastatic potential of tumor cells via improved mitochondrial integrity and performance.

Concerning the molecular mechanisms mediating improved mitochondrial energy production and enhanced invasion of PHD3-underexpressing tumor cells, we found that downregulation of PHD3 enhanced mitochondrial expression of MCL-1, a member of the BCL-2 family of antiapoptotic proteins, both in tumor cells *in vitro* and also in freshly isolated human colorectal cancer samples. MCL-1 is expressed in colorectal cancer (24). Like other BCL-2 family members, it exerts antiapoptotic effects by binding BCL-2 homology 3– (BH3) only proteins (26). MCL-1 is, however, distinct from other BCL-2 proteins in that it exerts crucial functions concerning the maintenance of normal mitochondrial physiology and energy metabolism. A truncated isoform of MCL-1 localizes to the mitochondrial matrix (28), where it supports oxidative phosphorylation and ATP production (26, 27). This unique function, which goes beyond the antiapoptotic activity of full-length MCL-1 protein, makes MCL-1 indispensable for the survival of cancer cells (26, 40). Furthermore, MCL-1 reportedly enhances the migratory and invasive potential of colorectal tumor cells, independently from its antiapoptotic effects (25, 31). Consistently, we observed that improved ATP production, as well as enhanced migration and invasion of PHD3-deficient tumor cells, could be reverted by simultaneous knockdown of MCL-1. Remarkably, we found that pharmacologic inhibition of MCL-1 with the BH3-mimetic GX15-070 likewise reverted facilitated ATP production of PHD3-underexpressing tumor cells. This might appear paradoxical as GX15-070 acts as a BH3-mimetic, targeting the antiapoptotic activity of BCL-2 proteins (29), and is thus not expected to affect the mitochondrial function of MCL-1. The efficacy of GX15-070 in reducing the ATP production of PHD3-underexpressing cells might however be explained by the fact that GX15-070 treatment downregulates the overall abundance of MCL-1 protein (41). Obviously, we do not want to rule out that GX15-070 affects ATP production in PHD3-underexpressing cells via mechanisms distinct from the effects of mitochondrial MCL-1 (42, 43). Taken together, our analyses identified MCL-1 as a novel molecular player, regulating tumor cell metabolism and invasion downstream PHD3. These findings link the oxygen sensor PHD3 to a current concept in tumor cell metabolism, namely, that mitochondria are crucial in regulating persistent migration and invasion of epithelial cancer cells (32), and, therefore, mechanistically involved in tumor metastasis (44).

It is tempting to speculate whether PHD3 can interact with MCL-1 directly or whether additional molecular mediators are involved. In view of current insights into the molecular targets of PHD3, the latter appears likely. For instance, activating transcription factor 4 (ATF4) has been identified as an HIF-independent interaction partner of PHD3 (45, 46), which is stabilized upon downregulation of PHD3 (46). ATF4 is not only capable of upregulating MCL-1 (47), but likewise regulates tumor cell metabolism and promotes tumor cell survival (48). Thus, although the precise mode of interaction between PHD3 and MCL-1 is beyond the scope of our study, it is conceivable that the effects of PHD3 downregulation on MCL-1 are at least partly mediated via ATF4 in a HIF-independent manner.

We do not wish to imply that the MCL-1–dependent effects described in this study represent an exclusive mechanism, facilitating metastatic spread of PHD3-underexpressing tumors. In fact, PHD3 reportedly exerts tumor-suppressive effects in various

tumor types and via various distinct mechanisms: in gastric cancer cells, it interferes with tumorigenicity via suppression of the β-catenin/T-cell factor signaling pathway (33). In colorectal cancer, it blunts tumor cell aggressiveness by inhibiting NF-κB signaling independently of its hydroxylase activity (13). In hypoxic brain tumors, it exerts growth-suppressive effects via attenuated signaling and function of the EGFR, independently of the established PHD3 targets HIF and NF-κB (14, 49). Collectively, these studies suggest that the tumor-suppressive effects of PHD3 rely on various molecular targets and pathways, including a number of non-HIF targets (50).

Regardless of the multifactorial nature of PHD3-mediated effects in tumor cells, this study uncovers an important link between PHD3 and the antiapoptotic protein, MCL-1, and corroborates the concept that PHD3 plays a central role in orchestrating molecular pathways affecting tumor cell migration and metastatic spread. These findings are of putative clinical relevance concerning the application of pharmacologic PHD inhibitors in clinical settings, especially in view of potentially deleterious side effects of pan-PHD inhibitors in patients suffering occult or dormant tumor disease.

Disclosure of Potential Conflicts of Interest

J.T. Opferman is a consultant/advisory board member for AbbVie. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: M. Schneider

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Prolyl Hydroxylase 3 Attenuates MCL-1–Mediated ATP Production to Suppress the Metastatic Potential of Colorectal Cancer Cells

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