PROLYL-4-HYDROXYLASE DOMAIN 3 (PHD3) IS A CRITICAL TERMINATOR FOR CELL SURVIVAL OF MACROPHAGES UNDER STRESS CONDITIONS

DOCTORAL THESIS

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Dedicated to my Father

Affidavit

I hereby declare that my doctoral thesis entitled “Prolyl-4-hydroxylase domain 3 (PHD3) is a critical terminator for cell survival of macrophages under stress conditions” has been written independently with no other sources and aids than quoted.

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Lija Swain

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Table of Contents

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List of figures

Abbreviations

°C	Degrees Celsius
2-OG	2-oxoglutarate
aa	Amino acid
AhR	Aryl hydrocarbon receptor
Angptl2	Angiopoietin-like protein 2
APS	Ammonium persulfate
ARNT	Aryl hydrocarbon receptor nuclear translocator
Asn	Asparagine
ATF	Activating transcription factor
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
bHLH	Basic helix-loop-helix
BMDM	Bone marrow derived macrophages
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CO_2	Carbondioxide
C-TAD	C-terminal transactivation domain
DAPI	4',6-diamidino-2-phenylindole
dd	Double distilled
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-strand DNA
ECL	Enhanced luminol-based Chemiluminescent
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGLN	Egg laying defective nine
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum

Fig	Figure
FIH	Factor inhibiting HIF
Fizz	Found in inflammatory zone
g	gram
GLUT	Glucose transporter
HCLK2	Human homologue of the *Caenorhabditis elegans* biological clock protein CLK-2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HRE	Hypoxia response element
HRP	Horse radish peroxidase
hrs	Hours
IFNγ	Interferon γ
IGFs	Insulin-like growth factors
IgG	Immunoglobulin G
IL	Interleukin
iNos	Inducible nitric oxide synthase
KCl	Potassium chloride
kDa	Kilo Dalton
KIF1β	Kinesin-like protein β
K_M	Michaelis-Menten constant
LILERB2	Leukocyte immunoglobin-like receptor B2
LPS	Lipopolysaccharide
LysM	Lysozyme M
LZIP	Leucine zipper domain
M-CSF	Macrophage colony-stimulating factor
min	Minutes
ml	millilitre
mM	millimolar
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MS12	Minisatellites 12
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
N-TAD	N-terminal transactivation domain

ODD	Oxygen-dependent degradation domain
PAGE	Polyacrylamide gel electrophoresis
PAS domain	Per-Arnt-Sim domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK-1	Pyruvate dehydrogenase kinase-1
PFA	Paraformaldehyde
Pfk	Phosphofructokinase
PHD	Prolyl-4-hydroxylase domain enzyme
PI	Propidium Iodide
PirB	Paired immunoglobin-like receptor B
PKM2	Pyruvate kinase M2
pVHL	von Hippel-Lindau tumor suppressor protein
qRT-PCR	Quantitative real-time PCR
RNase	Ribonuclease
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SNAP	S-nitroso-N-acetylpenicillamine
Stauro	Staurosporine
SYBR	SYBR green
TAE	Tris acetate EDTA
TCA	Tricarboxylic acid cycle
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
T_m	Melting temperature
TMB	3,3’,5,5’-Tetramethylbenzidine
TNFα	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
V	Volt
VEGF	Vascular endothelial growth factor
wt	Wild type

Summary

On molecular level, cells sense changes in oxygen availability through the prolyl-4-hydroxylase domain enzymes (PHDs), which in turn regulate the protein stability of the α-subunit of the transcription factor hypoxia-inducible factor (HIF). By using molecular oxygen PHDs 1 - 3 hydroxylate two specific proline residues thereby marking HIFα for proteasomal degradation. Among the three PHD isoforms the constitutively expressed PHD2 is the main regulator of HIFα stability and thus hypoxia-inducible gene expression in normoxia. PHD3 is highly induced under hypoxic conditions and serves as a negative feedback regulator. Especially PHD3 has been additionally associated with apoptotic cell death. I hypothesized that PHD3 plays a role in cell-fate decisions in macrophages. Therefore, myeloid-specific PHD3 knockout mice ($PHD3^{-/-}$) were created and analyzed. PHD3-deficient bone marrow-derived macrophages (BMDM) showed no altered HIF-1α or HIF-2α stabilization or increased HIF target gene expression in normoxia or hypoxia. Macrophage M1 and M2-polarization was unchanged likewise. Compared to macrophages from wild type littermates $PHD3^{-/-}$ exhibited a significant reduction in TUNEL positive cells after serum withdrawal. Under the same conditions the $PHD3^{-/-}$ macrophages also showed less Annexin V staining which detects the membrane disruption indicating a reduced early apoptosis. Application of apoptotic inducers such as S-nitroso-N-acetyl penicillamine (SNAP) or staurosporine also showed $PHD3^{-/-}$ cells to be less apoptotic. Additionally, calcein uptake results indicate that $PHD3^{-/-}$ macrophages are more viable. When the supernatant (medium) from the cultured macrophages was exchanged between the genotypes after 24 hrs of culture, then $PHD3^{-/-}$ macrophages showed more Annexin V staining suggesting that at least in part a secreted factor is involved in the PHD3 induced apoptosis mechanism. In an unbiased transcriptome screen the expression of a secretory glycoprotein angiopoietin-like protein 2 (Angptl2) expressions was found to be reduced in $PHD3^{-/-}$ BMDM under stress conditions. Addition of recombinant Angptl2 rescued the anti-apoptotic phenotype demonstrating that it is involved in the PHD3-mediated response towards apoptotic stimuli in macrophages. Additionally $Angptl2^{-/-}$ BMDM showed decreased apoptosis compared to wild type which support the lower expression of Angptl2 in $PHD3^{-/-}$ BMDM followed by decreased apoptosis. My data suggests that the anti-apoptotic effects in the $PHD3^{-/-}$ BMDMs are at least partially mediated by an altered production and response to Angptl2.

1. Introduction

1.1 Hypoxia

Molecular oxygen (O_2) is essential for life; it plays an important role in human physiology. Subsequently, oxygen is indispensable for the molecular metabolism and adequate oxygen supply is the prerequisite for the appropriate functionality of each cell, tissue and organ (Carreau *et al.*, 2011). Oxygen is available at the molecular level to tissues and cells by diffusion. The oxygenation in different tissues varies between 30 – 50 mmHg which is comparable to 4 – 7% of the atmospheric pressure at sea level (Almendros *et al.*, 2010; Qiao *et al.*, 2007). Insufficient oxygen availability to tissues and cells is termed as hypoxia which can be caused by both physiological and pathophysiological reasons. A physiological condition that may lead to an increase of oxygen consumption and thus leads to hypoxia even at sea level, is for example heavy exercise (Garvey *et al.*, 2012). In contrast to this, a pathophysiological condition, e.g. differentiation of stem cells inside the stromal niche during infection leads to inadequate supply of oxygen causing hypoxia which needs cellular adaptation to overcome the hypoxic effects (Carreau *et al.*, 2011; Dunwoodie, 2009; Lee *et al.*, 2001). Also a ventilation/perfusion mismatch resulting from progressive airflow limitation and emphysema can cause hypoxia leading to chronic obstructive pulmonary disease (Kent *et al.*, 2011). Pathological hypoxia can be caused by local ischemia due to the loss of blood supply or in massive tumors because of impaired vascularization (Acker and Plate, 2003). Since oxygen is essential for the functioning of each and every tissue and organ the cell has regulatory mechanisms to adapt to the changes in oxygen availability. The key regulators of this response are the hypoxia-inducible transcription factors (HIFs) which activate oxygen-dependent gene expression (Fong, 2009).

1.2 Hypoxia-inducible factors

Hypoxia-inducible factors (HIFs) are transcription factors which accumulate under low oxygen concentrations and regulate the expression of hypoxia-inducible genes. HIFs are the principal mediators of homeostasis of cells and tissues experiencing hypoxia (Semenza, 2004). They regulate more than thousand target genes including those promoting angiogenesis, cell differentiation, anaerobic metabolism,

and apoptosis (Semenza, 2012). HIFs are heterodimeric transcription factors consisting of an α-subunit and a HIF-1β-subunit, which is also known as aryl hydrocarbon receptor nuclear translocator (ARNT). Three major HIFα family members are known: HIF-1α, HIF-2α and HIF-3α. Both subunits, α and β are composed of basic helix-loop-helix proteins (bHLH) of the Per-Arnt-Sim (PAS) family. HIF-1β forms a complex together with HIF-1α, HIF-2α and HIF-3α (Wang *et al.*, 1995; Makino *et al.*, 2002), these dimers bind to a DNA motif in the hypoxia response elements (HREs) that leads to the transcriptional activation (Wang *et al.*, 1995; Wenger, 2002). HIF-1β mRNA and protein levels are unaltered with changes in oxygen availability. In contrast, in the presence of oxygen the HIFα subunits undergo the process of hydroxylation, polyubiquitination and proteasomal degradation, resulting in a half life of HIFα of about 5 min (Salceda and Caro, 1997). Low oxygen concentration leads to an insufficient hydroxylation and thereby to a HIFα subunit stabilization (Huang *et al.*, 1998; Salceda and Caro, 1997; Roy *et al.*, 2004). HIF-1 drives the initial response to hypoxia whereas HIF-2 plays an important role in chronic hypoxic exposure (Koh *et al.*, 2011). HIF-3 acts as a negative regulator of HIF-1 and HIF-2 (Makino *et al.*, 2002; Yamashita *et al.*, 2008).

1.2.1 HIF-1α

The human HIF-1α protein is constitutively and ubiquitously expressed and has a molecular weight of around 120 kDa (Wang and Semenza, 1993). It is a member of the basic helix-loop-helix (bHLH) PAS protein family. HIF-1α contains two transactivation domains; the N-terminal (N-TAD) and the C-terminal (C-TAD) and also contains an oxygen-dependent degradation domain (ODD) that regulates oxygen dependent stability (Pugh et al., 1997). The C-TAD interacts with co-activators like CBP/p300 to activate transcription. When N803 present in the C-TAD is hydroxylated it blocks the recruitment of transcriptional coactivator CBP/p300 (Ruas et al., 2002; Lando et al., 2002). The ODD domain contains two conserved proline residues, P402 and P564 that are hydroxylated by prolyl-4-hydroxylase domain enzymes (PHDs) and one lysine residue, K532 which is acetylated by acetyltransferase named arrest-defective-1 in an oxygen-dependent manner (Ke and Costa, 2006; Koh *et al.*, 2011) (Fig. 1). The stability and activity of HIF-1α are regulated by post-translational modifications such as hydroxylation, acetylation and ubiquitination (Kallio *et al.*, 1999; Wang *et al.*, 1995). Under normoxia, hydroxylation of two proline

residues (P402 and P564), one asparagine residue (N803) and acetylation of a lysine residue (K532) triggers HIF-1α for poly-ubiquitination leading to degradation by the proteasome pathway (Ke and Costa, 2006; Koh *et al.*, 2011; Mahon *et al.*, 2001). HIF-1α is stabilized and active during short periods (2 – 24 hrs) of intense hypoxia or anoxia, subsequently negative feedback regulators lead to a decrease in the protein level.

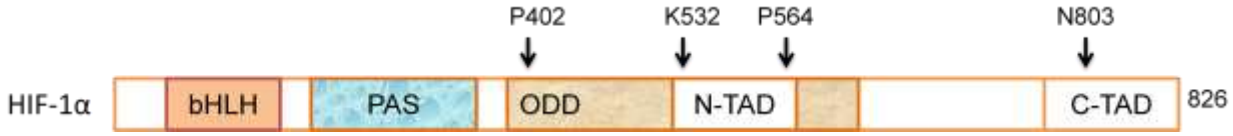


Figure 1: Domain structure of human HIF-1α

The protein contains two transactivation domains (C-TAD and N-TAD). HIF-1α contains an ODD that mediates oxygen-regulated stability through the hydroxylation of two proline (P402 and P562) residues, an asparagines residue (N803) and the acetylation of a lysine (K 532).

1.2.2 HIF-2α

HIF-2α has 48% of amino acid sequence and structure similarity to HIF-1α, but they have different target genes and mechanism of regulation. It is predominantly expressed in lung, epithelium and carotid body (Ema *et al.*, 1997). HIF-2α also contains transactivation domains at N-terminal (N-TAD) and C-terminal (C-TAD) in the C-terminal half (Koh and Powis, 2012). The open reading frame of HIF-2α encodes 870 aa with a molecular weight of the protein of 118 kDa (Fig. 2). HIF-2α is hydroxylated at much lower efficiency than HIF-1α (Koivunen *et al.*, 2004) and therefore senses the chronic hypoxia exposure (Koh *et al.*, 2011). In human HIF-2α is hydroxylated at the two conserved proline residues P405 and P531 (Koh *et al.*, 2011) and the asparagine residue N 851 in the C-TAD domain is hydroxylated (Mahon *et al.*, 2001)(Fig. 2).

Figure 2: Domain structure of human HIF-2α

The protein contains two transactivation domains (C-TAD and N-TAD) and an oxygen-dependent degradation domain (ODD) containing conserved proline residue sites (P405 and P531) and asparagines site (N803).

1.2.3 HIF-3α

HIF-3α is not as widely studied as HIF-1α and HIF-2α, the role of HIF-3α under hypoxia and its mode of action is far more unclear. The HIF-3α open reading frame encodes 626 aa resulting in a protein with a molecular weight of 73 kDa which is predominately expressed in adult thymus, lung, brain, heart, and kidney. It is the shortest HIFα-isoform composed of the bHLH and PAS domains but lacking the carboxy-terminal domain (Gu *et al.*, 1998), having only one TAD motif. It has a unique leucine zipper domain (LZIP) (Gu *et al.*, 1998) (Fig. 3). Multiple splice variants of HIF-3α exist. The most studied variant is the inhibitory PAS domain protein (IPAS) (Makino *et al.*, 2002). Under normoxia HIF-3α is degraded by hydroxylation in an oxygen dependent manner (Zhang *et al.*, 2014) but in hypoxia it can inhibit the action of HIF-1α and HIF-2α by acting as a competitor against HIF-1α or HIF-2α by binding to Arnt or forming a dimer with HIF-1α itself (Makino *et al.*, 2002; Yamashita *et al.*, 2008; Hara *et al.*, 2001).

Figure 3: Domain structure of human HIF-3α

HIF-3α contains besides the basic helix loop helix (bHLH) and Per-Arnt-Sim (PAS) domains only one transactivation domain (N-TAD), an oxygen dependent domain (ODD) and a leucine zipper domain (LZIP).

1.2.4 HIF-1β

HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator (Arnt), belongs to the bHLH protein family containing a PAS domain but it lacks the amino terminal N-TAD domain (Fig.4). HIF-1β consists of 789 aa with a molecular weight of 86 kDa and is constitutively expressed irrespectively of oxygen availability and is stable under normoxic conditions. HIF-1β forms heterodimers with many bHLH-PAS family proteins such as aryl hydrocarbon receptor (AhR) and HIF-1α, HIF-2α and HIF-3α. (Wang *et al.*, 2006; Kallio *et al.*, 1997).

Figure 4: Domain structure of human HIF-1β

HIF-1β contains besides the basic helix loop helix (bHLH) and Per-Arnt-Sim (PAS) domains one C-terminal transactivation domain (C-TAD).

1.3 Regulation of HIFα

The protein stability of HIFα is regulated by the prolyl-4-hydroxylase domain enzymes (PHDs) by hydroxylation at specific proline residues, followed by ubiquitination and proteasomal degradation (Jaakkola *et al.*, 2001). Additionally, HIF-1α and HIF-2α are also regulated by asparaginyl hydroxylation leading to transcriptional inactivation by factor inhibiting HIF (FIH) (Lando *et al.*, 2002). Both hydroxylase domain enzymes, PHDs and FIH, require oxygen, 2-oxoglutarate, ascorbate and iron (Fe^{2+}) as co-factors (Jaakkola *et al.*, 2001; Lando *et al.*, 2002) (Fig. 5).

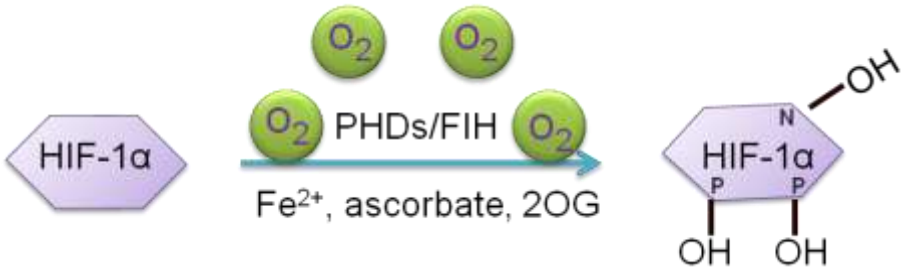


Figure 5: Hydroxylation of HIF-1α

Under the consumption of oxygen, hypoxia-inducible factor (HIF)-1α is hydroxylated at specific prolyl residues/asparagine residues by prolyl hydroxylase domain enzymes (PHDs)/factor inhibiting HIF (FIH) in the presence of Fe^{2+} 2-oxoglutarate (2-OG) and ascorbate.

HIFα is hydroxylated by PHDs at two specific proline residues situated in the ODD domain which promotes interaction/binding of HIFα with the von Hippel-Lindau protein (pVHL) to the ODD domain of HIF. Binding of pVHL to the ODD forms a substrate recognition module of an E3 ubiquitin ligase complex

which facilitates the polyubiqutination of HIFα thereby marking it for degradation by the 26S proteasome (Kallio *et al.*, 1999; Ohh *et al.*, 2000). Under hypoxic conditions, because of the unavailability of oxygen as co-factor, the PHDs are inhibited from hydroxylating HIFα, which in turn inhibits the binding of pVHL hence leading to the stabilization of HIFα. Accumulated HIFα is then translocated into the nucleus where it heterodimerizes with HIF-1β. The complex is able to activate the expression of hypoxia target genes by binding to cis-acting HRE located in the enhancer or promoter regions (Epstein *et al.*, 2001; Jaakkola *et al.*, 2001; Pugh and Ratcliffe, 2003). Hence, stabilization of HIFα affects various pathways in the cell for example angiogenesis, cell proliferation, metabolism, and apoptosis (Fig. 6). A high number of genes related to angiogenesis are upregulated under hypoxia. Vascular endothelial cell growth factor (VEGF) is one among them which recruits endothelial cells to the site of hypoxia and induces proliferation. The induction of VEGF in hypoxia is a sign of pro-angiogenesis and vessel formation (Josko *et al.*, 2000). Also genes such as matrix metalloproteinases (MMPs) involved in matrix metabolism and vessel maturation are induced in hypoxia. Cells adapt to hypoxia by switching the metabolism from oxygen-dependent tricarboxylic acid cycle (TCA) cycle to oxygen independent glycolysis. To meet the required ATP synthesis the cells increase glucose uptake. Therefore, under hypoxia cells induce the expression of glycolytic enzymes such as pyruvate kinase M (PKM), Phosphofructokinase (Pfk), Hexokinase, and glucose transporters such as Glut 1 and Glut 3, which are indeed HIF target genes (Wenger, 2002; Mora *et al.*, 2005; Chen *et al.*, 2003). HIF also induces the expression of growth factors such as adrenomedullin, insulin like growth factor 2 and transforming growth factor-α. Hifα stabilization also leads to the induction of apoptosis related genes such as BNip3, Bcl-2 and caspase-3 (Flamant *et al.*, 2010; Webster *et al.*, 2005; Regula, 2002).

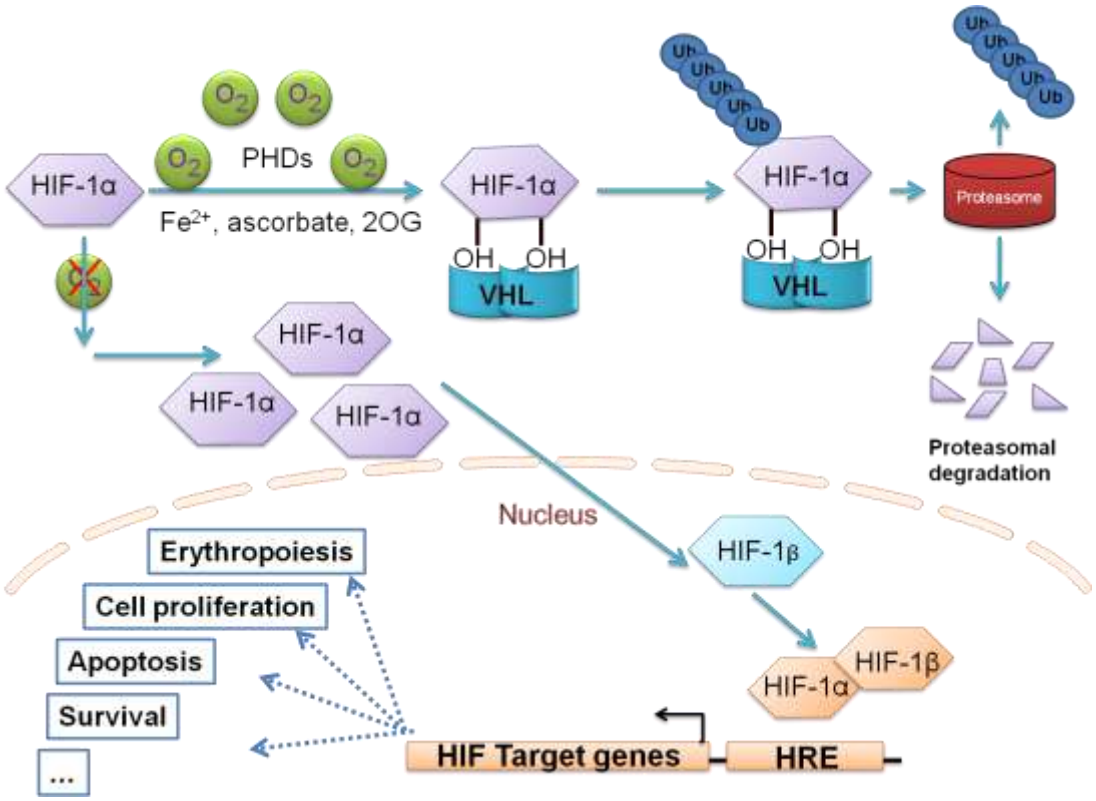


Figure 6: **HIF-1α regulation by PHDs**

In normoxia, hypoxia inducible factor (HIF)-1α is hydroxylated by prolyl hydroxylase domain enzymes (PHDs) in the presence of O_2, Fe^{2+}, 2-oxoglutarate (2-OG) and ascorbate. Hydroxylated HIF-1α is recognised by the von Hippel-Lindau (pVHL) protein, which marks HIF-1α with polyubiquitin, this leads to the recognition for proteasomal degradation. Under hypoxia, proline hydroxylation is inhibited, VHL is no longer able to bind and target HIF-1α for proteasomal degradation, which leads to HIF-1α accumulation and translocation to the nucleus. HIF-1α dimerises with HIF-1β, binds to hypoxia-response elements (HREs) within the promoters or enhancers of target genes and induces transcriptional activity. A range of cell functions are regulated by the target genes, such as cell proliferation, apoptosis, survival and erythropoiesis.

The second major mechanism controlling HIFα regulation is done by another oxygen regulated enzyme, FIH. FIH hydroxylates HIF at asparagines within the C-TAD domain. This hydroxylation inhibits the interaction with the transcription co-activators p300/CREB- and hence inhibits transcriptional activation of all target genes. Like PHDs, FIH also requires oxygen, 2-oxoglutarate, ascorbate and iron (Fe^{2+}) as co-factors (Lando *et al.*, 2002; Mahon *et al.*, 2001).

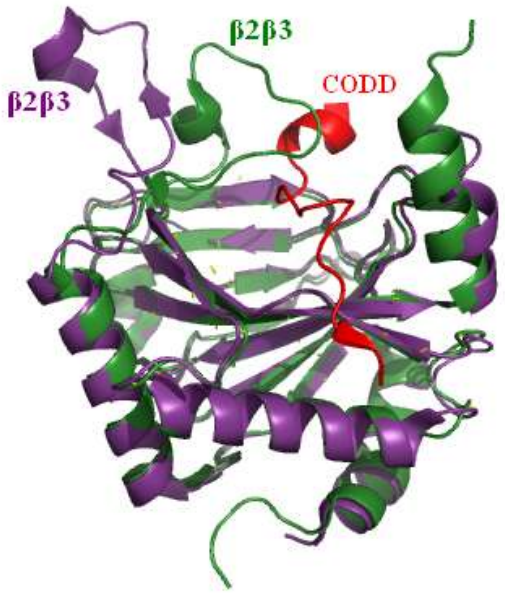
1.4 Prolyl-4-hydroxylase Domain Enzyme (PHD)

PHDs are enzymes that use di-oxygen to hydroxylate their substrates and simultaneously convert 2-oxoglutarate (2-OG) into succinate and release CO_2 as byproduct (McDonough *et al.*, 2006; Elkins *et al.*, 2003). During hydroxylation, PHDs use the oxygen molecule, dissolve the covalent bond and split it into two, one oxygen atom hydroxylates the proline residue and the other reacts with 2-OG to generate succinate and CO_2 (Bruick and McKnight, 2001; Masson and Ratcliffe, 2003). All the co-factors are essential for the hydroxylation activity of PHDs. For example, inhibition of the PHDs by 2-OG analogs can increase the half life of HIFα (Jaakkola *et al.*, 2001). Fe^{2+} at the active site of the PHDs is bound to two histidine residues and one aspartic acid forming a 2-histidine-1-carboxylate coordination motif. Ascorbate helps to maintain iron in the ferrous (Fe^{2+}) state which is important to achieve the full enzyme activity (Epstein *et al.*, 2001; Bruick and McKnight, 2001). The apparent K_M value for all the PHD enzymes is in between 230-250 µM which exceeds even the concentration of oxygen in air at sea level making these enzymes suitable oxygen sensors (Hirsila *et al.*, 2003). In *Drosophila melanogaster* and *Caenorhabditis elegans* similar to PHDs a single family member is present which is known as Fatiga and egg laying defective nine (EGLN) respectively (Epstein *et al.*, 2001; Centanin *et al.*, 2005). In mammals, there are four members that belong to the PHD protein family, PHD1 also known as EGLN2, PHD2 also known as EGLN1, PHD3 also known as EGLN3 and the recently discovered P4HTM (prolyl 4-hydroxylase, transmembrane). In addition to being oxygen sensors, PHDs are also known to be involved in the hydroxylation of non-HIF substrates and also in hydroxylase independent functions (for examples please see table 1.2) (Cummins *et al.*, 2006; Takeda and Fong, 2007; Ozer *et al.*, 2005).

All PHDs can hydroxylate HIFα *in-vitro*; however there are differences in the hydroxylation potential, PHD2 has the highest potential followed by PHD3, and PHD1. PHD2 is the most important enzyme for hydroxylating HIFα in normoxia. (Huang *et al.*, 2002). Under hypoxia, mRNA and protein expression of PHD2 is mildly induced, expression of PHD3 is strongly induced under hypoxia whereas expression of PHD1 remains unaffected (Epstein *et al.*, 2001; Metzen *et al.*, 2003). PHD2 is mostly localized in the cytoplasm, PHD1 is localized in the nucleus and PHD3 is localized both in cytoplasm and nucleus. PHD2 shuttles between the cytoplasm and the nucleus for degradation of HIFα in both compartments (Metzen *et*

al., 2003). All enzymes are expressed widely in many tissues but they exhibit tissue specific overexpression. PHD1 is abundant in the testis, PHD2 in skeletal muscles and adipose tissue, PHD3 in the heart and placenta (Lieb *et al.*, 2002).

The structure of PHDs consists of two domains: the variable N-terminal domain (≈21–58 aa) has homology to MYND zinc finger domains and the conserved catalytic C-terminal domain (≈181–426 aa) has homology to other 2-OG dioxygenases. The catalytic core domain of PHDs consists of eight β-strands/double stranded β-helices supported by three conserved α-helices and other β-strands and loops that pack along the core (McDonough *et al.*, 2006)(Fig. 7).



Adapted from (McDonough *et al.*, 2006)

Figure 7: Crystal structure of PHD2

PHD2 consists of a C-terminal domain and a N-terminal domain formed by eight β-strands in a "jelly-role"or double stranded β helix supported by three conserved α-helices and other β-strands and loops that pack along the core. This core contains the three Fe^{2+} binding ligands.

1.4.1 PHD1

PHD1 also named as EGLN2, is constitutively expressed but predominantly expressed in testis and localized in the nucleus. Full length human PHD1 consists of 407 aa and has a molecular weight of around 43 kDa. Resulting from two alternative translational initiations, PHD1 has two isoforms with molecular weights of 40 and 43 kDa. (Epstein *et al.*, 2001; Lieb *et al.*, 2002). PHD1 has been shown to be involved in the mitochondrial energy metabolism (Aragones *et al.*, 2008). Loss of PHD1 decreases oxidative stress in liver cell metabolism resulting in increased hypoxic survival (Schneider *et al.*, 2010) and increases the intestinal barrier function and thus is protective against colitis (Tambuwala *et al.*, 2010).

1.4.2 PHD2

PHD2 also known as EGLN1, consists of 426 aa and has a molecular weight of 46 kDa and is highly expressed in skeletal muscles and adipose tissue. PHD2 is the most important enzyme to hydroxylate HIFα in normoxia (Huang *et al.*, 2002). Therefore human hereditary mutations of PHD2 results in a decreased enzyme activity and are associated with familial erythrocytosis, one such mutation is P317R (Percy *et al.*, 2006). The complete knockout of PHD2 is lethal (Takeda *et al.*, 2006). PHD2 has a higher specificity towards HIF-1α compared to HIF-2α and is transcriptionally regulated by HIF. Therefore it is mildly induced under hypoxia. PHD2 is known to play a major physiological role during placental and cardiac development (Takeda *et al.*, 2006). In mice haplodeficiency of PHD2 can normalize the vasculature of expanding tumors, leading to improved tumor oxygenation and delay in metastasis (Mazzone *et al.*, 2009).

1.4.3 PHD3

Human PHD3 also known as EGLN3 is a 239 aa long protein with a molecular weight of 27 kDa and is mostly expressed in the heart and placenta. PHD3 protein is transcriptionally upregulated by HIF (Pescador *et al.*, 2005) and is strongly hypoxia inducible (Appelhoff *et al.*, 2004). The specific role of PHD3 upregulation under hypoxia is not clear. However, studies suggest that the hypoxic induction of PHD3 may play a predominant role in regulating HIFα via a feedback loop (Appelhoff *et al.*, 2004). It has

already been demonstrated that acute induction of hypoxia leads to a transient rise in HIF-1α protein levels, following a brief time period these levels subsequently return back to baseline despite continued hypoxia. This might have resulted from the hypoxia-induced PHD3 expression, which enables cells to effectively increase HIF-1α hydroxylation even under hypoxia.

PHD3 has been reported to be involved in the development of the sympatho-adrenal system (Bishop *et al.*, 2008) and induces apoptosis in cultured rat sympathetic neurons by withdrawal of nerve growth factor (Lipscomb *et al.*, 1999). Also it has been shown to induce apoptotic cell death in neurons and pheochromocytoma cell line (PC12) cells (Lipscomb *et al.*, 2001; Lee *et al.*, 2005). In relation to myeloid cells, PHD3 has been shown to regulate the life span of neutrophils under hypoxia (Walmsley *et al.*, 2011). In macrophages, loss of PHD3 enhances the innate immune response by accumulating pro-inflammatory macrophage function (Kiss *et al.*, 2012). Other than myeloid cells, PHD3 negatively regulates the NFκB pathway (Fu and Taubman, 2013; Xue *et al.*, 2010). Upregulation of PHD3 is associated with p53 induced growth arrest and apoptosis in Rat sarcoma (RAS)-transformed embryo fibroblasts (Madden *et al.*, 1996). PHD3 stimulates pyruvate kinase M2 co-activation of HIF-1 (Luo *et al.*, 2011) and it regulates Bcl-2 mediated apoptosis in H9c2 cells (Liu *et al.*, 2010). PHD3 targets and interacting proteins are listed below in the table 1.1.

Table 1.1 adapted from (Place and Domann, 2013)

Targets	Function	Interaction	Reference
HIFα	Degradation	Hydroxylation	(Elkins *et al.*, 2003; Appelhoff *et al.*, 2004)
ATF-4	Regulator of transcription factor	Binding	(Wottawa *et al.*, 2010; Koditz *et al.*, 2007)
PKM2	HIF-1 activation	Hydroxylation	(Luo, Hu et al. 2011)
Siah2	Degradation of PHD3	Binding	(Nakayama *et al.*, 2009)

Bcl-2	Reduce formation of Bax-Bcl-2 complex	Binding	(Liu *et al.*, 2010)
hCLK2	DNA damage	Hydroxylation	(Xie *et al.*, 2012)
IKKβ	Negative regulator of NFκB	Binding	(Xue *et al.*, 2010)
Kinesin like protein1Bβ	Induction of apoptosis	Hydroxylation	(Schlisio *et al.*, 2008)

1.4.3.1 Known role of PHD3 in pro-survival effect

PHD3 has been shown in many studies to have a pro-survival role by affecting apoptotic pathways under hypoxic conditions in several cell types. For example, PHD3 has also been known to play a role in cell viability under hypoxic conditions in tumor samples where its upregulation correlates with increased aggressiveness in cancers (Chen *et al.*, 2011b), also in head and neck carcinoma cells PHD3 appears to promote cell cycle progression from G1-S phase (Hogel *et al.*, 2011). PHD3 has been shown to increase the life span of neutrophils under hypoxia (Walmsley *et al.*, 2011). One mechanism, known for this increased survival is the effect of PHD3 on cell metabolism. Both PHD3 and PKM2 have been shown to complex with nuclear HIF-1α, inducing transcription of genes involved in glycolysis (Luo *et al.*, 2011). This leads to a higher production of ATP through the glycolytic pathway during hypoxia. A similar study showed an interaction between PKM2 and PHD3 in the cytosol, where it regulates flux of carbon through glycolysis (Chen *et al.*, 2011a). Thus, under hypoxia PHD3 may promote cell survival by modulating metabolic genes.

1.4.3.2 Known role of PHD3 in pro-apoptotic effect

In spite of several studies pointing towards the role of PHD3 in cell survival, evidence for the role of PHD3 in inducing apoptosis is pre-dominant in the literature. It has been shown in neurons during sympathoadrenal development that loss of PHD3 resulted in a decrease of apoptotic cells (Bishop *et al.*, 2008). Withdrawal of nerve growth factor from cultures of neuronal-derived cell lines leads to upregulation

of PHD3 (Lee *et al.*, 2005; Lipscomb *et al.*, 1999). Another similar study shows that, nerve growth factor withdrawal from a rat pheochromocytoma cell line resulted in c-jun mediated PHD3 upregulation inducing cell death (Straub *et al.*, 2003). Kinesin KIF1Bβ has been shown to act downstream of PHD3 to mediate pro-apoptotic effects (Schlisio *et al.*, 2008). Apart from neurons, also in muscle cells PHD3 has been shown to interact with Bcl-2, inhibiting the formation of the Bax-Bcl-2 complex which finally results in cell apoptosis (Liu *et al.*, 2010). Further, PHD3 hydroxylates human Biological Clock Protein (hCLK2) and promotes apoptosis through activation of the p53 mediated DNA damage response pathway (Xie *et al.*, 2012). In a cervical cancer cell line it has been shown that PHD3 localizes into large punctuated cytoplasmic aggregates followed by apoptosis (Rantanen *et al.*, 2008).

1.5 PHDs targets other than HIFs

PHDs can regulate other downstream targets than HIFs although it is not clear if it involves direct hydroxylation by the involved PHDs or not. For example PHD1 and PHD3 inhibit the IkB kinase (IKK) in the NFκB pathway (Xue *et al.*, 2010). Another role of PHD3 is the hydroxylation of pyruvate kinase isoform PKM2 which enhances the PKM2 binding to HIF-1α (Luo *et al.*, 2011). PHD2 may directly interact with Phosphodiesterase 4D to function as a regulator of the intracellular cAMP levels in cardiomyocytes (Huo *et al.*, 2012). A recent study showed that PHD3 regulates the ion channel TRPA1 by hydroxylation (Takahashi *et al.*, 2011). Table1.1 below summarizes all the targets and binding partners of the different PHDs known so far.

Table1.2 adapted from (Wong *et al.*, 2013)

Target	Abbreviation	PHD Isoform	Interaction	Reference
Inhibitor of kappaB kinase β	IKKβ	PHD1/2/3	Hydroxylation	(Fu and Taubman, 2010; Cummins *et al.*, 2006)
Pyruvate kinase muscle factor2	PKM2	PHD2	Binding	(Luo *et al.*, 2011)
LIN-10	LIN-10	Egl-9E	Binding/Hydroxylation	(Park *et al.*, 2012)

Transient receptor potential cation channel, member A1	TRPA1	PHD1/3	Hydroxylation	(Takahashi *et al.*, 2011)
FK506 binding protein 38	FKBP38	PHD2	Binding	(Barth *et al.*, 2009)
Seven in absentia homolog 2	Siah2	PHD3	Binding	(Nakayama *et al.*, 2007)
Activation transcription factor 4	ATF4	PHD1/3	Binding	(Koditz *et al.*, 2007; Hiwatashi *et al.*, 2011)
Human precursor RNA processing 19	hPRP19	PHD3	Binding	(Sato *et al.*, 2010)
Paired box gene 2	Pax2	PHD3	Hydroxylation	(Yan *et al.*, 2011)
Sprouty homolog 2	Spry2	PHD1/2/3	Hydroxylation	(Anderson *et al.*, 2011)Anderson, Nordquist et al., 2011)
TCP-1 ring complex	TRiC	PHD3	Binding	(Masson *et al.*, 2004)
Mitogen-activated protein kinase organizer 1	Morg1	PHD3	Binding	(Hopfer *et al.*, 2006)
Inhibitor of growth protein 4	ING4	PHD2	Binding	(Ozer *et al.*, 2005)
Iron-only hydrogenase-like protein1	IOP1	PHD2	Binding	(Huang *et al.*, 2007)
Cerebellar degeneration related protein 2	Cdr2	PHD1	Binding	(Anderson *et al.*, 2011; Balamurugan *et al.*, 2009)
Myogenin	Myogenin	PHD3	Hydroxylation	(Fu *et al.*, 2007)

Kinesin-like protein1B β	KIF1Bβ	PHD3	Hydroxylation	(Schlisio *et al.*, 2008)
Large subunit of RNA polymerase II	Rbp1	PHD1/2	Hydroxylation	(Mikhaylova *et al.*, 2008)

1.6 Hypoxia and myeloid cells

Myeloid cells are comprised of cells having common descendants from progenitors derived from hematopoietic stem cells in the bone marrow. Granulocytes and monocytes together are called myeloid cells. These are short living immune cells that respond and move to any change in tissue integrity or microbial invasion. They move to the affected tissue and phagocytose the microorganisms and damaged tissue debris or release antimicrobial molecules and pro-inflammatory factors. The role of macrophages and neutrophils in the regulation of microbial infection and inflammation is the central mechanism of innate immunity. In particular neutrophil apoptosis is important for the onset of granulocytic inflammation. HIF-1 is known to prolong the neutrophil viability by inhibiting apoptosis under hypoxia (Walmsley *et al.*, 2005) and in von Hippel-Lindau disease having mutation in one VHL allele, neutrophils showed reduced apoptosis and induced phagocytosis of bacteria (Walmsley *et al.*, 2008). In myeloid cells, HIF-1α knockout shows less efficient phagocytosis in eliminating bacteria during infection (Peyssonnaux *et al.*, 2005). HIF-dependent functions of myeloid cells in oxygen depleted areas are well known (Cramer *et al.*, 2003).

1.7.1 Hypoxia and macrophages

Macrophages are highly elastic immune cells which are derived from bone marrow progenitors, which proliferate continuously and release monocytes into the blood stream. Monocytes circulate in the blood stream for 1-2 days before migrating into the tissues to differentiate into macrophages, which exhibit various tissue specific functions and protect the body from infection by bacteria, viruses and other pathogens. Therefore, macrophages are found in all kind of tissues of the body. Huge extravasation of monocytes into the tissue is a sign of early onset of inflammation. Wound healing and various diseases in which macrophages exhibit a range of functions such as hypoxic survival, phagocytosis, bacterial killing,

antimicrobial activity, antigen presenting and tissue invasion (Fig. 1.8) (Ross and Rosen, 2002). Tissue hypoxia can be caused by various ways such as inflammation, wound healing or due to occlusion of the local blood supply. It has been shown in the literature that macrophages accumulate in large numbers in such hypoxic tissues and respond to the hypoxia by altered gene expression mediated by activation of both HIF-1 and HIF-2 target genes (Semenza, 2004; Burke *et al.*, 2003; Talks *et al.*, 2000). Numerous studies showed the changes in gene expression in macrophages in the hypoxic sites, for example altered VEGF, Glut-1, IL-1, TNF-α, IL-6, MMPs have been shown in the literature in macrophages under hypoxia (Lewis *et al.*, 2000).

Wounds have areas of hypoxia due to lack of perfusion caused by vascular damage. Initially neutrophils infiltrate the wound, increase their number and stay at the hypoxic site for 1-2 days post wound formation. This is followed by monocyte recruitment which peaks at days 2 till 5. Both macrophages and neutrophils phagocyte debris and secrete factors which attract additional immune cells to the site of the wound that facilitate neovascularization (Allen *et al.*, 1997). In an animal model of myocardial infarction, hypoxic areas are detected, where significantly higher numbers of macrophage accumulation has been shown (Azzawi *et al.*, 2005). Studies showed that HIF-1α and HIF-2α are upregulated in these macrophages found in infarcted tissues (Jurgensen *et al.*, 2004).

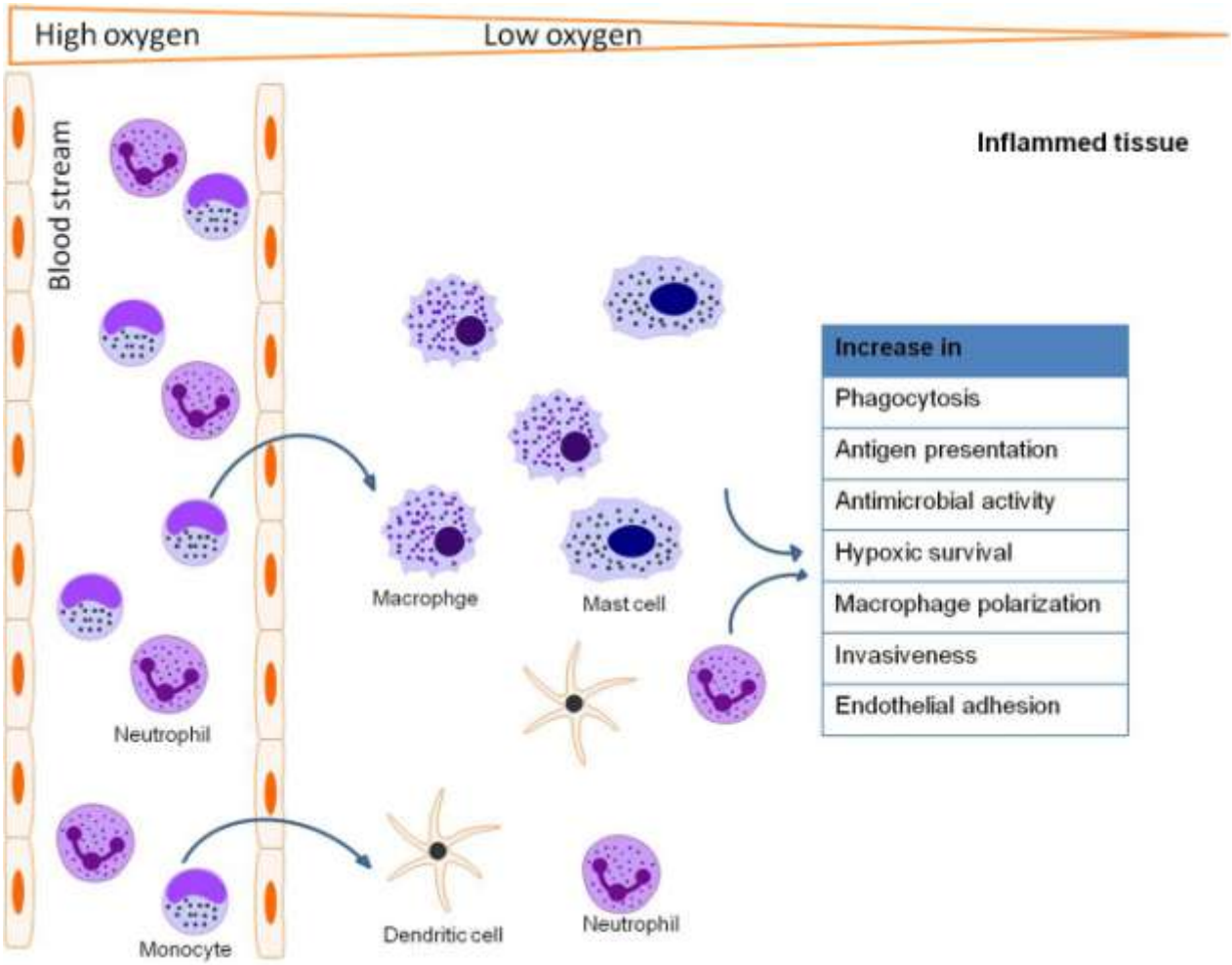


Figure adapted and modified from (Eltzschig and Carmeliet, 2011)

Figure 8: Myeloid cell response to hypoxia/ inflammation

The schematic explains the invasion of immune cells to the hypoxic or inflamed site. Inflamed hypoxic tissue mount a host defense response which amplifies the recruitment of innate immune cells such as neutrophils, mast cells or macrophages to the site of infection.

1.8 Angiopoietin-like protein 2 (Angptl2)

Angiopoietins belong to the family of proteins which regulate angiogenesis and hematopoietic stem cell differentiation (Yancopoulos *et al.*, 2000; Suda *et al.*, 2005) . It has been reported that tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie2) is the receptor for the members of the angiopoietin family (Davis *et al.*, 1996; Suri *et al.*, 1996). Recently, a protein family structurally similar to angiopoietins

was identified and is designated as angiopoietin-like proteins (Angptls). Angptls contain an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain (Hato *et al.*, 2008). They do not bind to the receptor Tie2, suggesting that Angptls have a different signaling mechanism than that of angiopoietins (Hato *et al.*, 2008). It has recently been reported that LILRB2 and PIRB are receptors for several Angptl family members (Zheng *et al.*, 2012; Oike *et al.*, 2004). The family of Angptls consists of seven members, Angptl1-7.

Angptl2 is constitutively expressed in several cell types including monocytes and macrophages and in tumor cells (Okada *et al.*, 2010; Aoi *et al.*, 2011; Tazume *et al.*, 2012). It has been reported that Angptl2 plays a crucial role in mediating obesity, tumor metastasis, rheumatoid arthritis and in inducing atherosclerotic diseases through chronic inflammation (Tabata *et al.*, 2009; Tazume *et al.*, 2012; Endo *et al.*, 2012; Aoi *et al.*, 2011; Okada *et al.*, 2010).

1.9 Angptl2 and inflammation

Literature reports several studies about the role of Angptl2 in both chronic and acute inflammation. For example, Angptl2 has been reported to mediate persistent low grade inflammation by activating NFκB signaling through α5β1 integrin receptor (Tabata *et al.*, 2009). Another similar study suggests that Angptl2 derived from infiltrating macrophages accelerates abdominal aortic aneurysm progression by inducing chronic inflammation and degradation in the aneurismal vessel wall (Tazume *et al.*, 2012). Studies also show correlation between chronic inflammation and Angptl2 expression level which induces tumor metastasis (Aoi *et al.*, 2011). This study was done by inducing skin squamous cell carcinoma (SCC) in a mouse model and expression of Angptl2 in this mouse model correlates with the frequency of carcinogenesis which is found to be caused by the accumulation of ROS due to chronic inflammation. Higher ROS level can inhibit the DNA repair enzyme machinery leading to increased risk of carcinogenesis (Aoi *et al.*, 2011). Also during acute inflammation, Angptl2 has been shown to be induced by endotoxin in the eye (Kanda *et al.*, 2012). Angptl2 is also induced during fin regeneration in adult zebrafish, further supporting a role of Angptl2 in inflammation (Kubota *et al.*, 2005; Boehler *et al.*, 2011).

Interestingly, reports also showed a stress related induction in the expression and secretion of Angptl2. In adipocytes, ER stress increases Angptl2 expression and secretion (Tabata *et al.*, 2009). Most interestingly, Angptl2 mRNA levels in tumor cells are significantly increased under hypoxia and starvation (Endo *et al.*, 2012), suggesting a link between cellular stress and hypoxia signaling pathways.

1.10 Aim of the study

The stability of the transcription factor HIFα is regulated by PHD enzymes in an oxygen dependent manner. Under normoxia PHD1-3 enzymes utilize molecular oxygen to hydroxylate HIFα and mark it for proteasomal degradation. Contrary, under hypoxic conditions HIFα is stabilized and can modulate hypoxia-dependent cell functions. Hypoxia is a common feature of infected and inflamed tissues, tumors, wounds, and atherosclerotic lesions. Myeloid cells are attracted to these hypoxic areas and a strong accumulation of macrophages can be observed. They are able to adapt rapidly to hypoxia by altered gene and protein expression profiles by activating hypoxia inducible factor signaling pathways and this influences the outcome of the diseases. The involvement of HIF in modulating inflammation has been well described in the literature. As macrophage function and hypoxia are important players in health and disease I aimed to investigate how macrophages are influenced by targeting HIF-signaling. PHDs are known to be expressed in myeloid cells and interfering with any of these PHD isoforms might alter the response of the immune system. As the PHD isoforms differ in enzyme activity, the isoform specific function of PHDs in myeloid cells needs to be investigated separately. A huge number of recent studies support the role of PHD3 in cell differentiation, survival, apoptosis, and metabolism. In myeloid cells loss of PHD3 is known to enhance the innate immune response (Kiss et al., 2012) and regulate the life span of neutrophils under hypoxia (Walmsley et al., 2011). However the specific role of PHD3 in macrophages is less explained so far. Therefore, the aim of this thesis was to investigate the role of PHD3 in macrophage specific function in a newly generated myeloid specific PHD3 knockout mouse line. To investigate the broad role following sub goals were set.

a) Isolation, differentiation and characterization of PHD3$^{-/-}$ BMDMs - I first aimed to isolate and differentiate BMDM and do general characterization of these *in-vitro* 8 days differentiated macrophages. These characterizations include confirming the knockdown by protein expression and gene expression analysis, doing morphological analysis and examining the macrophage differentiation efficiency.
b) To examine the polarization of macrophages - Macrophages can differentiate into two subpopulations which carry out distinct and time overlapping functions. Therefore another sub aim

was to investigate if the PHD3$^{-/-}$ BMDM differentiate into a distinct subpopulation both with and without stimulations by gene expression analysis and cytokine protein expression analysis.

c) To identity the effect of PHD3$^{-/-}$ on BMDM viability - For PHD3 a connection to apoptotic cell death mechanisms has been reported in various cell type. Therefore I was interested to investigate the effect of PHD3$^{-/-}$ in BMDMs using assays such as TUNEL assay, Annexin V assay, and calcein uptake assay.

d) Molecular studies of PHD3$^{-/-}$ BMDM - To further identify the molecular mechanisms of PHD3 on macrophage function I performed a transcriptome screen and analyzed the identified candidate Angptl2 in more detail.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

Table 2.1: List of used chemicals and materials

Name of the chemical	Provider
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromid (MTT)	Enzo Life Sciences, Lörrach, Germany
3-trimethylammoniopropyl methanethiosulphonate bromide (TAPS-sulphonate)	Wako Pure chemicals, Osaka, Japan
Accutase	PAA Laboratories, Cölbe, Germany
Bovine serum albumin	Applichem, Darmstadt, Germany
Complete Mini EDTA-free Protease Inhibitor	Roche Applied Science, Mannheim, Germany
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH, Karlsruhe, Germany
Dimethyloxaloylglycine (DMOG)	Frontier Scientific, Carnforth, UK
EDTA	PAN™ biotech GmbH, Passau, Germany
Fetal calf serum	PAN™ biotech GmbH, Passau, Germany
GeneRuler™ 1kb Plus DNA Ladder	Fermentas GmbH, St. Leon-Rot, Germany
Glycine	Carl Roth GmbH, Karlsruhe, Germany
HEPES	PAN™ biotech GmbH, Passau, Germany
Horse serum	PAN™ biotech GmbH, Passau, Germany
Interferon γ (IFNγ)	Peprotech, Hamburg, Germany
Interleukin (IL)-4	Peprotech, Hamburg, Germany
KCl	Carl Roth GmbH, Karlsruhe, Germany
L-glutamine	PAN™ biotech GmbH, Passau, Germany

Lipopolysaccharide (LPS)	Enzo Life Sciences, Lörrach, Germany
Methanol	Carl Roth GmbH, Karlsruhe, Germany
NaCl	Carl Roth GmbH, Karlsruhe, Germany
NaOH	Carl Roth GmbH, Karlsruhe, Germany
PageRuler™ Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot, Germany
Penicillin/streptomycin for cell culture	PAN™ biotech GmbH, Passau, Germany
Phalloidin	Invitrogen, Germany
S-nitroso-N-acetylpenicillamine (SNAP)	Santa Cruz
Sodium Citrate	Carl Roth GmbH, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
Sodium Pyruvate	PAN™ biotech GmbH, Passau, Germany
Staurosporine	Enzo Life Sciences, Lörrach, Germany
Super signal ECL	Merck Millipore, Darmstadt, Germany
Tetramethylethylenediamine (TEMED)	Carl Roth GmbH, Karlsruhe, Germany
Triton X	Carl Roth GmbH, Karlsruhe, Germany
Triton X-100	Carl Roth GmbH, Karlsruhe, Germany
Trizol	Invitrogen, Darmstadt, Germany
Trypan blue	Sigma, United Kingdom
β-mercaptoethanol	Carl Roth GmbH, Karlsruhe, Germany
Annexin V	Biolegend,London, United Kingdom
Propidium iodide	Biolegend,London, United Kingdom

2.1.2 Buffers

Table 2.2 List of buffers used

Buffer	Composition
Western blot transfer buffer	25 mM Tris 192 mM glycine 800 ml H_2O 10% SDS 200 ml methanol
DEPC-treated water	2 ml diethylpyrocarbonate 1 l H_2O
Enhanced luminol-based chemiluminescent substrate (ECL)	100 mM Tris/HCl, pH 8.5 90 mM coumaric acid 250 mM luminol 0.009% H_2O_2
Lysis Buffer	400 mM NaCl 10 mM Tris/HCl, pH 8 1 mM EDTA, pH 8 0.1% TritonX100 H_2O
Phosphate buffered saline (PBS)	137 mM NaCl 2.7 mM KCl 4.3 mM $Na_2HPO_4 \cdot 7H_2O$ 1.4 mM KH_2PO_4, pH 7.4
5x SDS electrophoresis buffer	125 mM Tris 1.25 M glycine 0.5% SDS, pH 8.3
2x SDS-sample buffer	100 mM Tris/HCl, pH 6.8 4% SDS 0.2% bromphenol blue 20% glycerol 5% β-mercaptoethanol
10x TAE	0.4 M Tris acetate 10 mM EDTA, pH 8.3
0.1x TE	1 mM Tris 0.1 mM EDTA, pH 8.0
6x DNA sample buffer	30% glycerine 0.25% bromphenol blue 0.25% Xylene cyanole FF H_2O
Tris/HCl	40 mM Tris, pH 5.0
Alkaline tail lysis buffer	25 mM NaOH 0.2 mM EDTA H_2O

2.1.3 Antibodies

Table 2.3 List of antibodies used for western blots

Antibody against	Source	Provider	Dilution	Catlog number
β-actin	mouse	Sigma	1:10000	A 5441
HIF-1α	mouse	Novus Biologicals	1:1000	NB-100-479
HIF-2α	goat	R&D Systems	1:500	AF2997
PHD2	mouse	Novus Biologicals	1:1000	NB 100-2219
PHD3	rabbit	Novus Biologicals	1:500	NB100-303
Secondary antibodies				
rabbit HRP	goat	SantaCruz	1:1000	Sc-2004
mouse HRP	goat	SantaCruz	1:1000	Sc-2005

Table 2.4 List of antibodies used for FACS

Antibodies	Provider
anti-Alpha5 integrin	Biolegend, London, United Kingdom
anti-integrin β1	Biolegend, London, United Kingdom
anti-CD11b	BD Bioscience, Heidelberg, Germany
anti-Pir-A/B	Biolegend, London, United Kingdom
anti-F4/80	Biolegend, London, United Kingdom
Rat IgG2a	Biolegend, London, United Kingdom
IgG	Biolegend, London, United Kingdom
Rat IgGK1	Biolegend, London, United Kingdom

2.1.4 Cell culture medium

Table 2.5 List of cell culture medium

Medium	Composition/Provider
Culture medium	DMEM 10% FCS 1% sodium pyruvate 1% HEPES 0.5% Penicillin/streptomycin 1% L-glutamine
DMEM	PAN™ biotech GmbH, Passau, Germany
Pluznik	DMEM 10% FCS 5% Horse serum 30% L929 conditioned medium 1% sodium pyruvate 0.5% Penicillin/streptomycin β-mercaptoethanol (1:1000)

2.1.5 Primers

Table 2.6 List of primers used for qRT-PCR

mRNA	Primer name	Primer sequence	Annealing temperature
PHD3	mPHD3 for	5'-GGCCGCTGTATCACCTGTAT-3'	**60°C**
	mPHD3 rev	5'-TTCTGCCCTTTCTTCAGCAT-3'	
MS12	mS12 for	5'-GAAGCTGCCAAGGCCTTAGA-3'	**58°C**
	mS12 rev	5'-AACTGCAACCAACCACCTTC-3'	
TNFα	mTNFa for	5'-GACCCTCACACTCAGATCATCTTC-3'	**58°C**
	mTNFa rev	5'-CCACTTGGTGGTTTGCTACGA-3'	
iNos	miNOS for	5'-AAGTCCAGCCGCACCACCCT-3'	**58°C**
	miNOS rev	5'-TCCGTGGCAAAGCGAGCCAG-3'	

Arginase	mArginase for	5'-AGGACAGCCTCGAGGAGGGG-3'	**58°C**
	mArginase rev	5'-CCCTGGCGTGGCCAGAGATG-3'	
Fizz	mFizz for	5'-TCCCAGTGAATACTGATGAGA-3'	**54°C**
	mFizz rev	5'-CCACTCTGGATCTCCCAAGA-3'	
PHD1	mPHD1 for	5'-GCTAGGCTGAGGGAGGAAGT-3'	**60°C**
	mPHD1 rev	5'-TCTACCCAGGCAATCTGGTC-3'	
PHD2	PHD2_ex3/5 for	5'-TTGCTGACATTGAACCCAAA-3'	**58°C**
	PHD2_ex5 rev	5'-GGCAACTGAGAGGCTGTAGG-3'	
PKM2	mPKM2 for	5'-AACCGAAGTACGCCCGAGGAC-3'	**58°C**
	mPKM2 rev	5'-ATCGGGAAGCAGGCCCAATGGT-3'	
Pfk	mPfkl for	5'-ACGAGGCCATCCAGCTCCGT-3'	**59°C**
	mPfkl rev	5'-TGGGGCTTGGGCAGTGTCCT-3'	
Pdk1	mpdk1 for	5'-GTTCACGTCACGCTGGGCGA-3'	**66°C**
	mpdk1 rev	5'-CCAGGCGTCCCATGTGCGTT-3'	
Ym-1	mYm1 for	5'-GCCAGCAGAAGCTCTCCAGAAGCAA-3'	**59°C**
	mYm1 rev	5'-ACTGAACGGGGCAGGTCCAAACT-3'	
IL-6	mIL6 for	5'-GCTGGTGACAACCACGGCCT-3'	**58°C**
	mIL6 rev	5'-TGCACAACTCTTTTCTCATTTCCACGA -3'	
Angptl2	mAngptl2 for	5'-ACCTCAACAGGTACAAGCGG-3'	**60°C**
	mAngptl2 rev	5'-CTCACGATGCCTCCGTCTAC-3'	
CD36	mCD36 for	5'-GCATCACCCCTCCAGAATCC-3'	**60°C**
	mCD36 rev	5'-TGGTCCCAGTCTCATTTAGCC-3'	
Primers for genotype PCR			

PHD3	PHD3 for	5'-CCACGTTAACTCTAGAGCCACTGA-3'	**58°C**
	PHD3 rev	5'-ATGGCCGCTGTATCACCTGTAT-3'	
LysM-cre	LysMcre for	5'-GTTCGCAAGAACCTGATGGACA-3'	**60°C**
	LysMcre rev	5'-GTTCGCAAGAACCTGATGGACA-3'	

2.1.6 Commercial kits

Table 2.6 List of kits used

Application	Name of the kit	Provider
Angptl2 ELISA	ELISA kit for the detection of Angptl2	Uscn Life sciences Inc, Wuhan, China
cDNA synthesis	First Strand cDNA synthesis kit	Fermentas GmbH, St. Leon-Rot, Germany
Cytokine detection	BD catometric Bead array mouse inflammation kit instruction	BD biosciences, Germany
PCR	PCR Master Mix K0171	Thermoscientific
real-time PCR	Brilliant II SYBR Green qPCR	Bioline, Luckenwalde, Germany
Transcriptome assay	One-Color Microarray-Based Gene Expression analysis kit	Agilent technologies
TUNEL Assay	*In-situ* cell death detection kit, fluorescein	Roche Diagnostics, Mannheim Germany
Viability Assay	Calcein-AM cell viability assay	Trevigen

2.1.7 Software

Table 2.7 List of software used

Program	Application	Source
CorelDraw	Image editor	Corel Corporation
Image J	Image analysis	Imagej.nih.gov
Axio-vision	Image analysis	Carl Zeiss
Blast	Sequence alignment	Ncbi.nlm.nih.gov
Graphpad Prism	Statistical analysis	GraphPad Software Inc.
Multi Gauge	Western blot analysis	Fujifilm Corporation
EndNote	Managing of bibliographic references	Thomson, Wintertree Software Inc.
BD FCAP Array software 3.0	FACS analysis	BD Biosciences
BD FACSDiva	FACS analysis	BD Biosciences

2.1.8 Instruments

Table 2.8 List of instruments used

Application	Name of the instrument	Manufacturer
Centrifugation	Centrifuge 5415R	Eppendorf AG Hamburg, Germany
	Centrifuge 5810R	Eppendorf AG Hamburg, Germany
	Micro Centrifuge	Carl Roth GmbH and Co. KG, Karlsruhe, Germany
Cleanbench	HERA safe KS 12	Thermo Electron Corporation, Langenselbold, Germany
Flow cytometer	BD FACS Canto II flowcytometer	BD Biosciences
Fluorescence microscope	Axio Observer D1	Zeiss, Germany
Gel Doc	Gene flash UV imager	Syngene bio imaging, UK

Heating Block	Block thermo stat BT2000	Kleinfeld Labortechnik, Germany
Incubation shaker	Minitron	INFORNS HT AG, Bottmingen, Switzerland
Incubator for cell culture	CB159	Binder GmbH, Tuttlingen, Germany
Luminometer	Centro LB 960	Berthold Technologies GmbH & Co. KG, Bad Wildbad Germany
Magnetic stirrer	Ikamag® RH	IKA Labortech, Staufen, Germany
Micro plate reader	Model 680	Bio-Rad, München, Germany
Microscope	Motic® AE30	TED, Pella Inc, USA
Oxygen-controlled work station	*In Vivo*$_2$ 400	Ruskin Technologies, Bridgend, UK
PCR cycler	Primus 96 Thermocycler	Peqlab, Erlangen, Germany
Photometer	Smart SpecTM Plus	Bio-Rad, München, Germany
Power supply		
Real-time PCR cycler	Mx3000P	Stratagene, La Jolla, USA
Rotator	Rotator RS-24	G.Kisker GbR, Steinfurt, Germany
UV-transilluminator	InGenius	Syngene, Camebridge, UK
Vortex	Vortex gemie 2	Scientific industries, Germany
Water bath	Water bath	Medigen, Germany
Western blot	PerfectBlueSemi-Dry elektroblotter	Fujifilm, Düsseldorf, Germany
Western blot imaging	LAS 3000 Imager	Fujifilm, Düsseldorf, Germany
Western blot transfer	PerfectBlue Double gel system Twin	Peqlab, Erlangen, Germany

2.2 Methods

2.2.1 Myeloid-specific conditional PHD3 knockout (PHD3$^{-/-}$) mice

The generation and detailed characterization of *Phd3* $^{flox/flox}$ mice were reported previously (Takeda *et al.*, 2006). The mice were kindly provided by Prof. Guo-Hua Fong, Center for Vascular Biology, University of Conneticut Health Center, Farmington, USA. All animals in this study were backcrossed to C57BL/6 mice at least five times. *Phd3* $^{flox/flox}$ mice were crossed with *LysMcre* mice (Clausen *et al.*, 1999) to generate *Phd3* $^{flox/flox}$ x *LysMcre*$^{+/-}$ mice within two generations. *Phd3* $^{flox/flox}$ x *LysMcre*$^{+/-}$ mice were then crossed with *Phd3* $^{flox/flox}$ mice to obtain PHD3$^{-/-}$ (*Phd3* $^{flox/flox}$ x *LysMcre*$^{+/-}$) mice and littermate control wt mice (*Phd3* $^{flox/flox}$ x *LysMcre*$^{-/-}$).

2.2.2 Isolation of gDNA

Small pieces of ear/tail clipping from mice were taken in 75 µl of alkaline tail lysis buffer. Then the samples were incubated for 1 hour at 95°C for tissue lysis and subsequently transferred to 4°C for 10 min. 75 µl of 40 mM Tris/HCl buffer was added. Out of this, 5 µl of sample were taken for performing PCR.

2.2.3 Polymerase chain reaction

For the amplification of DNA, the well described polymerase chain reaction (PCR) method was used. Genomic DNA from mouse tail was used as template. PCR was performed in a total reaction volume of 25 µl containing 6.5 µl ddH_2O, 12.5 µl PCR master mix, 5 µl DNA template, 0.5 µl forward primer (10 pmol/µl), and 0.5 µl reverse primer (10 pmol/µl). The reaction was carried out in the Primus 96 thermocycler. The used PCR cycling parameters are described in Table 2.9.

Table 2.9 Parameters used for PCR cycling

Step		Temperature	Duration	Number of cycles
Initial denaturation		95°C	3 min	1
PCR	Denaturation	95°C	30 sec	40
	Annealing	T_m	1 min	
	Extension	72°C	2 min	
Final extension		72°C	10 min	1

2.2.4 Differentiation and cultivation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) are primary macrophages, derived from bone marrow cells in vitro in the presence of colony stimulating and growth factors. Macrophage colony-stimulating factor (M-CSF) is a lineage-specific growth factor that is responsible for the proliferation and differentiation of committed myeloid progenitors into cells of the macrophage/monocyte lineage.

In this protocol, bone marrow cells were grown in petridishes in the presence of M-CSF, which is secreted by L929 cells and is used in the form of L929-conditioned medium. The efficiency of the differentiation was assessed using fluorescence-activated cell sorting (FACS) analysis of F4/80 surface antigen expression (Weischenfeldt and Porse, 2008). The differentiated BMDMs are suitable for various experiments, for example morphological, gene expression, and physiological analysis.

For the isolation of bone marrow cells mice were sacrificed by cervical dislocation at the age of 8-12 weeks. Bone marrow cells were isolated from the femur by flushing 10 ml of culture medium through the bone. The flushed cells were then cultured on cell culture dishes in culture at 37°C and 5% CO_2 in a humidified incubator as described before (Burgess *et al.*, 1985). After 24 hrs non-adherent monocytes were harvested, seeded in pluznik medium and differentiated for 3-8 days. Adherent BMDM were detached with 3.5 ml accutase and resuspended in culture medium.

2.2.5 Isolation of RNA

For RNA isolation 0.7 x 10^6 BMDM were seeded in 2 ml culture medium in 6-well plates and incubated for 4, 24 and 48 hrs at 37°C and 5% CO_2 in normoxia (20% O_2) or hypoxia (1% O_2) using the *In Vivo* 400 work station. Cells were washed once with PBS and harvested in 1 ml of Trizol per well. The samples were then collected in a 1.5 ml eppendorf tube and were incubated for 5 min at room temperature. 200 µl of chloroform was added and mixed well by inverting the tubes for 6 times followed by incubation for 3 min. Samples were centrifuged at 12000xg at 4°C for 15 min. Then the aqueous upper layer was transferred into a new eppendorf tube. 500 µl isopropanol was added to each sample and vortexed, incubated for 10 min at room temperature, then again incubated for 10 min at -20°C and centrifuged at 12000g at 4°C for 10 min. The supernatant was discarded, the pellet was washed using 1 ml 75% ethanol. After centrifuging at 7000g 4°C for 10 min, the supernatant was carefully discarded and the pellet was dried. After drying it was dissolved in 15 µl of RNase free water. The RNA solution was incubated at 56°C for 10 min. Then the samples were stored at -80°C.

2.2.6 RNA concentration measurement

After RNA isolation, the concentrations of the samples were determined by measuring the absorption at 260 nm and 280 nm using the BioAnalyzer. The threshold for a sufficient purity of RNA samples was determined by the 260 nm/280 nm ratio of 1.8, presence of proteins in the samples decreases the ratio.

2.2.7 cDNA synthesis

The ability to synthesize DNA from an RNA template, using reverse transcriptase is explored for the process of cDNA synthesis. 1 µg of RNA was used for the synthesis of cDNA using the First strand cDNA synthesis kit. For the synthesis of cDNA a mixture of 11 µl consisting of 1 µg RNA, H_2O and 1 µl Oligo-dT was prepared and incubated for 65° C for 5 min then the samples were immediately transferred onto ice for 5 min. To each of the tubes a 9 µl mastermix composed of 2 µl dNTP, 1 µl Ribolock, 2 µl reverse transcriptase, and 4 µl RT buffer was added. The samples were incubated at 37° C for one hour. The reaction was stopped by inactivating the reverse transcriptase by incubating at 70° C for 5 min.

2.2.8 Quantitative real-time PCR

A quantitative real-time polymerase chain reaction (qRT-PCR) is a PCR technique, which is used to amplify and simultaneously quantify a DNA template. In quantitative PCR, the amount of the amplified product is linked to fluorescence intensity using a fluorescent reporter molecule, a DNA-intercalating dye that glows once it binds to double-stranded DNA. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as Primer dimer). This can potentially interfere with, or prevent, accurate quantification of the intended target sequence. As a reference dye ROX^{TM} was added to the samples to normalize the fluorescence signal intensity between the reactions. With reference to a standard dilution, the dsDNA concentration in the PCR can be determined. The quantification can be either is done by an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes using any housekeeping gene. This quantitation approach is termed the comparative C_t method. C_t (threshold cycle) is a relative measure of the concentration of target in the PCR reaction. This method of analysis involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene.

Transcript levels were analyzed by qRT-PCR with the Brilliant II SYBR Green qPCR Master Mix in an MX3005Pro light cycler. For the reaction 1 µl cDNA, 12.5 µl SYBR Advantage qPCR Premix (2x), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM) and 0.5 µl ROX reference dye LMP were mixed and dH_2O added to a total volume of 25 µl. The condition of the reaction is described in table 2.10

Table 2.10 Parameters used for qRT-PCR cycling

	Step	Temperature	Duration	Cycles
Denaturation	Denaturation	95°C	30 sec	1
PCR	Denaturation	95°C	10 sec	40
	Annealing	T °C	20 sec	
	Extension	72°C	20sec	
Amplification of the dissociation curve	Denaturation	95°C	1 min	1
	Annealing	T_m °C	30 sec	
	Extension	72°C	30 sec	

2.2.9 Protein extraction

For protein extraction $4x10^6$ cells were seeded in 10 ml culture medium in tissue culture grade petridishes and incubated for 24 hrs at 37°C and 5% CO_2 in normoxia (20% O_2) or hypoxia (1% O_2). For harvesting, the cells were washed once with PBS and 175 µl of lysis buffer were added to each 10 cm dish. An EDTA-free protease-inhibitor was added to the lysis buffer to prevent protein degradation. The cells were scraped from the dishes and the cell lysate was transferred into a 1.5 ml eppendorf tube. The samples were placed on ice for about 10 min. The lysed cells were pelleted by centrifugation at 12000 rpm for 20 min at 4°C and the supernatant was transferred into a new 1.5 ml eppendorf tube. The protein concentration was measured by performing a Bradford protein concentration assay.

2.2.10 Bradford Protein concentration assay

The Bradford protein concentration assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. During this assay under acidic conditions the red form of the dye is converted into its bluer form upon binding to the protein. Under acidic conditions, the dye is

predominantly in the doubly protonated red cationic form which has an maximum absorbance at 470 nm. When the dye binds to proteins, it is converted to a stable unprotonated blue form which has an absorbance at 595 nm (Compton and Jones 1985). The linear range of Bradford assay is from 0 µg/ml to 2000 µg/ml. A standard curve was prepared from a series of standards between 0.5 µg and 5 µg bovine serum albumin (BSA). For the Bradford assay, 1 µl of the sample was added to 200 µl of 1:5 times diluted Protein Assay Reagent from Bio-Rad and the absorbance was measured at 595 nm. The concentration of the unknown protein samples was determined using the standard curve.

2.2.11 SDS-Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is a technique to separate biological macromolecules for example proteins or nucleic acids according to their molecular weight. To eliminate the effect of folding structures, proteins are treated with sodium dodecyl sulphate (SDS), an anionic detergent, to denaturate the protein by breaking the secondary and non–disulfide–linked tertiary structures. SDS additionally applies a negative charge to each protein in proportion to its mass. The negative charge of the proteins by SDS makes the proteins run towards the positive electrode during electrophoresis. The reducing agent β-mercaptoethanol further denatures the proteins by reducing disulfide linkages and breaking up quaternary protein structures. The sample buffer contains a tracking dye which has a higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the solution through the gel during the electrophoretic run. The preparation of a gel needs two different layers of acrylamide. The lower layer resolving gel is responsible for actually separating polypeptides by size. The upper layer stacking gel has the sample wells, it sweeps up the sample that they are compressed into micrometer thin layers when they reach the separating gel to have a uniform start before the separation. The pH conditions in the stacking gel result in the samples to stack between the fast migrating chloride ions and the slower migrating glycine from the sample buffer at the starting point above the resolving gel. Usually 10-12% of acrylamide is used for the preparation of the gel.

Double the volume of SDS sample buffer was added to 80-100 µg of protein. Before loading the samples to the 12% SDS polyacrylamide gel, the samples were boiled at 95°C for 10 min. The prestained

PageRuler™ marker was loaded along with the samples onto the gel. The electrophoresis was run at a constant current of 40-45 mA with maximum voltage set at 300 V.

Table 2.11 Composition of stacking and resolving gel

	Stacking gel	Resolving gel 10%	Resolving gel 12%
H_2O	6.8 ml	11.9 ml	9.9 ml
30% acrylamide mix	1.7 ml	10 ml	12 ml
1 M Tris pH6.8	1.25 ml	-	-
1.5 M Tris pH8.8	-	7.5 ml	7.5 ml
10% ammonium persulfate	1 ml	0.3 ml	0.3 ml
10% SDS	1 ml	0.3 ml	0.3 ml
TEMED	0.01 ml	0.012 ml	0.12 ml

2.2.12 Western blot Development

The separated proteins on the gel were transferred onto a nitrocellulose membrane. For transfer of proteins, the gel and the nitrocellulose membrane were equilibrated in western blot transfer buffer. The gel and the nitrocellulose membrane were then sandwiched between filter papers already soaked in blotting buffer. The transfer was performed using PerfectBlue Semi-Dry Electroblotter with a constant current setting of 2 mA/cm^2 for 1 hour. The membrane was analyzed for the protein expression of interest by using primary antibody against it. The primary antibodies were then detected by horseradish peroxidase conjugated secondary antibodies which are raised against the Fc region of the primary antibody.

After transfer the membrane was washed with PBS for 10 min. Then the confirmation of the quality of transfer was controlled by Ponceau S staining. The membrane was washed three times with PBS to get rid of the Ponceau S staining. The blot was blocked with 5% milk in PBS for 1 hour at room temperature to prevent unspecific binding of the antibodies. Dilution of the primary antibody was prepared in PBS

containing 5% non fat milk and was applied on to the blot and incubated overnight at 4°C. The used dilutions of the antibodies are listed in Tab. 2.3.

After overnight incubation of the primary antibody at 4°C, the membrane was washed thrice for 10 min with PBS. The secondary antibody which is conjugated to horseradish peroxidase is diluted in PBS containing 5% non fat milk. The membrane was incubated in secondary antibody for 2 hrs at room temperature. The membrane was washed three times with PBS for 10 min each. 10 ml of ECL solution was applied on to the membrane for development. The blot was developed by exposing the membrane onto X-ray films or was imaged using the LAS3000 Imager from Fujifilm for different exposure times.

2.2.13 TUNEL ASSAY

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay detects DNA fragmentations by labeling the terminal end of nucleic acids. The hallmark of apoptosis is DNA degradation, which in early stages, is selective to the internucleosomal DNA linker regions. The DNA cleavage may yield double-stranded and single-stranded DNA breaks (nicks). Both types of breaks can be detected by labeling the free 3'-OH terminal with modified nucleotides (*e.g.,* biotin-dUTP, DIG-dUTP, fluorescein-dUTP) in an enzymatic reaction. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the template-independent polymerization of deoxyribonucleotides to the 3'-end of single- and double-stranded DNA.

0.5 x 10^6 BMDM were seeded in 6-well plates on coverslips in 2 ml culture medium and were incubated for 48 hrs at 37°C and 5% CO_2 in normoxia (20% O_2) or hypoxia (1% O_2). The cells were then washed with PBS and fixed using 4% PFA for 20 min. The cells were permeabilized using 0.1% TritonX-100 in freshly prepared 0.1% sodium citrate for 2 min on ice and were then stained for 60 min at 37ºC in a dark and moist chamber using enzyme and labelling solution provided by the fluorescein *in-situ* cell death detection kit. Cells treated with DNAse served as positive control. The cells were then washed with PBS and were imaged using the Axio Observer fluorescence microscope.

2.2.14 Annexin V Staining

Phospholipids of the cell membrane are asymmetrically distributed between the inner and outer leaflets of the membrane. Phosphatidylcholine and sphingomyelin are exposed on the external leaflet of the lipid bilayer, while phosphatidylserine is located on the inner surface. During apoptosis, this asymmetry is disrupted and phosphatidylserine becomes exposed on the outside surface of the plasma membrane. Because the anticoagulant protein Annexin V binds with high affinity to phosphatidylserine, the fluorochrome-conjugated Annexin V can been used to detect apoptotic cells by flow cytometry.

BMDM were seeded in 35 mm dishes in culture medium with and without serum. After 48 hrs the cells were detached using accutase for 15 min and cells were counted. 1.5 x 10^6 cells per sample were subsequently analyzed. After washing with PBS cells were stained with Annexin V and propidium iodide for 20 min at 4 ºC. Then the cells were resuspended in Annexin V binding buffer and were anlysed using the BD FACS Canto flow cytometer. In some experiments, cells were treated with 100 µM S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) or 500 nM staurosporine or 5-10 µg/ml LPS for 12 hrs.

2.2.15 Flow cytometry

Flow cytometry is a laser-based method for cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. FACS provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. A wide range of fluorophores can be used as labels in flow cytometry.

$0.5x10^6$ cells were cultured in 35 mm petridishes for 24 and 48 hrs at 37°C and 5% CO_2 in normoxia (20% O_2) or hypoxia (1% O_2). $0.2x10^6$ cells were taken in a FACS tube for further analysis. Then the cells were washed with PBS and were incubated with anti-Pir-A/B, anti-F4/80, anti-integrin α5, anti-integrin β1, anti-

CD11b and the respective control antibodies rat IgGk1, rat IgG2a, IgG for 20 min at 4°C. After washing with PBS cells were resuspended in 100 µl of binding buffer and analyzed using the flow cytometer.

2.2.16 Calcein-AM cell viability assay

Calcein-AM is a non-fluorescent, hydrophobic compound that easily permeates intact, living cells. The hydrolysis of Calcein-AM by intracellular esterase produces calcein, a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. Cells, preferably grown in black-walled plates, can be stained and quantified in less than two hrs. $0.2x10^5$ cells were grown in 200 µl of culture medium in black walled 96 well plates for 4, 12, 24 and 48 hrs at 37°C at 20% O_2 or 1% O_2. The cell viability was determined using the Calcein-AM cell viability assay kit. Before measurement, the plate was taken out from 37°C and was centrifuged at 250 g for 5 min. Supernatant was discarded and 100 µl of 1X calcein AM DW buffer was added to each well. Then again the plate was centrifuged at 250g for 5 min. The calcein AM DW buffer was removed and 50 µl of fresh calcein AM DW buffer was added. To each well, 50 µl of freshly diluted 2X AM working solution was added. The plate was then incubated for 30 min at 37°C. The fluorescence was measured using a 490 nm excitation filter and a 520 nm emission filter. The fluorescence intensity is proportional to the number of viable cells.

2.2.17 Angptl2 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a method which uses antibodies to identify a specific substance. Antigens from the sample are attached to a surface coated with a specific antibody. A second antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate. The microtiter plate had been pre-coated with an antibody specific to Angptl2. Standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to Angptl2. Then Avidin conjugated to horseradish peroxidase (HRP) was added to each micro plate well. After 3,3',5,5'-Tetramethylbenzidine(TMB) substrate solution is added, only those wells that contain Angptl2, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color

change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of Angptl2 in the samples is then determined by comparing the O.D. of the samples to the standard curve. $0.75x10^6$ cells were grown in 200 µl of culture medium in black walled 6 well plates for 4, 12, 24,and 48 hrs at 37°C at 20% O_2 or 1% O_2. The supernatant was harvested for the ELISA. 100 µl of prepared standards or samples were added to a 96 well coated plate. The wells were then incubated for 2 hrs at 37°C.The supernatant was then discarded and 100 µl of detection reagent A was added. The wells were then incubated for 1 hour at 37°C. The supernatant was again discarded and the wells were washed three times with washing buffer. Then 100 µl of detection reagent B was added. The wells were then incubated for 30 min at 37°C. The supernatant was discarded and the wells were washed five times with washing buffer. 90 µl of substrate solution were added and incubated for 15-25 min at 37°C. 50 µl of stop solution were added and the absorbance of the samples was measured at 450 nm using a micro plate reader.

2.2.18 Transcriptome screen

A microarray chip consists of DNA spots on a solid chip. It is used to measure the expression of a large number of genes simultaneously. Each spot on a microarray contains multiple identical strands of DNA. The DNA sequence on each spot is unique and represents one gene. Thousands of spots are arrayed in orderly rows and columns on a solid surface. The precise location and sequence of each spot is recorded in a computer database. Microarrays can be the size of a microscope slide, or even smaller. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

0.7×10^6 BMDM were seeded in 2 ml culture medium in 6-well plates and incubated for 48 hrs at 37°C and 5% CO_2 in 20% O_2 or 1% O_2. Cells were washed once with PBS and harvested in Trizol. RNA was isolated (section 2.2.5) and 1 µg RNA was transcribed using the First Strand cDNA Synthesis Kit (section 2.2.7). RNA integrity was determined (section 2.2.6). RNA was then added to the Spike mix provided with the Agilent Technologies, One-Color Microarray-Based Gene Expression analysis kit. Samples were then labeled according to the Agilent – Low Input Quick Amp Labeling, one color protocol. cDNA was synthesized from the labeled mRNA. Amplified RNA (aRNA) was then synthesized using T7 RNA polymerase enzyme. Quantification of the labeled aRNA was done before hybridization onto the

microarray slides. The hybridization was then carried out on the Agilent Microarray Hybridization slide at 65ºC for 17 hrs. All subsequent steps were followed as instructed by Low Input Quick Amp Labeling protocol. The whole transcriptome screen and analysis of the raw data was performed in collaboration with the Transkriptions Analyse Labor (TAL, University Medical Center Göttingen).

2.2.19 Statistics

All the statistical evaluations were performed using graph pad prism 4 software. Statistical analyses were performed using Student`s two-tailed t-test. Data are shown as means ± SD. Values of $p<0.05$ were considered statistically significant. Alpha level was set to 0.05, p values are: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3. Results

3.1 Generation of myeloid-specific PHD3 knockout mice

The results of the study are in part published (Swain *et al.*, 2014). Figures of the publication are also used in part for the thesis. The goal of my study was to characterize and investigate the role of PHD3 in BMDM. In my experiments I used a conditional PHD3-deficient mouse model. The PHD3 knockout (referred to as $PHD3^{-/-}$) mouse model was established by using $PHD3^{flox/flox}$ mice, in which exon 2 of the PHD3 gene was flanked by loxP sites (Fig. 9). $PHD3^{flox/flox}$ mice were crossed with M lysozyme Cre (LysMCre) mice which express the Cre recombinase under the control of the mouse lysozyme M gene regulatory region. This cross generates littermates that had either no copies ($PHD3^{flox/flox}$ x $LysMCre^{-/-}$) or one copy of the Cre rebombinase ($PHD3^{flox/flox}$ x $LysMCre^{+/-}$). $PHD3^{flox/flox}$ x $LysMCre^{+/-}$ mice have a reduced PHD3 expression in the myeloid cell lineage (monocytes, mature macrophages and granulocytes).

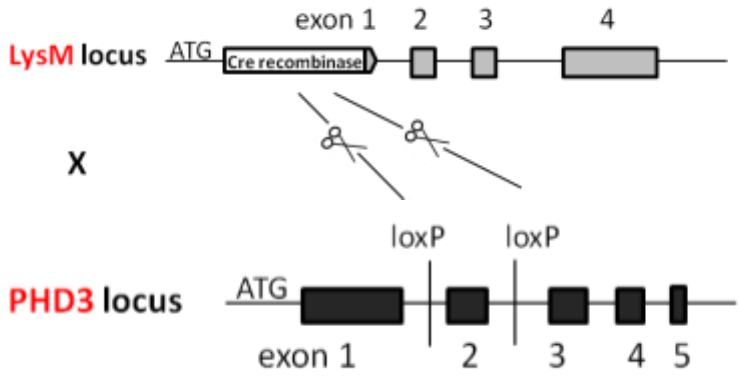


Figure 9: **Myeloid-specific PHD3-deficient mouse line generation scheme**

Myeloid-specific PHD3-deficient mice were generated by crossing $PHD3^{flox/flox}$ mice, in which exon 2 is flanked by loxP (flox) sites, with mice expressing the Cre recombinase under control of the lysozyme (LysM) gene regulatory region.

Littermates were genotyped by performing PCRs with gDNA (Fig. 10) to confirm the presence of the loxP sites within the PHD3 gene (product size 840 bp and 400 bp in floxed and wt mice, respectively) and to determine the presence or absence of the Cre recombinase (product size 700 bp and 350 bp in cre positive and wt mice, respectively).

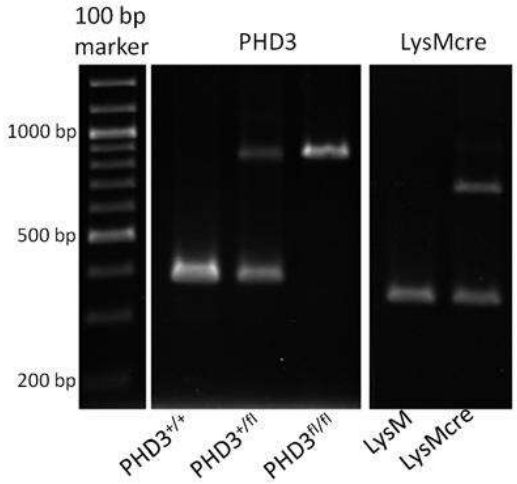


Figure 10: **Genotype determination using PCR**

$PHD3^{fl/fl}$ x $LysMCre^{+/-}$ (referred to as $PHD3^{-/-}$), $PHD3^{+/fl}$ x $LysMCre^{+/-}$ and $PHD3^{+/+}$ x LysM $Cre^{+/-}$ mice were genotyped by performing PCRs with gDNA. Representative PCRs of mice with different genotypes are shown.

3.2 Consequences of a PHD3 deficiency in macrophages for their differentiation and morphology

It has been shown earlier by Kiss et al that the macrophage differentiation process of bone marrow cells was altered in BMDM isolated from constitutive PHD3 knockout mice over time (Kiss *et al.*, 2012). To investigate if the conditional knockout of PHD3 also influences the differentiation of macrophages over time, bone marrow cells of both genotypes, i.e. wt and $PHD3^{-/-}$ were differentiated to mature macrophages with M-CSF conditioned medium. To quantify the differentiation efficacy of the macrophages over time, the cells were stained with anti-F4/80 and anti-CD11b antibodies and analyzed using flow cytometry. F4/80 is a marker for mature macrophages. CD11b marks cells that originated from the monocyte population and neutrophils. The macrophage differentiation process of bone marrow cells was indeed altered in wt and $PHD3^{-/-}$ cells over time. $PHD3^{-/-}$ bone marrow cells differentiated at earlier time points, i.e. day 3, 4 and 5, demonstrated by elevated numbers of F4/80 and CD11b positive cells (Fig. 11). 8 days after starting the differentiation the difference was no longer detectable and cells of both genotypes were fully differentiated.

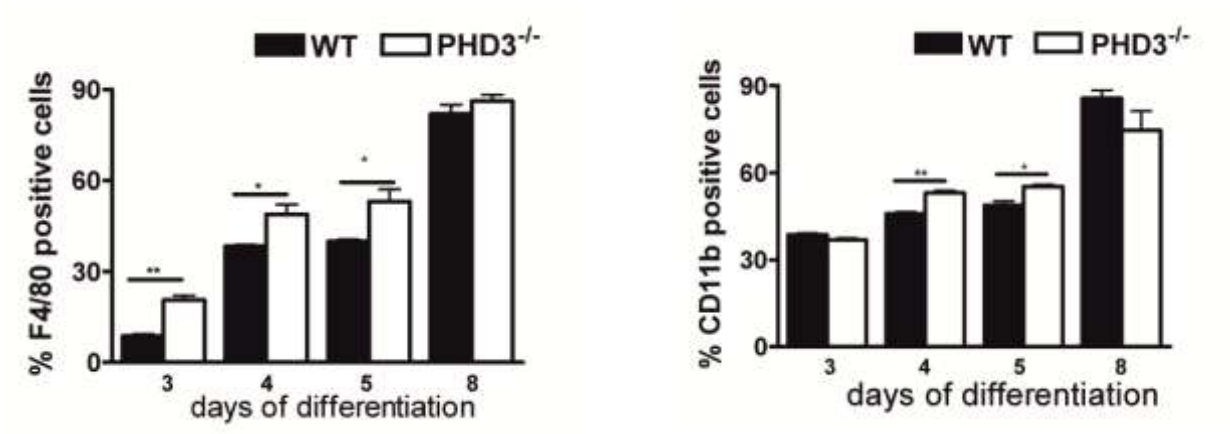


Figure 11: **Differentiation of wt and PHD3$^{-/-}$ BMDM over time**

Macrophages were differentiated from bone marrow cells of 8 - 12 weeks old wt and PHD3$^{-/-}$ mice using M-CSF containing medium over 3-8 days. PHD3-deficiency in myeloid cells promoted the differentiation of BMDM as shown by F4/80 and CD11b-stainings using FACS analysis. Shown are mean values ± SD, n=3, *p<0.05, **p<0.01.

To study if the knockout of PHD3 in macrophages affects the morphology of the macrophages, cell staining was performed. Cells isolated from both wt and PHD3$^{-/-}$ mice were differentiated over 3 or 8 days with M-CSF conditioned medium. After 3 or 8 days of differentiation the cells were stained with phalloidin (F-actin marker) and DAPI (nucleus marker) (Fig. 12). The cells were then imaged using a fluorescence microscope for morphological differences. Bone marrow cells of both genotypes differentiated to mature macrophages and showed no obvious difference in cell morphology after 3 or 8 days of differentiation. In all subsequent experiments, if not stated otherwise, BMDM, which were differentiated for 8 days, were used.

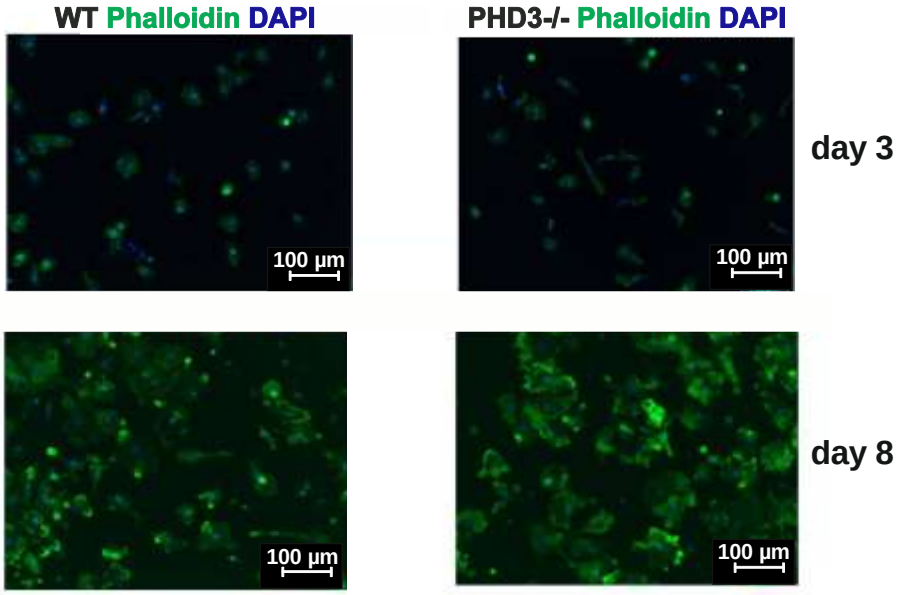


Figure 12: **Morphology of wt and PHD3$^{-/-}$ BMDM during differentiation**

Wt and PHD3$^{-/-}$ BMDM were differentiated for 3 or 8 days with M-CSF conditioned medium. Subsequently, cells were stained with phalloidin and DAPI. PHD3-deficiency in myeloid cells did not affect the morphology of the cells.

3.3 Consequences of PHD3-deficiency for macrophage gene expression

To confirm the knockout of PHD3 and gain insight into the knockout efficiency, qRT-PCRs were performed. Wt and PHD3$^{-/-}$ BMDM were incubated in normoxia (20% O_2) or hypoxia (1% O_2) for 24 hrs. Subsequently, the mRNA expression level of PHD3 was analyzed (Fig. 13). The hypoxic induction of PHD3 is in line with the literature and is part of a negative feedback mechanism to limit the acute hypoxic response (Stiehl *et al.*, 2006). PHD3$^{-/-}$ BMDM showed a 75% and 60% reduction of PHD3 RNA levels in normoxia and hypoxia respectively. Thus, in hypoxia the knockout efficiency was still maintained even after the induction of PHD3

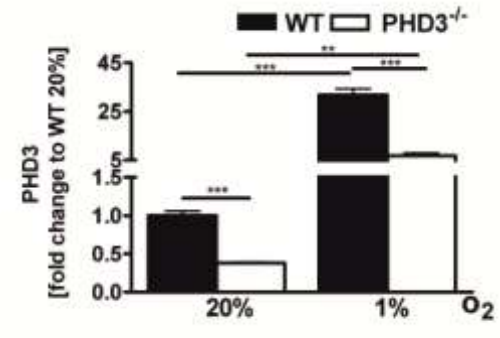


Figure 13: **PHD3 mRNA expression of wt and PHD3$^{-/-}$ macrophages**

PHD3 RNA levels were quantified in BMDM isolated from wt and PHD3$^{-/-}$ mice and exposed to 20% or 1% O_2 for 24 hrs. Shown are mean values ± SD, n=3, **p<0.01, ***p<0.001.

The mRNA expression of the other two isoforms of PHDs, i.e. PHD1 and PHD2 was investigated to analyze if the knockout of PHD3 affects their expression. qRT-PCR was performed with samples obtained from wt and PHD3$^{-/-}$ BMDM, which were incubated in normoxia (20% O_2) or hypoxia (1% O_2) for 24 hrs (Fig. 14). Comparable to PHD3, PHD2 is inducible in hypoxia. This effect was observed in wt and PHD3$^{-/-}$ BMDM. In none of the conditions analyzed there was a difference when comparing PHD1 and PHD2 RNA levels in wt versus PHD3$^{-/-}$ BMDM.

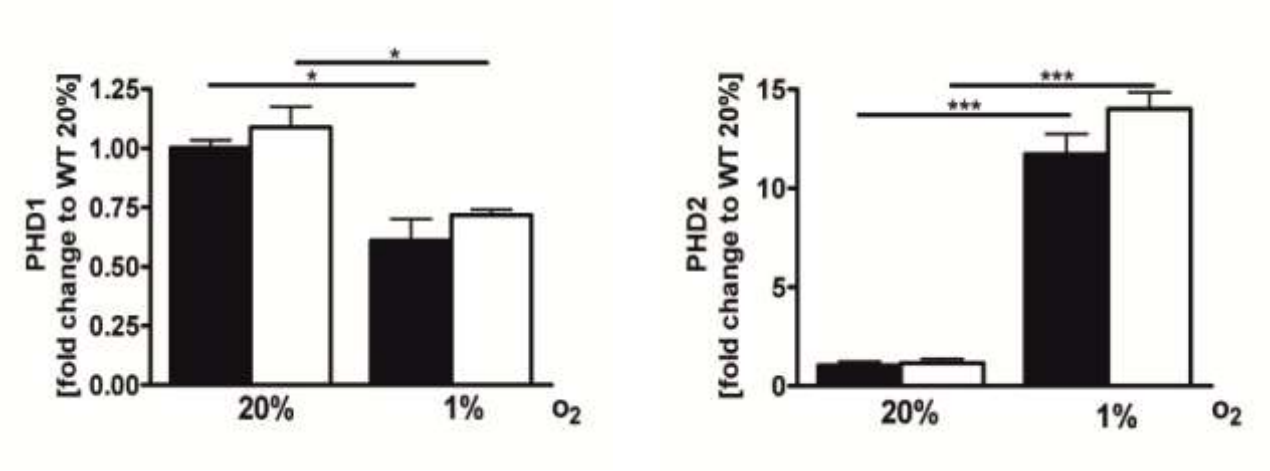


Figure 14: **mRNA expression of PHD1 and PHD2 of wt and PHD3$^{-/-}$ macrophages**

PHD1 and PHD2 RNA levels were quantified by qRT-PCR in wt (black bars) and PHD3$^{-/-}$ (white bars) BMDM, which were incubated for 24 hrs in normoxia (20% O_2) or hypoxia (1% O_2). Shown are mean values ± SD, n=3, *p<0.05, ***p<0.001.

Western blots were performed to confirm the data obtained by analyzing mRNA expression of PHD2 and PHD3 at the protein level. Wt and PHD3$^{-/-}$ BMDM were cultured under 20% and 1% O_2 conditions for 24 hrs. Expression of PHD3, PHD2 and HIF-1α was analyzed at protein level both in normoxic and hypoxic conditions (Fig. 15). PHD1 was not analyzed based on the lack of a suitable antibody. In line with the RNA data, at protein level a significant decrease of PHD3 was seen in the PHD3$^{-/-}$ BMDM. This difference was more obvious after incubating the cells for 24 hrs at hypoxia compared to normoxia based on the hypoxic induction of PHD3. PHDs regulate the stability of HIF-1α and HIF-2α. A knockout of PHD2 alone is indeed sufficient to stabilize HIFα in normoxia. Knockout of PHD3 in the BMDM did however not result in a significant change in PHD2, HIF-1α and HIF-2α protein levels in normoxia. The hypoxic induction of all

three proteins was not altered in the PHD3$^{-/-}$ BMDMs likewise indicating that the reduction of PHD3 expression alone is not sufficient to induce HIFα stabilization.

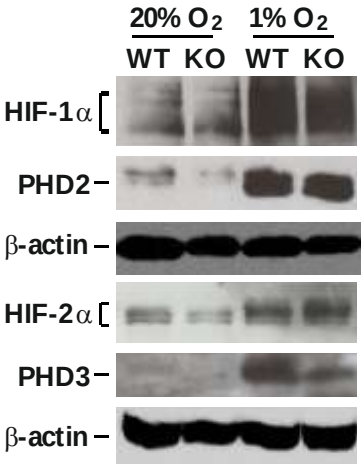


Figure 15: Protein expression of PHD3, PHD2, HIF-1α and HIF-2α of wt and PHD3$^{-/-}$ BMDM

BMDM were isolated from wt or PHD3$^{-/-}$ (KO) mice. Cells were incubated for 24 hrs in normoxia (20% O_2) or hypoxia (1% O_2). Subsequently, cells were lysed and HIF-1α, PHD2, HIF-2α, PHD3 and β-actin protein levels were analyzed by Western blots.

3.4 HIF-dependent target gene expression is unchanged in PHD3$^{-/-}$ BMDM

To further confirm that HIFα stabilization and HIF activity were not affected by knocking out PHD3 the mRNA expression of HIF target genes were investigated. To this end, wt and PHD3$^{-/-}$ BMDM were incubated in normoxia (20% O_2) or hypoxia (1% O_2) for 24 hrs and the mRNA expression levels of the HIF target genes pyruvate kinase M2 (PKM2), phosphofructokinase (Pfk) and pyruvate dehydrogenase kinase-1 (PDK-1) were quantified. The normoxic RNA expression as well as the hypoxic induction of these HIF target genes was unaltered in the PHD3$^{-/-}$ BMDM (Fig. 16). These results indicate that knocking down PHD3 is not sufficient to affect HIF dependent pathways in BMDM.

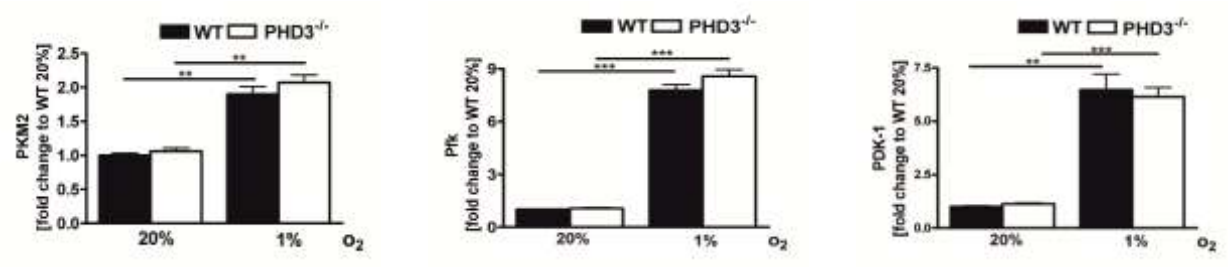


Figure 16: **mRNA expression of HIF target genes of wt and PHD3$^{-/-}$ BMDM**

The expression of the HIF target genes PKM2, Pfk and PDK-1 on mRNA level was analyzed after cultivating BMDM for 24 hrs at 20% O_2 or 1% O_2 by qRT-PCR. Shown are mean values ± SD, n=3, **p<0.01, ***p<0.001.

To gain insight if there are any other differentially expressed genes in PHD3$^{-/-}$ versus wt BMDM, an unbiased transcriptome screen was performed in collaboration with the Transkriptions Analyse Labor (TAL, University Medical Center Göttingen). BMDM were incubated in normoxia (20% O_2) or hypoxia (1% O_2). In the following unbiased transcriptome screen I could indeed not find any significantly differentially expressed gene comparing wt versus PHD3$^{-/-}$ BMDM in normoxia and hypoxia (Fig. 17). There was as expected a whole set of RNAs, which were upregulated in hypoxia in wt and PHD3$^{-/-}$ BMDM. This classical hypoxic response however was not altered by the knockout of PHD3.

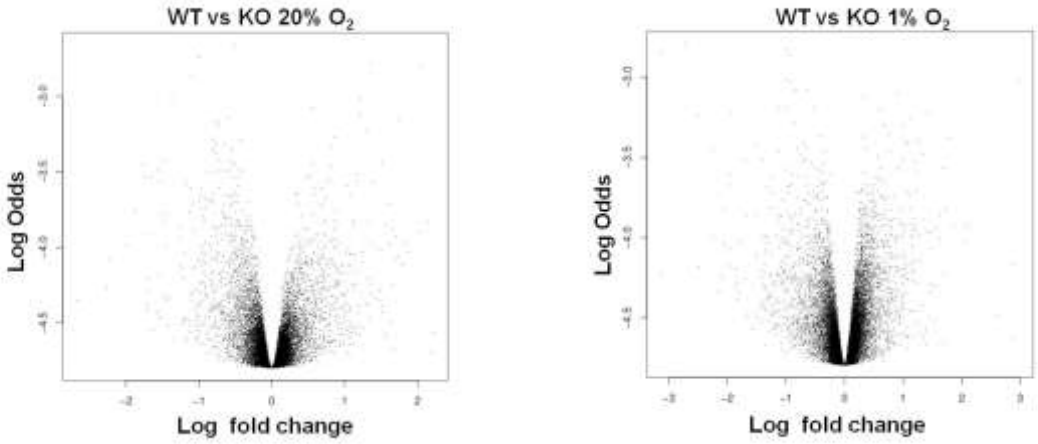


Figure 17: **Volcano plot for differentially expressed genes under normoxia and hypoxia**

PHD3$^{-/-}$ and wt BMDM, which were isolated from three independent mice for each genotype, were cultivated for 48 hrs at 20% O_2 or 1% O_2. Subsequently, cells were lysed and RNA isolated. RNA samples were analyzed in a transcriptome microarray assay. The volcano blots were generated based on the microarray results.

Among the RNAs which were identified to be upregulated in hypoxia there were classical HIF-target genes like Bnip3, Egln3, arginase etc. (Table 3.1). This analysis served as internal quality control for the screen.

Table 3.1	List of genes, which were identified to be highly induced in hypoxia in wt BMDM	Fold change
Ppp1r3g	protein phosphatase 1, regulatory (inhibitor) subunit 3G	7.82
Rcor2	REST corepressor 2	5.30
Ankrd37	ankyrin repeat domain 37	5.50
Fam162a	family with sequence similarity 162, member A	4.17
Bnip3	BCL2/adenovirus E1B interacting protein 3	4.57
Egln3	EGL nine homolog 3 (C. elegans)	6.28
Egln3	EGL nine homolog 3 (C. elegans)	6.33
Gipr	gastric inhibitory polypeptide receptor	7.61
Mboat2	membrane bound O-acyltransferase domain containing 2	5.54
Prelid2	PRELI domain containing 2	4.86
Rgs11	regulator of G-protein signaling 11	4.91
Triobp	TRIO and F-actin binding protein	5.55
Mgarp	mitochondria localized glutamic acid rich protein	8.33
Ak4	adenylate kinase 4	6.15
Bnip3	BCL2/adenovirus E1B interacting protein 3	4.31
Gm5486	predicted gene 5486	4.36
Smtnl2	smoothelin-like 2	7.31
Ero1l	ERO1-like (S. cerevisiae)	4.55
Ppp1r3c	protein phosphatase 1, regulatory (inhibitor) subunit 3C	5.10
Prr15	proline rich 15	5.54
Adm	adrenomedullin	4.28
Skint3	selection and upkeep of intraepithelial T cells 3	4.49
Slc16a3	solute carrier family 16 (monocarboxylic acid transporters), member 3	4.17
Glt25d2	glycosyltransferase 25 domain containing 2	4.48
Bnip3	BCL2/adenovirus E1B interacting protein 3	4.68
Arg1	arginase, liver	8.29
Xpnpep2	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	5.23
Gm3367	predicted gene 3367	6.12
Muc2	mucin 2	6.05
Dapk2	death-associated protein kinase 2	4.55
Plod2	procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	5.25
F13a1	coagulation factor XIII, A1 subunit	5.63
Ak4	adenylate kinase 4	4.67
Nos2	nitric oxide synthase 2, inducible	4.02
Nrbp2	nuclear receptor binding protein 2	4.29
5430405G05Rik	RIKEN cDNA 5430405G05 gene	4.11

Sh3gl3	SH3-domain GRB2-like 3	4.22
Kbtbd11	kelch repeat and BTB (POZ) domain containing 11	4.21
Apln	apelin	4.11
Plekha6	pleckstrin homology domain containing, family A member 6	4.34

Additionally, I was able to identify a cluster of small G-protein related factors, which were altered in hypoxia in both genotypes (Table 3.2). The hypoxic regulation of these factors was not described so far. The Rho GTPase activating protein 29 (ArhGAP29) was found to be induced in hypoxia. It is markedly involved in controlling Rho activity and cytoskeletal dynamics in hypoxia. This finding was in the meantime verified in other cell lines by members of the Institute of Cardiovascular Physiology and initiated an independent project on the hypoxic regulation of ArhGAP29 and the implication of this for the regulation of the actin cytoskeleton.

Table 3.2	**List of Rho-GTPase associated genes, which were identified to be differentially expressed in hypoxia**
Arhgap19	Rho GTPase activating protein 19
Arhgdig	Rho GDP dissociation inhibitor (GDI) gamma
Arap2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2
Srgap3	SLIT-ROBO Rho GTPase activating protein 3
Arhgap29	Rho GTPase activating protein 29
Kalrn	kalirin, RhoGEF kinase
Arhgap28	Rho GTPase activating protein 28
Arhgap15	Rho GTPase activating protein 15
Fgd2	FYVE, RhoGEF and PH domain containing 2
Arhgap21	Rho GTPase activating protein 21
Arhgap27	Rho GTPase activating protein 27
Plekhg6	pleckstrin homology domain containing, family G (with RhoGef domain) member 6
Arhgef19	Rho guanine nucleotide exchange factor (GEF) 19
Syde1	synapse defective 1, Rho GTPase, homolog 1 (*C. elegans*)

It has already been reported that apart from HIFα under specific circumstances PHDs can regulate additionally NFκB activity (Scholz *et al.*, 2013; Cummins *et al.*, 2006). To test, if the NFκB response was altered as a consequence of the PHD3 knockout, wt and PHD3$^{-/-}$ BMDMs were cultured under control and starved conditions (no FCS in normoxia) for 24 hrs or stimulated with 100 ng LPS for 12 hrs. Subsequently

cells were lysed and NFκB activity was analyzed. The assay revealed that in the PHD3$^{-/-}$ BMDM there was no obvious difference in NFκB activity compared to the wt cells under resting conditions or after serum starvation (Figure 18). After stimulation with LPS, however, the wt BMDM demonstrated higher NF-kB activity compared to the PHD3$^{-/-}$ BMDM, which is in line with the above referenced work that has uncovered mechanisms by which hypoxia modulates the activation of NFκB through decreased oxygen-dependent suppression of the key regulators of this pathway (Scholz *et al.*, 2013; Cummins *et al.*, 2006).

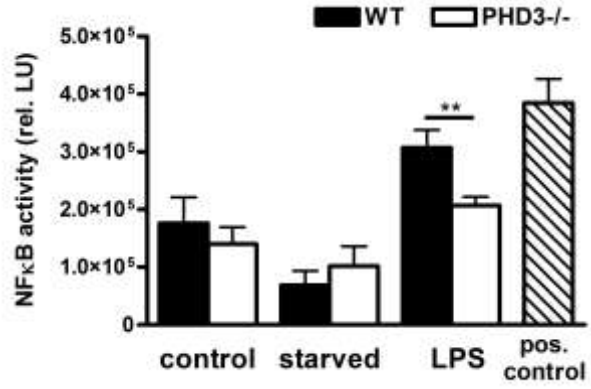


Figure 18: **NFκB activity in wt and PHD3$^{-/-}$ macrophages**

PHD3$^{-/-}$ and wt BMDM were cultured with or without serum for 24 hrs or stimulated with 100 ng LPS for 12 hrs. Subsequently NFκB activity was analyzed. As a positive control for the NFκB activity assay a cell extract of HeLa cells treated with LPS, was used. Shown are mean values ± SD, n=3, **p<0.01.

3.5 Macrophage polarization is unaffected in PHD3$^{-/-}$ BMDM

Macrophages display remarkable plasticity and can change their phenotype upon stimulation (Mosser and Edwards, 2008). The most prominent macrophage populations are the so called M1 and M2-polarized macrophages. M1-polarized macrophages are pro-inflammatory and tissue destructive, whereas M2-polarized macrophages are anti-inflammatory and involved in tissue remodelling as well as angiogenesis. Macrophage polarization can be affected by HIF-1α and HIF-2α (Takeda *et al.*, 2010) (Takeda et al). To analyze if PHD3 affects macrophage polarization we treated wt and PHD3$^{-/-}$ BMDM, which were differentiated for 8 days, with LPS and the T_H1 cytokine IFNγ or the T_H2 cytokine IL-4 to functionally characterize M1 and M2-polarization, respectively (Fig. 19 a and b). Successful M1 and M2-polarization after stimulation was verified by increased RNA levels of the M1 markers IL-6, iNOs and TNFα as well as the M2 markers arginase, Ym-1 and Fizz, respectively. Expression levels of the M1 and M2 marker RNAs were not different comparing wt and PHD3$^{-/-}$ BMDM.

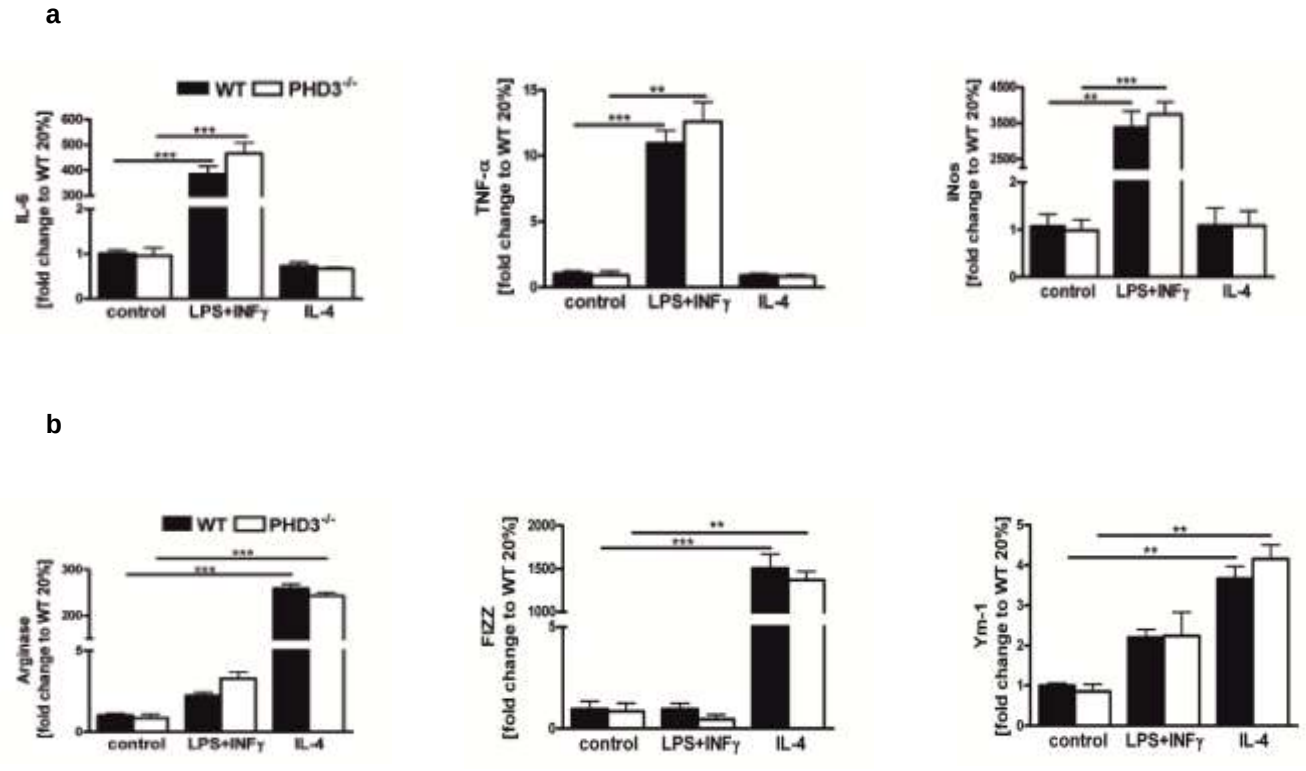


Figure 19: Macrophage polarization of PHD3$^{-/-}$ macrophages

PHD3$^{-/-}$ and wt BMDM, which were differentiated for 8 days, were stimulated with LPS+INFγ (M1-polarization) or IL-4 (M2-polarization) for 6 hrs, subsequently RNA levels for the (a) M1-polarization markers IL-6, iNos and TNFα (b) the M2-polarization markers Arginase, Ym-1 and FIZZ were quantified by qRT-PCR. Shown are mean values ± SD, n=3, **p<0.01, ***p<0.001.

The above findings were further confirmed at protein level, by investigating the concentrations of IL-6 and TNFα in the cell culture supernatants. Similar to the above described experiments wt and PHD3$^{-/-}$ BMDM, which were differentiated for 8 days, were treated with LPS and the T_H1 cytokine IFNγ. Then the culture supernatant was harvested and FACS analyses were performed to investigate the cytokine concentrations. IL-6 and TNFα protein levels were significantly induced in the cell culture supernatants after stimulation with LPS+IFNγ (Fig. 20). In line with the RNA data, the concentrations of IL-6 and TNFα were not different comparing wt and PHD3$^{-/-}$ BMDM.

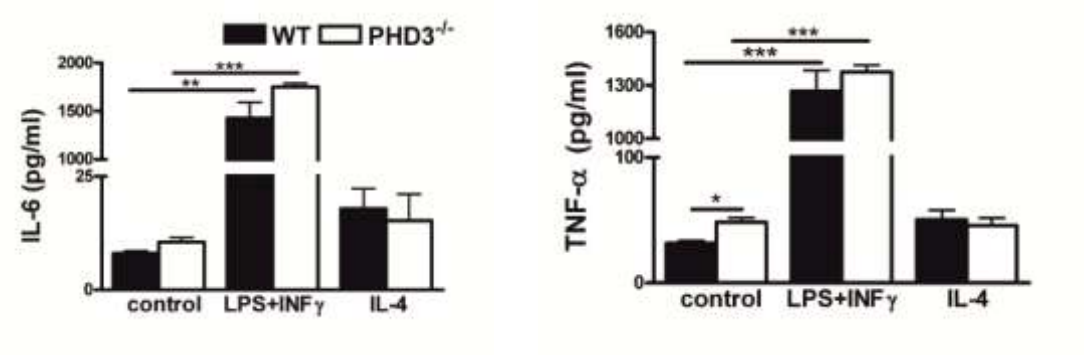


Figure 20: **IL-6 and TNFα secretion of PHD3$^{-/-}$ macrophages**

PHD3$^{-/-}$ and wt BMDM were stimulated with LPS+INFγ (M1-polarization) or IL-4 (M2-polarization) for 6 hrs, subsequently protein levels for the M1-polarization markers IL-6 and TNFα were quantified by FACS analysis. Shown are mean values ± SD, n=3, **p<0.01, ***p<0.001.

3.6 PHD3$^{-/-}$ BMDM are protected from apoptotic cell death

PHD3 has been associated with cell survival decisions in the context of apoptosis in various cell and animal models (Bishop *et al.*, 2008) (Wottawa *et al.*, 2010) (Jaakkola and Rantanen, 2013). Therefore, we next determined the role of PHD3 in macrophage cell-survival pathways. To analyze, if apoptosis is altered in the PHD3$^{-/-}$ BMDM I deprived cells for 48 hrs from serum (Fig. 21). Starving the cells induced apoptosis in wt BMDM as determined by quantifying TUNEL positive cells which marks the fragmented DNA/damaged DNA in the cell. Most interestingly in PHD3$^{-/-}$ BMDM, apoptosis was just slightly induced under starvation compared to wt cells. Thus, the PHD3$^{-/-}$ macrophages were significantly protected under stress condition when compared to the respective wt macrophages.

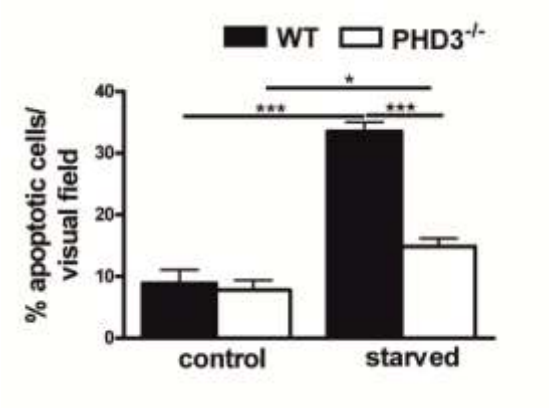


Figure 21: **TUNEL assay comparing wt and PHD3$^{-/-}$ BMDM apoptosis**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without serum (starved) for 48 hrs, subsequently the number of apoptotic cells was quantified by TUNEL assay. A knockout of PHD3 in macrophages significantly protects the cells from apoptosis when serum starved. Shown are mean values ± SD, n=3, *p<0.05, ***p<0.001.

The above obtained result was further confirmed by Annexin V assays. With this assay the membrane integrity of the cells is analyzed to investigate the amount of apoptotic cells. Both wt and PHD3$^{-/-}$ BMDM were cultured for 48 hrs in control or starved conditions. Afterwards the cells were stained with an anti-Annexin V antibody and were analyzed using FACS. In line with the TUNEL assay, the Annexin V assay showed that starving the cells induced apoptosis in wt BMDM whereas PHD3$^{-/-}$ macrophages were significantly protected. (Fig. 22)

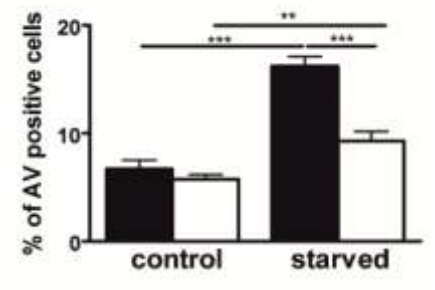


Figure 22: **Annexin V staining of wt and PHD3$^{-/-}$ BMDM**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without serum (starved) for 48 hrs, subsequently the number of Annexin V positive cells were quantified. PHD3$^{-/-}$ in macrophages significantly protects the cells from apoptosis when serum starved. Shown are mean values ± SD, n=6, **p<0.01, ***p<0.001.

The partial protection of PHD3$^{-/-}$ BMDM after serum starvation was further confirmed by performing Calcein-AM assays to see if the anti-apoptotic effect is reflected in altered viability of the cells. Indeed, PHD3$^{-/-}$ BMDM had a higher viability under control conditions and after 48 hrs of starvation compared to wt cells (Fig. 23).

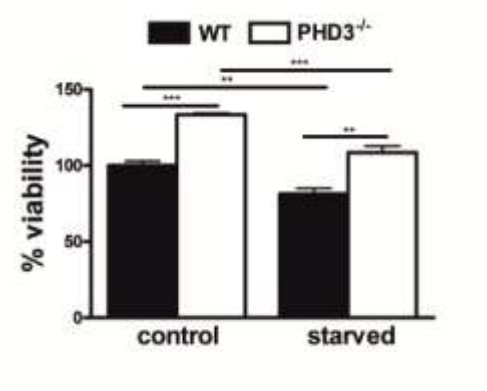


Figure 23: **Viability assay wt and PHD3$^{-/-}$ BMDM**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without serum (starved) for 48 hrs and then the cell viability was determined using the Calcein-AM viability assay. PHD3$^{-/-}$ BMDM showed significantly increased viability both in control and starved conditions. n=6, mean ± SD, **p<0.01, ***p< 0.001.

To analyze if the PHD3 mediated anti-apoptotic effect is just limited to serum deprivation, macrophages were cultured in the presence of the known pro-apoptotic agents S-nitroso-N-acetylpenicillamine (SNAP, 100 µM), staurosporine (stauro, 500 nM) or LPS (5 or 10 µg/mL) and rates of apoptosis were determined (Fig. 24). The result obtained showed that in the presence of the pro-apoptotic agents tested PHD3$^{-/-}$ macrophages were significantly protected as compared to the respective wt BMDM.

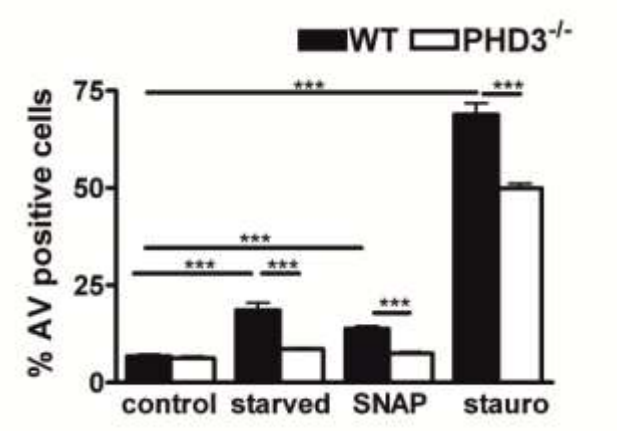


Figure 24: Annexin V assay staining of wt and PHD3$^{-/-}$ BMDM with apoptotic inducers treatment

The number of Annexin V-positive cells were quantified after cultivating PHD3$^{-/-}$ and wt BMDM with or without (starved) serum for 48 hrs or treating the cells with 100 µM SNAP, 500 nM stauro or 5-10 µg/mL LPS for 12 hrs. n=5, mean ± SD, ***p< 0.001.

In addition to the treatment with pro-apoptotic agents in normoxia the PHD3-mediated phenotype was also confirmed under hypoxic conditions. To analyze the impact of hypoxia on serum starvation induced apoptosis I exposed wt and PHD3$^{-/-}$ BMDM to normoxia (20% O_2) or hypoxia (1% O_2) during serum

starvation. Both wt and PHD3$^{-/-}$ BMDM were cultured for 48 hrs in control or starved conditions at 20% O_2 and 1% O_2 (Fig. 25).Then TUNEL assays were performed and the cells were analyzed to determine the amount of apoptotic cells. In normoxia as well as in hypoxia PHD3$^{-/-}$ BMDM were significantly protected from apoptosis compared to wt macrophages. In hypoxia however, the protective effect was slightly but not significantly weakened in the PHD3$^{-/-}$ BMDM. This correlates to the hypoxic induction of PHD3 as demonstrated above, which was present albeit less significantly in the PHD3$^{-/-}$ macrophages.

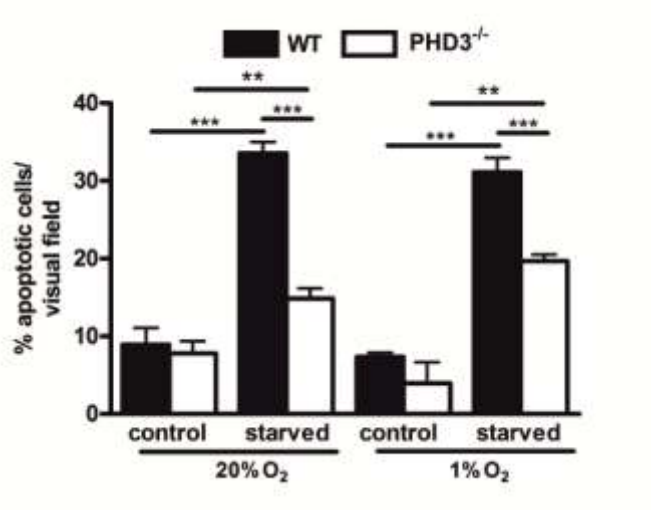


Figure 25: TUNEL assay of wt and PHD3$^{-/-}$ BMDM comparing normoxic versus hypoxic effects

TUNEL-positive cells were quantified after cultivating PHD3$^{-/-}$ and wt BMDM with or without serum (starved) for 48 hrs at 20% O_2 or 1 % O_2. n = 3, mean ± SD, **p<0.01, ***p< 0.001.

To gain insight into the apoptotic phenotype an unbiased transcriptome analysis was performed. Using a mouse genome array we compared RNA transcript abundance in wt and PHD3$^{-/-}$ BMDM, which were deprived of serum for 48 hrs. Among the transcript changes, which differed in their extent in response to the serum deprivation between wt and PHD3$^{-/-}$ BMDM, I identified in total 26 transcripts that were up-regulated (Table 3.3) and 19 transcripts that were down-regulated in response to serum starvation (Table 3.4).

Table 3.3: Genes of the RNAs, which were identified to be induced and differentially expressed comparing wt and PHD3$^{-/-}$ BMDM after serum starvation

Gene ID	Symbol	Description
56473	Fads2	fatty acid desaturase 2
17392	Mmp3	matrix metallopeptidase 3
14990	H2-M2	histocompatibility 2, M region locus 2
26360	Angptl2	angiopoietin-like 2
76009	5830415G21Rik	RIKEN cDNA 5830415G21 gene
14858	Gsta2	glutathione S-transferase, alpha 2 (Yc2)
246049	Slc36a2	solute carrier family 36 (proton/amino acid symporter), member 2
14238	Foxf2	forkhead box F2
68027	Tmem178	transmembrane protein 178
18548	Pcsk1	proprotein convertase subtilisin/kexin type 1
17384	Mmp10	matrix metallopeptidase 10
20408	Sh3gl3	SH3-domain GRB2-like 3
19746	Rhd	Rh blood group, D antigen
13051	Cx3cr1	chemokine (C-X3-C) receptor 1
19124	Procr	protein C receptor, endothelial
15490	Hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7
207683	Igsf11	immunoglobulin superfamily, member 11
16197	Il7r	interleukin 7 receptor
79201	Tnfrsf23	tumor necrosis factor receptor superfamily, member 23
56018	Stard10	START domain containing 10
228543	Rhov	ras homolog gene family, member V
217265	Abca5	ATP-binding cassette, sub-family A (ABC1), member 5
12614	Celsr1	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)
627270	Gm10055	predicted gene 10055
320121	6720473M08Rik	RIKEN cDNA 6720473M08 gene
194126	Mtmr11	myotubularin related protein 11

Table 3.4: Genes of the RNAs, which were identified to be reduced and differentially expressed comparing wt and PHD3$^{-/-}$ BMDM after serum starvation

Gene ID	Symbol	Description
20511	Slc1a2	solute carrier family 1 (glial high affinity glutamate transporter), member 2
69590	Gpx8	glutathione peroxidase 8 (putative)
12035	Bcat1	branched chain aminotransferase 1, cytosolic
17472	Gbp4	guanylate binding protein 4
64074	Smoc2	SPARC related modular calcium binding 2
20350	Sema3f	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F
109264	Me3	malic enzyme 3, NADP(+)-dependent, mitochondrial
241230	St8sia6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6
105785	Kdelr3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3
16429	Itln1	intelectin 1 (galactofuranose binding)
12478	Cd19	CD19 antigen
12837	Col8a1	collagen, type VIII, alpha 1
69675	Pxdn	peroxidasin homolog (Drosophila)
20692	Sparc	secreted acidic cysteine rich glycoprotein
20204	Prrx2	paired related homeobox 2
12831	Col5a1	collagen, type V, alpha 1
22402	Wisp1	WNT1 inducible signaling pathway protein 1
98932	Myl9	myosin, light polypeptide 9, regulatory
18054	Ngp	neutrophilic granule protein

3.7 Confirming the differential expression of genes by qRTPCR

The transcriptome screen revealed 45 genes to be differentially expressed in starved PHD3$^{-/-}$ compared to wt BMDM. To further check the quality of the screen, qRT PCR analyses of some of the identified genes were done (Fig. 26). The RNA of the proprotein convertase subtilisin/kexin type 1 (Pcsk1) and the protein C receptor (Procr) were found to be induced by serum starvation. This was verified by the independent qRT-PCR analyses. Additionally, the differential induction of Pcsk1 and Procr comparing wt and PHD3$^{-/-}$ BMDMs was verified. Whereas the induction of Pcsk1 after serum starvation was significantly higher in the wt BMDMs compared to the PHD3$^{-/-}$ BMDMs, the opposite was found for Prcor. The differential expression

of WNT1 inducible signaling pathway protein (WISP1) and paired related homeobox 2 (Prrx2), which were both reduced after serum starvation, was verified likewise.

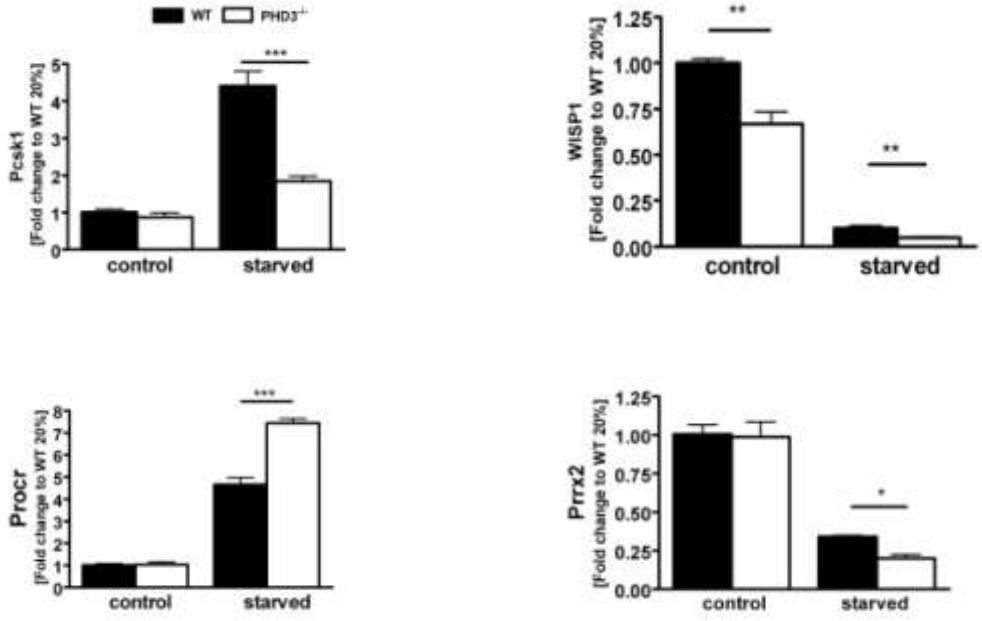


Figure 26: qRT-PCR analysis confirming the differential expression of candidate genes

PHD3$^{-/-}$ and wt BMDM were cultivated with or without serum for 48 hrs (starved). Subsequently genes from the transcriptome screen Pcsk1, WISP1, Procr and Prrx2 mRNA were analyzed by qRT-PCR. n = 6, mean ± SD, *p<0.05, ***p<0.001.

3.8 Angptl2 is differentially expressed and involved in the differential apoptotic response

Pro- and anti-apoptotic pathways can be induced by secreted factors. To test, if secreted factors are involved in the differential induction of apoptosis of wt and PHD3$^{-/-}$ BMDMs, I performed experiments with wt or PHD3$^{-/-}$ conditioned medium. Treating wt and PHD3$^{-/-}$ BMDM during serum deprivation with conditioned medium of serum-deprived PHD3$^{-/-}$ and wt cells, respectively, revealed a loss of the anti-apoptotic phenotype in the PHD3$^{-/-}$ BMDM (Fig. 27). This indicated that at least in part a secreted factor is involved in the PHD3-mediated response towards an apoptotic stimulus.

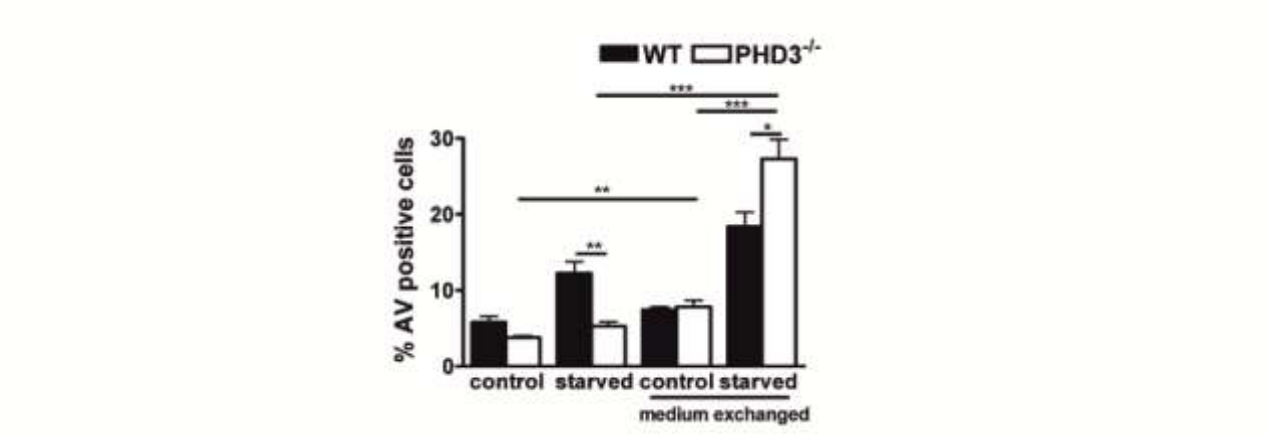


Figure 27: **wt conditioned medium induces apoptosis of PHD3$^{-/-}$ macrophages**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without serum in total for 48 hrs. After 24 hrs the medium was exchanged across the genotypes and cultured for 24 additional hrs. Induction of apoptosis was compared using Annexin V staining and FACS analysis. n = 5 mean ± SD, **p<0.01, ***p< 0.001.

The result shown in Fig. 27 made me to go back to the transcriptome screen and look specifically for soluble/secreted factors. Further gene cluster analysis of the 45 genes identified in the above described transcriptome array revealed a cluster of genes (i.e. Angptl2, Col8A1, Col5A1, KDELR3, MYL9 and PXDN), which are known to be co-regulated. Angiopoietin-like proteins (Angptls) are secreted proteins, which like angiopoietin itself have an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. So far 7 Angptls have been identified. None of these bind to the angiopoietin receptor Tie2 or to the homologous Tie1 receptor. Angptl2 has been found to affect vascular cells and monocytes. In line Angptl2 functions as a chronic inflammatory mediator in obesity (Tabata *et al.*, 2009), atherosclerotic disease (Tabata *et al.*, 2009), rheumatoid arthritis (Tabata *et al.*, 2009) (Okada *et al.*, 2010), and cancer (Endo *et al.*, 2012). Although the receptor signaling of Angptl2 is not fully understood, some of these biological effects seem to be at least in part mediated via binding to $\alpha5\beta1$ integrin and the paired immunoglobin-like receptor B (PirB) or human orthologue immunoglobin-like receptor B2 (LILERB2) (Tabata *et al.*, 2009). The differential expression of Angptl2 in wt and PHD3$^{-/-}$ BMDM after serum starvation was verified by independent qRT-PCR (Fig. 28). To analyze, if hypoxia affects the expression of Angptl2, I additionally exposed the wt and PHD3$^{-/-}$ BMDM during serum starvation to 20% O_2 or 1% O_2. Exposure of the macrophages to hypoxia during starvation significantly increased the expression of Angptl2 in the wt but not in the PHD3$^{-/-}$ BMDM.

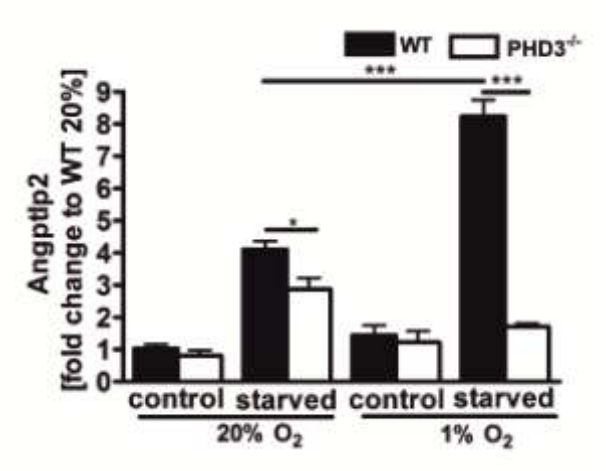


Figure 28: **Angptl2 expression of wt and PHD3$^{-/-}$ macrophages**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without (starved) serum for 48 hrs in normoxia (20% O_2) or hypoxia (1% O_2). Subsequently Angiopoietin-like protein 2 (Angptl2) mRNA was analyzed by qRT-PCR. n = 6, mean ± SD, *p<0.05, ***p< 0.001.

In the above described experiments I was able to show that the protection from apoptosis in the PHD3$^{-/-}$ BMDM is not just restricted to the induction of apoptosis after serum starvation. SNAP and stauro treatment resulted in an altered induction of apoptosis in the PHD3$^{-/-}$ BMDM likewise. Therefore, Angptl2 RNA levels were quantified in wt and PHD3$^{-/-}$ BMDM after treatment with SNAP or stauro to proof that the altered Angptl2 response of the PHD3$^{-/-}$ BMDM does occur after treatment with several different apoptotic stimuli. Serum starvation was included in this experiment as positive control. Like in starvation, the Angptl2 RNA levels were less in the PHD3$^{-/-}$ BMDM compared to wt cells after SNAP and stauro treatment (Fig. 29).

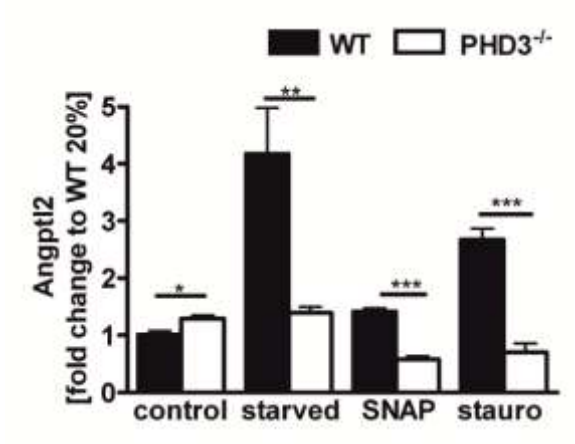


Figure 29: **Angptl2 expression under the treatment of apoptotic inducers**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without serum (starved) or treated with 100 µM S-nitroso-N-acetylpenicillamine (SNAP) or 500 nM staurosporine (stauro). Subsequently Angiopoietin-like protein 2 (Angptl2) mRNA was analyzed. n = 6, mean ± SD, *p<0.05, **p<0.01, ***p< 0.001. Serum starvation was included in the experiment as positive control.

The differential expression of Angptl2 in wt and PHD3$^{-/-}$ BMDM was verified at protein level using an ELISA. For this wt and PHD3$^{-/-}$ BMDM were cultured for 48 hrs both in control and starved conditions. The supernatants were harvested to estimate the Angptl2 concentration in these samples. In line with the mRNA data the Angptl2 protein concentration was less in PHD3$^{-/-}$ BMDM cell culture supernatants compared to wt (Fig. 30).

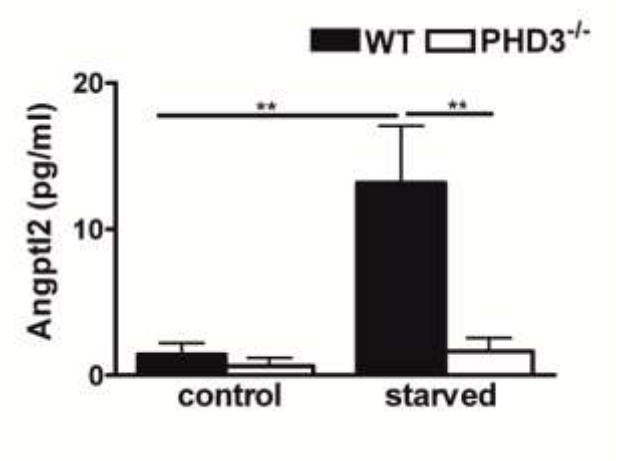


Figure 30: **Angptl2 expression and secretion at protein level**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without (starved) serum for 48 hrs. Subsequently, cell culture supernatants were analyzed for Angiopoietin-like protein 2 (Angptl2) protein levels by ELISA. n = 6, mean ± SD, **p<0.01.

The function of Angptl2 in macrophages is not fully described so far. Moreover, there were at the time no data available, if Angptl2 can affect apoptosis of macrophages. Therefore I analyzed in cooperation with Prof. Y. Oike (Department of Molecular Genetics, Kumamoto University, Japan) the induction of serum starvation-induced apoptosis in wt and Angptl2 knockout (Angptl2$^{-/-}$) BMDM. The group of Prof. Y. Oike was able to produce Angptl2$^{-/-}$ mice in the past (Tabata, Kadomatsu et al. 2009). These animals were used for the analyses. In line with the anti-apoptotic effect and diminished expression of Angptl2 in the PHD3$^{-/-}$ cells, BMDM, which were isolated and differentiated from previously described Angptl2$^{-/-}$ mice, exhibited less AV-positive cells after serum starvation or stauro treatment (Fig. 31).

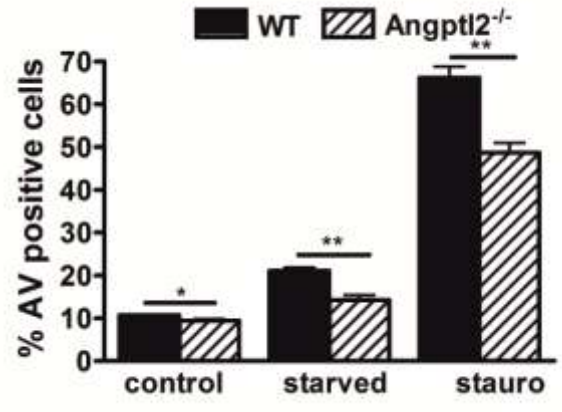


Figure 31: **Effect of recombinant Angptl2 on wt BMDM**

Angptl2$^{-/-}$ and wt BMDM were cultivated with or without serum (starved) for 48 hrs or exposed to 500 nM staurosporine (stauro) for 12 hrs. Subsequently, induction of apoptosis was compared by Annexin V staining and FACS analysis. n = 4-5, mean ± SD, *p<0.05, **p<0.01.

3.9 Treatment of BMDM with recombinant Angptl2

In the final experiments I tested, if reconstitution of the diminished Angptl2 response in the PHD3 deficient macrophages would rescue the apoptosis phenotype (Fig. 32). Therefore 2 or 4 µg recombinant Angptl2 was added to wt or PHD3$^{-/-}$ BMDM during serum starvation. 48 hrs later, the number of Annexin V-positive cells was analyzed. Adding the recombinant Angptl2 to the PHD3$^{-/-}$ BMDM indeed reversed the anti-apoptotic effect demonstrating that Angptl2 is at least partially involved in the PHD3-mediated response towards an apoptotic stimulus. Most interestingly, wt BMDM did not respond with increased apoptosis after adding 2 µg recombinant Angptl2 under resting conditions or after starvation. After adding 4 µg recombinant Angptl2 there was even an anti-apoptotic effect after starvation These data indicate that not only the production of Angptl2 is altered in PHD3$^{-/-}$ BMDM but also the cellular response to it.

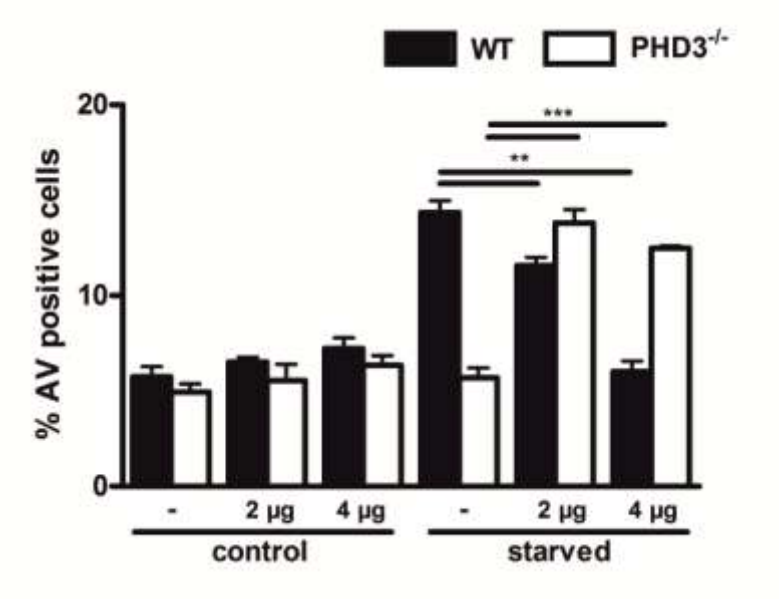


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Figure 32: **Treatment of wt and PHD3$^{-/-}$ BMDM with recombinant Angptl2**

PHD3$^{-/-}$ and wt BMDM were cultivated with or without serum (starved) in total for 48 hrs. Recombinant Angiopoietin-like protein 2 (Angptl2) was added as indicated. Induction of apoptosis was analyzed by Annexin V staining and FACS analysis. n = 5, mean ± SD, **p<0.01, ***p<0.001.

As described above, $\alpha 5\beta 1$ and PirB were previously identified to bind to Angptl2. This does not include that this orphan ligand does not signal via other receptors. Since the results with the recombinant Angptl2 indicate that the response towards it might be altered in the PHD3$^{-/-}$ BMDM, I analyzed the cell surface

expression of $\alpha5\beta1$ and PirB in wt versus PHD3$^{-/-}$ BMDM (Fig. 33). Both receptors could be identified by FACS analysis. The receptor expression levels, however, were not different comparing the BMDM of both genotypes.

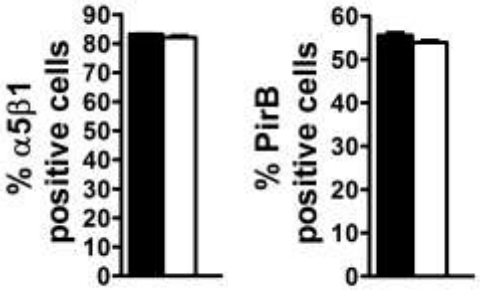

Figure 33: Expression of Angptl2 receptors in wt and PHD3$^{-/-}$ macrophages

$\alpha5\beta1$ and PirB receptor expression was analyzed in PHD3$^{-/-}$ (white bars) and wt (black bars) BMDM by FACS analysis. n= 5, mean ± SD

4. Discussion

Adequate supply of oxygen is an essential prerequisite for living organisms. Insufficient oxygen availability is known as hypoxia. The oxygen sensors PHD 1-3 sense hypoxia and respond to it by regulating the master transcription factor HIF by prolyl hydroxylation at specific prolyl residues, marking the HIFα subunit for proteasomal degradation. HIF-1α is predominantly hydroxylated by PHD2 and HIF-2α by PHD3. Although the PHD shares many similarities, they differ in enzymatic activity, expression pattern and specific function. Among the three PHD isoforms, the constitutively expressed PHD2 is the main regulator of HIFα stability in normoxia. In contrast PHD3, which is induced under hypoxia, creates a feedback loop to further regulate HIFα under hypoxia and upon reoxygenation. To gain further insight into the specific function of different PHD isoforms is important for the ongoing development of small molecule PHD inhibitors that are currently tested in preclinical and clinical trials for the treatment of anemia and for cytoprotection. Interfering with any of these PHD isoforms might alter the response of the immune system. It has been shown earlier that, tissue inflammation can lead to hypoxia, resulting in stabilization of HIF-1α or HIF-2α in the inflamed tissues. Therefore, the interplay and the close connection between PHDs and immunomodulators have to be studied in more detail. Additionally, hypoxia and HIFs are known to be crucial modulators of systemic inflammation (Imtiyaz *et al.*, 2010; Zinkernagel *et al.*, 2007). PHDs are also known to be expressed in leukocytes and macrophages (Peyssonnaux *et al.*, 2005). Myeloid-derived cells are rapidly mobilized to hypoxic areas of ischemic tissues and are acknowledged to contribute to post-ischemic regeneration. They adapt rapidly through the activation of the HIF signaling pathway. Targeting the PHD/HIF-pathway in the myeloid cell lineage would therefore be a promising strategy to investigate the isoform and cell specific function of PHDs. In the past few years the importance of myeloid cells for angiogenesis and neovascularization in hypoxia-associated disorders, like arterial occlusion or atherosclerotic plaques, became evident. Loss of PHD3 is known to enhance the innate immune response (Kiss *et al.*, 2012) and regulate the life span of neutrophils under hypoxia (Walmsley *et al.*, 2011). The specific role of PHD3 in macrophages however was less explained so far. For this purpose a myeloid-specific PHD3 knockout mouse has been established.

4.1 HIF target gene expression is unchanged in PHD3$^{-/-}$ BMDM

To investigate the role of PHD3 in BMDMs myeloid-specific PHD3$^{-/-}$ mice were used. The myeloid-specific mouse line was generated using the conditional Cre/loxP knockout strategy. The Cre recombinase was expressed under the control of LysM promoter. Through this conditional knockout strategy I generated BMDMs with a knockout efficiency of roughly 75% (Fig. 13). The motive of using a conditional knockout is that it more or less resembles the pharmacological inhibition of the PHD enzymes. PHD enzymes are currently tested for their potential to be used as drug targets to stimulate HIF-dependent gene expression (Myllyharju, 2008). This strategy is developed for applications in the context of ischemia tissue protection and severe anemia (Katschinski, 2009). The strong hypoxic induction of PHD3 is even conserved in BMDMs which has already been described in myeloid cells in several publications (Rohrbach *et al.*, 2005; Appelhoff *et al.*, 2004; Walmsley *et al.*, 2011) .

Knocking out PHD3 in myeloid cells affects neither the morphology nor the differentiation of 8 days differentiated BMDMs (Fig. 11 and Fig. 12). However the process of differentiation was altered in PHD3$^{-/-}$ BMDMs observed by F4/80 and CD11b staining (Fig. 11). The PHD3$^{-/-}$ BMDM showed a higher differentiation efficiency over time. This is in line with an earlier study (Kiss *et al.*, 2012) analyzing constitutive PHD3$^{-/-}$ BMDMs. My results confirmed that PHD3 might be involved in the differentiation process of macrophages.

The PHD3$^{-/-}$ BMDM do not alter the expression of the other HIF-regulating isoforms of PHDs, i.e. PHD1 and PHD2, suggesting that no compensatory effect occurs (Fig. 14). In addition the knockout does not show increased HIF-1α and HIF-2α protein levels in normoxia and hypoxia (Fig. 15). Further, HIF target gene expression in the knockout macrophages do not seem to be regulated both in normoxic and hypoxic conditions, but as expected hypoxic induction of HIF target genes was confirmed (Fig. 16). Even in the unbiased transcriptome screen, I could indeed not find any significantly differentially expressed HIF target gene in the PHD3$^{-/-}$ BMDM (Fig. 17). My results show that knocking out PHD3 in myeloid cells is not sufficient to affect HIF dependent pathways.

4.2 Macrophage polarization is unaffected in PHD3$^{-/-}$ BMDM

Plasticity and diversity are the known hallmarks of the monocyte-macrophage differentiation pathway. Macrophages have huge versatility and can change their phenotype upon stimulation (Mosser and Edwards, 2008). It has been described in the literature that macrophages can differentiate into two subpopulations, which carry out distinct but time overlapping functions. These subpopulations can be broadly categorized as classically activated macrophages (M1) which consist of the pro-inflammatory macrophage subset, induced by interferon-γ and/or LPS and alternatively activated macrophages (M2) which consist of an anti-inflammatory macrophage subset induced by exposure to IL-4. M1 polarized macrophages are tissue destructive and remove the damaged tissue whereas M2 polarized macrophages induce tissue remodeling and angiogenesis (Fernando *et al.*, 2014; Mosser and Edwards, 2008). The ability of macrophages to reprogram their function blurs in a way the distinction between innate and adaptive immune response. Moreover, evidence shows that the orchestration of macrophage function and polarization has a key role in different pathological conditions (Mantovani *et al.*, 2004).

Literature suggests that both HIF-1α and HIF-2α affect macrophage polarization. It is known that hypoxia can lead to macrophage inflammatory functions, e.g the production of cytokines by macrophages increases under hypoxia. LPS treatment induces stabilization of HIFα in macrophages (Frede *et al.*, 2006; Takeda *et al.*, 2010). It has been shown that 5 day differentiated constitutively knocked out PHD3 macrophages are M1 polarized (Kiss *et al.*, 2012). In contrast to this, my quantitative real-time PCR analysis showed no significant difference in induction of either M1 or M2 markers in PHD3$^{-/-}$ macrophages (Fig. 19), nor does my protein expression analysis showed any difference in induction of cytokines upon exposure LPS, IFNγ and IL-4 in PHD3$^{-/-}$ macrophages (Fig. 20). This suggests, that 8 days differentiated myeloid specific PHD3$^{-/-}$ BMDMs are not significantly affected in their proinflammatory function.

4.3 NFκB activity is unaffected in PHD3$^{-/-}$ BMDM

NFκB regulates the expression of pro-inflammatory molecules including cytokines and chemokines (Balkwill and Mantovani, 2001). PHDs have been shown to impact in the regulation of NFκB (Cummins *et al.*, 2006a). It has been demonstrated that PHDs are repressors of NFκB activity, via their potential to hydroxylate the IKB kinase (Cummins *et al.*, 2006). Further it has been described that PHD3 can associate with IKKβ independently of its hydroxylase function, thereby blocking the interaction between IKKβ and HSP90, which is required for IKKβ phosphorylation and release of NFκB (Xue *et al.*, 2010). In addition, loss of function studies indicate that PHD3 serves as a co-activator of NFκB signaling activity (Fujita *et al.*, 2012). It was therefore then worthwhile to speculate that loss of PHD3 in my BMDMs might regulate NFκB activity (Hatada *et al.*, 2000).

In my study, I could show that PHD3$^{-/-}$ BMDM do not have any obvious difference in NFκB activity compared to the wt cells (Fig. 18). Activation of macrophages with LPS treatment, however, showed less NFκB activity in PHD3$^{-/-}$ BMDM, which is in line with the above referenced work that has uncovered mechanisms by which hypoxia modulates the activation of NFκB through decreased oxygen-dependent suppression of the key regulators of this pathway (Cummins *et al.*, 2006; Scholz *et al.*, 2013).

4.4 PHD3 plays a pro-apoptotic role in BMDM

A huge number of recent studies support the role of PHD3 in cell differentiation, survival, apoptosis, and metabolism. A clear role for PHD3 in neuronal apoptosis suggests that PHD3 mutations may be involved in the progression of neuronal malignancies. On the other hand, inhibition of PHD3 activity leads to survival of cancer cells (Lee *et al.*, 2005). Apart from the role of PHD3 in other cell types, a major role of PHD3 on cell survival in myeloid cells is well described (Walmsley *et al.*, 2011; Kiss *et al.*, 2012). Also my analysis of cell survival in the conditional PHD3$^{-/-}$ BMDM cells showed a pronounced apoptotic phenotype (Fig. 21). A major impact of PHD3 on cell survival is in line with the findings, that deficient PHD3 neutrophils are not significantly altered regarding their pro-inflammatory function but regarding their apoptotic phenotype (Walmsley *et al.*, 2011). At the molecular level this has been associated with a

PHD3-dependent regulation of KIF1β, HCLK2, SIVA and ATF-4 (Schlisio *et al.*, 2008; Walmsley *et al.*, 2011; Xie *et al.*, 2012; Koditz *et al.*, 2007).

My studies demonstrated that under stress condition such as starvation PHD3$^{-/-}$ BMDMs have significantly lower DNA damage (Fig. 21). These results indicate that knocking out PHD3 in BMDM protects the cells from apoptosis under stress condition. To further confirm the impact of PHD3 on BMDM, I analyzed the membrane integrity of the cells (Fig. 22) and could confirm that PHD3 plays a pro-apoptotic role in BMDMs. Further I did experiments to investigate the BMDM viability, which showed an increased viability of the PHD3$^{-/-}$ BMDMs (Fig. 23). Apart from the role of starvation for PHD3 mediated apoptosis my results also revealed that PHD3$^{-/-}$ BMDM are significantly more protected to other apoptotic inducers such as staurosporine which causes ER stress or SNAP which causes oxidative stress (Fig. 24). Taken together, the above results demonstrate for the first time that PHD3 is involved in cell survival decisions in macrophages.

4.5 Loss of PHD3 affects the expression of Angptl2

To get an insight into the underlying molecular mechanism of the pro-apoptotic role of PHD3 in BMDMs, I performed a transcriptome screen in collaboration with the Transkriptions Analyse Labor (TAL, University Medical Center Göttingen). In the unbiased screen, none of the known target genes of PHD3-dependent regulation like KIF1β, HCLK2, SIVA and ATF-4 (Köditz *et al.*, 2007; Schlisio *et al.*, 2008; Walmsley *et al.*, 2011; Xie *et al.*, 2012) were differentially regulated compared to wt BMDM. In the screen, 26 genes were found to be significantly induced under starvation (Table 3.3) and 19 genes were down regulated in a quantitatively different extent comparing wt versus PHD3$^{-/-}$ BMDM (Table 3.4). Among these 45 genes, further gene cluster analysis revealed a cluster (i.e. Angptl2, Col8A1, Col5A1, KDELR3, MYL9 and PXDN), which is known to be co-regulated and is associated with the regulation of the secretory pathway.

To further gain insight into the PHD3 mediated mechanism an experiment was performed involving the supernatant culture medium which contains the secreted proteins from the cells. The result revealed a loss of the anti-apoptotic phenotype in the PHD3$^{-/-}$ BMDM when treated with wt conditioned medium (Fig.

27). This indicated that at least in part a secreted factor is involved in the PHD3-mediated response towards an apoptotic stimulus.

One candidate of the transcriptome screen, Angptl2 seemed to be a promising factor involved in regulating the PHD3 mediated response. A significant difference in the level of the secretory glycoprotein Angptl2 both at RNA and protein level was found (Fig. 28-30). Angiopoietin-like proteins are structurally similar to the angiopoietin group of proteins which contain a coiled-coil domain in the N-terminus and a fibrinogen-like domain in the C-terminus. Angiopoietin-like proteins represent a multi-functional family of secreted factors with structural similarity to angiopoietin, a molecule with pro-angiogenic function (Kadomatsu *et al.*, 2011). Angptl2 is also known to induce chronic inflammation (Tazume *et al.*, 2012). Angptls do not bind to the Tie2 receptor, but it has recently been reported that the LILRB2 and PIRB are receptors for several Angptl family members (Zheng *et al.*, 2012; Oike *et al.*, 2004). Angptl2 is expressed in a variety of cells including monocytes, macrophages and in tumors (Okada *et al.*, 2010; Aoi *et al.*, 2011; Tazume *et al.*, 2012). Angptl2 has been reported to have a myriad of functions that are involved in inflammation, metabolism, angiogenesis and cancer (Kanda *et al.*, 2012; Tazume *et al.*, 2012; Tabata *et al.*, 2009). The data obtained in this study demonstrate that PHD3 affects the expression of Angptl2 both at protein and RNA levels (Figure 3.19-3.21). The results showed enhanced Angptl2 levels upon starvation but decreased Angptl2 expression in the PHD3$^{-/-}$ macrophages. Not only with starvation but also other stress inducers such as the NO donor SNAP and the ER stress inducer staurosporine induce the expression of Angptl2. In contrast to that, the treatment of BMDMs with SNAP and staurosporine results in decreased expression of Angptl2 in PHD3 deficient BMDMs which earlier proved to have decreased apoptosis (Fig. 28 and Fig. 29). Additionally, PHD3$^{-/-}$ BMDM also showed significantly less secretion of Angptl2 into the culture supernatant (Fig. 30). Above results suggest that differential expression and secretion of Angptl2 might play a role in influencing the apoptotic phenotype of the PHD3$^{-/-}$ BMDMs.

4.6 Loss of Angptl2 attenuates BMDM apoptosis

To analyze the functional role of the decreased expression of Angptl2 in PHD3$^{-/-}$ BMDM and the anti-apoptotic phenotype, our collaborator partner Prof. Yuichi Oike at Keio University, Japan investigated apoptosis in Angptl2$^{-/-}$ BMDM. Previously, it has been reported that in macrophages Angptl2 increases the

expression of pro-inflammatory cytokines and MMP-9 by activating an NFκB dependent cascade in infiltrating macrophages, which accelerates chronic inflammation and promotes destructive remodeling in the vessel wall (Tazume *et al.*, 2012). In this study the apoptosis of macrophage was not studied. In zebrafish however, Angptl2 knockdown showed minor vascular defects in embryos and also played a critical anti-apoptotic role in endothelial cells (Kubota *et al.*, 2005)

The impact of Angptl2 on macrophages during serum starvation was proven using the Angptl2$^{-/-}$ BMDM. These data demonstrated that Angptl2$^{-/-}$ BMDM showed decreased apoptosis compared to wt BMDM under serum-induced starvation (Fig. 31). Even upon treatment with other apoptotic stimuli such as SNAP and stauro Angptl2$^{-/-}$ BMDM were significantly protected against apoptosis. The above results support the conclusion that the altered Angptl2 expression in PHD3$^{-/-}$ BMDM is functionally involved in the apoptosis phenotype. Along this line, the decreased Angptl2 expression in PHD3$^{-/-}$ macrophages under serum starvation mirrors the shut-down of a death-supporting factor.

4.7 Angptl2 affects macrophage apoptosis

To confirm whether the loss of an autocrine effect of Angptl2 alters the apoptotic phenotype of BMDM, I evaluated the effect of adding exogenous Angptl2 to the culture medium. Addition of recombinant Angptl2 reverses the cell protection phenotype and results in increase apoptosis of PHD3$^{-/-}$ BMDM (Fig. 32). Taking into account the high induction of Angptl2 after serum starvation in wt BMDM, this can be interpreted as an activation of a protective feedback mechanism. Angptl2 treatment resulted in increased apoptosis of PHD3$^{-/-}$ BMDM (Fig. 32), suggesting that Angptl2 regulates BMDM apoptosis in an autocrine manner. Angptl2 treatment under serum starvation resulted in an anti-apoptotic effect in the wt BMDM. The anti-apoptotic function of Angptl2 is in line with previous reports in endothelial cells (Kubota *et al.*, 2005). Most interestingly, in PHD3$^{-/-}$ BMDM the response was reverted to a pro-apoptotic effect. Angptl2 treatment increased the serum-starvation induced cell death of PHD3$^{-/-}$ macrophages but did not affect cell viability under normal cell-culture conditions. This implies that Angptl2 is not per se a pro-apoptotic factor but interferes with the apoptosis pathway after the application of a stress stimulus.

It has been previously reported, that Angptl2 signals through integrin α5β1 and PIRB receptors (Okada *et al.*, 2010) and that the α5β1 and PIRB are receptors for several Angptl family members (Zheng *et al.*, 2012; Oike *et al.*, 2004). Analysis of receptors expression showed no difference in the expression level of these receptors excluding that the expression of these is involved in the altered Angptl2 response (Fig. 33).

5. Conclusion and Outlook

In the present thesis, results showed for the first time a functional connection between PHD3 and Angptl2. In this study myeloid-specific PHD3-deficient macrophages were investigated. The PHD3$^{-/-}$ BMDM did not show an altered expression of the two other HIF-regulating PHDs, i.e. PHD1 and PHD2. Also the knockout BMDM showed no altered/increased HIF-1α or HIF-2α protein levels in normoxia and hypoxia. Further, the results were substantiated by the fact that the HIF target gene expression was unaltered in the PHD3$^{-/-}$ BMDM compared to wt cells. Macrophage M1 and M2-polarization remained unaltered as well. PHD3 has been associated with apoptotic cell death mechanisms in various cell types. In this study, macrophages were investigated regarding differences in apoptosis using TUNEL and Annexin V staining. TUNEL staining revealed under starvation a decrease in number of apoptotic PHD3$^{-/-}$ macrophages as compared to wt. The same results were confirmed using the early apoptotic marker Annexin V. Treatment with apoptotic inducers such as SNAP and staurosporine confirmed that PHD3$^{-/-}$ macrophages are less apoptotic. In line calcein uptake results revealed that PHD3$^{-/-}$ cells are significantly more viable compared to wt BMDM. When the supernatant medium from the cultured macrophages was exchanged between the genotypes after 24 hrs of culture, PHD3$^{-/-}$ macrophages showed more Annexin V staining indicating that at least in part a secreted factor is involved in the PHD3$^{-/-}$ apoptosis mechanism. In an unbiased transcriptome screen comparing starved wt and PHD3$^{-/-}$ BMDM, I found a significant difference in the starvation-induced Angptl2 RNA. These data demonstrate that PHD3 affects the production of Angptl2 and additionally influences the response towards this apoptosis modulating factor. Further, treatment with recombinant Angptl2 rescued the anti-apoptotic phenotype demonstrating that it is involved in the PHD3-mediated response towards apoptotic stimuli in macrophages. My data was further strengthened by investigating apoptosis in Angptl2$^{-/-}$ BMDM, which showed decreased apoptosis compared to wt BMDM supporting the lower expression of Angptl2 in PHD3$^{-/-}$ BMDM followed by decreased apoptosis. Taken together, my data demonstrate for the first time that PHD3 plays a critical role for cell survival decisions in macrophages. This includes apoptotic cell death induced by serum starvation, which mimics in part ischemic conditions but also treatment with stauro or the endogenous inducers of apoptosis like the NO donor SNAP or LPS. The anti-apoptotic effect in the PHD3$^{-/-}$ BMDM is at least partially mediated by an

altered production and response to Angptl2. Findings such that inhibition of PHD3 can boost the innate immune defense might be exploited for clinical treatment strategies. Further research on loss of PHD3 and its effect on myeloid cell apoptosis is important to be investigated in more detail to determine the outcome on damaged tissue repair and tissue remodeling. Since macrophages are responsible for the early immune response, the data imply that PHD3 may shape this response by altering the life expectancies of macrophages in inflamed and ischemic tissues. Regardless of underlying molecular mechanisms, the present findings, that loss of PHD3 results in an increased lifespan of macrophages might have consequences for the clinical outcome of inflamed tissues.

6. References

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7. Curriculum vitae

PERSONAL DETAILS

Name: LIJA SWAIN

Date of Birth: 16.10.1984

Place of Birth: Bhubaneswar

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EDUCATION AND RESEARCH EXPERIENCE

Since 07/2011	**Pursuing PhD** under the supervision of Prof. Dr. Dörthe Katschinski at the Georg-August University Göttingen, thesis on "Prolyl-4-hydroxylase domain 3 (PHD3) is a critical terminator for cell survival of macrophages under stress conditions".
09/2010-03/2011	**Master thesis** under supervision Prof. Dr. Jens Rettig, at University of Saarland, Physiology, for my Master thesis on "Functional Characterization of molecular components involved in exocytotic machinery of cytotoxic T lymphocytes".
09/2009-03/2011	**Masters in Biomedical Engineering,** Furtwangen University, Schwenningen, Germany
12/2008-31/2009	**Training** under the supervision of Dr Swati Subodh at **"**The Center for Genomic Applications" project on "Unique Signature prediction of HIV and HBV genome"
03/2008-08/2008	**Bachelor's thesis**, Institute of Genomics and Integrative Biology, Delhi under the supervision of Dr. Beena Pillai on MicroRNA and synthetic biology topic "Clonning and Functional Assay of Drosha in Saccharomyces Cerevisiae".
08/2004-05/2008	**Bachelors in Technology in Biotechnology,** Maharishi Dayanada University Rohtak, India
08/2001-08/2003	**Class XII Council of Higher Secondary Education Bhubaneswar,** Maharishi College of natural laws, Orissa, India
03/2001	**Class X Central Board of Secondary Education,** D.A.V public school, Puri, Orissa, India

POSTER PRESENTATION

Keystone Symposia conference Cancer and Metabolism/ Advances in Hypoxic Signaling: From Bench to Bedside

Location: Fairmont Banff Springs, Banff, Alberta, Canada

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HypoxiaNet meeting, Sensing Hypoxia in the Cell and the Organism

Location: Essen, Germany

Date: 20 - 23 September 2012

92nd Annual Meeting of the German Physiological Society DPG 2013,

Location: Heidelberg, Germany

Date: March 2 – 5, 2013

Dealing with hypoxia: Regulatory aspects in cells, tissues and organisms

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Date: 7-12 January 2014

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