The Role of PINK1-dependent Mitophagy for Development of Pulmonary Hypertension

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DEDICATION

This dissertation is sincerely dedicated to the blessed memory of my beloved mother, Mina Najafi-Saraji (1958-2017) whose steadfast love and enormous sacrifice have contributed greatly to the man I have become.

I had promised to make her proud by the achievement of this academic goal and I hope her soul is happy now. I wish she could be here today to celebrate with me, and my graduation with a PhD degree.

I wish to express my heartfelt gratitude to my two pretty angels, Elisa and Hanna-Mina, for their patience, love, and support while I was doing this research project.

Declaration

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

Alireza Saraji
Giessen, Germany

Date, Place_____ Signature _____

I. Table of the contents

II. List of figures

III. List of tables

IV. List of abbreviations

ALK-1	Activin receptor-like kinase-1
AMP/ATP	Adenosine monophosphate - adenosine triphosphate ratio
AMPKα	Adenosine monophosphate-activated protein kinase alpha
Bax/ Bcl-2	B-cell lymphoma 2-associated X protein
BMPR	Bone morphogenetic protein receptor
BNIP3L	BCL2/Adenovirus E1B 19 KDa protein-interacting protein 3-like
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$[Ca^{2+}]$ i	Intracellular calcium concentration
Ca^{2+}m	Mitochondrial calcium
CCCP	Carbonylcyanide m-chlorophenylhydrazone
CCL2	CC chemokine ligand 2
cGMP	Cyclic guanosine monophosphate
C-lobe	Kinase C-terminal lobe
COPD	Chronic obstructive pulmonary disease
CTEPH	Chronic thromboembolic pulmonary hypertension
CTR	C-terminal region
DAPI	4'-6-diamidino-2-phenylindole
DCA	Dichloroacetate
DRP1	Dynamin-related protein 1
ECM	Extracellular matrix
EGF	Epidermal growth factor
eNOS	Endothelium-dependent nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
ESR	Electron spin resonance
ET-1	Endothelin-1
Fb	Fibroblast
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazon
FiO_2	Fraction of inspired oxygen
FIS-1	Fission protein-1
FOXO1	Forkhead box protein O1

FUNDC1	FUN14 domain containing 1
H^+	Proton
H_2O_2	Hydrogen peroxide
HEY1	Hairy/enhancer-of-split related with YRPW motif protein 1
HIF-1α	Hypoxia-inducible factor 1- α
HOX	Hypoxia
HPRT	Hypoxanthine phosphoribosyltransferase
HPV	Hypoxic pulmonary vasoconstriction
i1-i3	Insertions 1-3
IL-6 Tg	Transgenic mice overexpressing interleukin 6
IMM	Inner mitochondrial membrane
IPAH	Idiopathic pulmonary arterial hypertension
IPF	Idiopathic pulmonary fibrosis
KCNK3	Potassium channel subfamily K member 3
$K_v1.5$	Voltage-dependent potassium channel 1.5
LA	Left atrium
LC3B	Microtubule-associated protein 1A/1B-light chain 3
LPS	Lipopolysaccharide
LV	Left ventricle
MAPK	Mitogen-activated protein kinase
MCT	Monocrotaline
MCU	Mitochondrial Ca^{2+} uniporter
MEFs	Mouse embryonic fibroblasts
MFN2	Mitofusion 2
MID49	Mitochondrial dynamics proteins 49
MMP	Mitochondrial membrane potential
mPAP	Mean pulmonary artery pressure
MPP	Mitochondrial processing peptidase
mRNA	Messenger RNA
MTS	Mitochondrial targeting sequence
MWT	Medial wall thicknes
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase

NFAT	Nuclear factor of activated T
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
N-lobe	Kinase N-Terminal lobe
NMR	Nuclear magnet resonance
NO	Nitric oxide
NOX	Normoxia
NRF1	Nuclear respiratory factor 1
O_2	Oxygen
OMM	Outer mitochondrial membrane
OPA-1	Optic atrophy protein-1
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation
p-AMPKα	adenosine monophosphate-activated protein kinase (phosphorylated at Thr-172)
PAP	Pulmonary arterial pressure
PARKIN	Parkinson protein 2, E3 ubiquitin protein ligase
PARL	Presenilins-associated rhomboid-like protein
PARP	Poly ADP ribose polymerase
PAWP	Pulmonary arterial wedge pressure
PBS	Phosphate buffered saline
PDK	Pyruvate dehydrogenase kinase
PECs	Pulmonary endothelium cells
PGAMA5	Phosphoglycerate mutase family member 5
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
PH	Pulmonary hypertension
PHB2	Prohibitin-2
PHD	Prolyl hydroxylase
Pi	Phosphoric acid
PINK1	PTEN-induced putative kinase 1
pO_2	Partial pressure of O2
PPARγ	Peroxisome proliferator-activated receptor γ
pSOD	Pegylated superoxide dismutase

PVDF	Polyvinylidene fluoride
PVOD	Pulmonary veno-occlusive disease
Q	Perfusion
RA	Right atrium
ROS	Reactive oxygen species
RV	Right ventricle
RVH	Right ventricular hypertrophy
RV/LV+S	Ratio of right ventricular weight to the weight of the left ventricle and septum
RVSP	Right ventricular systolic pressure
scr siRNA	Scrambled small interfering RNA
SEM	Standard error
SIRT3	Silent mating type information regulation 2 homolog 3
Smac	Second mitochondria-derived activator of caspases
SMAD	Family of proteins related to Drosophila" mothers against decapentaplegic" (Mad) and Caenorhabditis elegans Sma
SNPs	Single nucleotide polymorphisms
SR	Sarcoplasmic reticulum
STAT3	Signal transducer and activator of transcription
TBK1	Tank Binding Kinase 1
TBS-T	Tris Buffer Saline + 0.1% Tween20
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
Tg	Transgenic
TGF-β	Transforming growth factor β
TM	Transmembrane domain
TNF	Tumor necrosis factor
TRPC3	Transient receptor potential cation channel 3
Ub	Ubiquitin
UCP	Uncoupling protein
UPS	Ubiquitin proteasome system
VDAC	Voltage-dependent anion channels
VEGF	Vascular endothelial growth factor

VSMCs	Vascular smooth muscle cells
WT	Wild type
β_2M	Beta2-microglobulin
$\Delta\psi_m$	Mitochondrial membrane potential

1. Introduction

1.1 Pulmonary hypertension (PH)

1.1.1. Definition and characteristics of PH

Pulmonary hypertension (PH) is a severe disorder that leads to right ventricular failure and premature death [1, 2]. Previously, PH was defined as an increase in mean pulmonary artery pressure (mPAP) to ≥25 mmHg [3]. However, at the 6th World symposium on PH in Nice (France, 2018), PH was defined as a mPAP >20 mmHg and "a mPAP >20 mmHg concomitant with a pulmonary vascular resistance (PVR) ≥ 3 Wood Units (WU) for pre-capillary forms of PH to account for the effect of cardiac output (CO) and pulmonary arterial wedge pressure (PAWP) on mPAP [PVR = (mPAP-PAWP)/CO]" [4, 5]. Historically, PH was first described by Ernst von Romberg in 1891. Later in 1951, Dresdale and colleagues originated the term, "primary PH", to describe conditions in patients with unknown reason of PH [6]. Currently, PH (in all of its forms) affects about 1% of the world population, and approximately 10% of people aged 65 years and beyond [7]. Some forms of PH, that is, idiopathic pulmonary arterial hypertension (IPAH) and heritable PAH, are more prevalent among women [4] however, due to the "estrogen paradox" in women the progression of the diseases seems to be more moderate and with a better outcome [8].

1.1.2. Classification of PH

PH was first classified into two groups, primary and secondary PH, at the WHO symposium held in Geneva 1973, according to their causes or risk factors [9]. It has taken four decades and several symposiums to come up with the current classification of PH, which is now widely accepted and applied in routine clinical practice around the world. One important update of PH classification was issued in the fifth world symposium held in Nice, France, 2013 [10]. This classification categorized different forms of PH into five groups, based on their pathogenesis and clinical manifestations. Finally, at the 6th world symposium held on 27th February 2018, Nice (France), the same classification was reconsidered with minor revision, as indicated in table 1 [5, 11]. The new classification categorized the different forms of PH based on clinical manifestation, pathophysiological, and hemodynamic characteristics, along with possible

therapeutic handlings [12-14]. In the present study, we focused on PH group III of the modern PH classification.

Table 1. Updated clinical classification of PH, Nice, France, 2018 [11].

1. PAH (pulmonary arterial hypertension)
1.1. Idiopathic PAH 1.2. Heritable PAH 1.3. Drug- and/or toxin-induced PAH 1.4. PAH associated with: 1.4.1 Connective tissue disease 1.4.2 HIV infection 1.4.3 Portal hypertension 1.4.4 Congenital heart disease 1.4.5 Schistosomiasis 1.5. PAH long-term responders to Ca^{2+}- channel blockers 1.6. PAH with overt features of venous/capillaries (PVOD/PCH) involvement 1.7. Persistent PH of the newborn syndrome
2. PH due to left heart disease
2.1. PH due to heart failure with preserved LVEF 2.2. PH due to heart failure with reduced LVEF 2.3. Valvular heart disease 2.4. Congenital/acquired cardiovascular conditions leading to post-capillary PH
3. PH due to hypoxia and/or lung disease
3.1. Obstructive lung disease 3.2. Restrictive lung disease 3.3. Other lung disease with mixed restrictive/obstructive pattern 3.4. Hypoxia without lung disease 3.5. Developmental lung disorders
4. PH due to pulmonary artery obstructions
4.1. Chronic thromboembolic PH (CTEPH) 4.2. Other pulmonary artery obstructions
5. PH with unclear and/or multifactorial mechanisms

5.1. Hematological disorders 5.2. Systemic and metabolic disorders 5.3. Others: Chronic renal failure with or without haemodialysis, fibrosing mediastinitis 5.4. Complex congenital heart disease

1.1.3. Animal models of PH

For experimental studies of PH, there are currently numerous animal models. The most common ones are the exposure of rodents to chronic hypoxia or subcutaneous injection of monocrotaline (MCT). Although these models of PH have contributed a lot to the knowledge in the field, none of these models is able to mimic all pathological characteristics of human PAH, for example, plexiform lesions are absent in the hypoxic PH model. [15]. It is noteworthy that plexiform lesions are a hallmark of IPAH [4, 16]. Recently, it was proposed that shunting between vasa vasorum within the adventitia of pulmonary arteries and bronchial arteries within the peribranchial connective tissue could be involved in plexiform lesions formation [17]. Presently, new animal models of PH have been suggested to simulate different groups or subgroups of PH [18]. In table 2, available animal models that mimic PH of group 1 and 3 (according to the current Nice classification) are listed (Table 2).

Table 2. Experimental animal models for PH of group 1 and 3 (according to the current Nice classification).

Animal models for PH group 1:

- **MCT-injection (rats)**: One of the most common methods used to induce PH in rats is subcutaneous injection of MCT (an alkaloid from the plant *Crotalaria spectabilis*) [19, 20]. MCT initially triggers endothelial injury in the pulmonary vasculature via the activation of proliferation and anti-apoptotic factors of pulmonary endothelial cells (PECs), along with dysregulation of nitric oxide (NO) signaling, resulting in the development of pulmonary vascular remodeling [21]. The histological features of MCT-induced PH include intimal hyperplasia, medial hypertrophy and adventitial thickening [15, 22].
- **Chronic hypoxia + SU-5416 (rats)**: Exposure of rats to chronic hypoxia together with injection of the vascular endothelial growth factor (VEGF) inhibitor, SU-5416, causes PH with pulmonary arterial changes similar to plexiform lesions. There are initial increases in apoptosis of PECs. SU-5416 selects apoptosis-resistant PECs in chronic hypoxia and thus, promotes intima remodeling in the pulmonary vasculature. [23-26].
- **Left-to-right shunt (piglets):** Surgical intervention to anastomose the left subclavian artery to the pulmonary artery trunk of growing piglets results in rapid and severe PH [27-29].
- **Schistosomiasis (mice):** In this model, transcutaneous infection of mice with the eggs of *S. mansoni* induces chronic infection and PH development. This model explores pulmonary vascular remodeling with plexiform-like lesion formation along with perivascular inflammation [30, 31].
- **Bone morphogenetic protein receptor 2 (*BMPR-2*$^{+/-}$) (mice):** Mice with a heterozygous mutant allele of *BMPR-2* (lacking exon 4 and 5) along with 3 weeks of chronic hypoxia exposure [11% of fraction of inspired O_2 (FiO_2)] develop mild PH characterized by thickening of the pulmonary arteries [32]. Additionally, other models including mutation with zinc finger nuclease in rat [33] and different cell-type specific knockout have been also created [34].
- **Interleukin-6 (IL-6) overexpression (mice):** Lung specific IL-6 overexpressing transgenic mice show elevated right ventricular systolic pressure (RVSP) along with right ventricular hypertrophy (RVH) and pathological changes in the vasculature, which is as a result of IL-6-induced increased proliferation and decreased apoptosis of pulmonary artery smooth muscle cells (PASMC) and PECs [35].
- **S100A4/Mts1 overexpression (mice):** About 5% of the metastasis-promoting protein S100A4 (Mts-1)-overexpressing mice develop pulmonary vascular remodeling resembling the plexogenic arteriopathy observed in human PH. This model is suitable for the study of vascular changes of severe PH including neointimal thickening and plexiform lesion formation [36-38].

Animal models for PH group 3:

- **Chronic hypoxic exposure (rodents):** Chronic exposure of rodents to hypoxia is the most common model for studying PH group 3 [15]. Mice are placed into a normobaric chamber (10% FiO_2) for 28 days. Chronic hypoxia induces rather mild PH without plexiform lesions [39-42].
- **Chronic exposure to cigarette smoke (Rodents):** Mice that are exposed to cigarette smoke for several months develop mild PH [43, 44].

Abbreviations: BMPR-2 - Bone morphogenetic protein receptor 2; IL-6 Tg - Transgenic mice overexpressing interleukin 6; S100A4 – A member of the S100 protein family (soluble in 100% saturated ammonium sulfate); RVH - right ventricular hypertrophy.

1.1.4. Pathogenesis of PH

PH is a multifactorial disease and is triggered by a wide spectrum of environmental factors, as well as genetic and epigenetic alterations. Common features of the different forms of precapillary PH (PH group I, III, IV, V) are vasoconstriction of small pulmonary vessels and vascular remodeling.

1.1.4.1. Vasoconstriction

Pulmonary vasoconstriction is induced by several triggers such as hypoxic pulmonary vasoconstriction (HPV) called von Euler-Liljestrand reflex, and an imbalance in various vasodilators and vasoconstrictors. HPV is a physiological reaction of the pulmonary vasculature to acute hypoxia, resulting in the redirection of blood flow from poorly ventilated to better ventilated lung areas to optimize blood oxygenation [45, 46]. Prolonged and global alveolar hypoxia, however, may contribute to PH. Moreover, an imbalance of the vasoconstrictor's endothelin-1 (ET-1) and thromboxane on the one side, and the vasodilators prostacyclin, and NO on the other side, plays an important role for development of PH [47]. In numerous studies, the elevation of vasoconstrictors, such as ET-1 [48], and a decreased level of the vasodilator, NO, along with its messenger cyclic guanine monophosphate (cGMP) was shown in IPAH patients [49-51]. Additionally, ET-1 can increase the proliferation of PASMC, e.g. after chronic hypoxic treatment [52]. Although these pathways are exploited for treatment of human PH, they only can slow down progression but not cure the disease [53, 54].

1.1.4.2. Vascular remodeling

Vascular remodeling is a key factor in all precapillary forms of PH (PH group I, III, IV, V), including IPAH and chronic hypoxia-induced PH [55, 56], which affects all the three layers of the pulmonary blood vessels including their major cell types, PASMC, PECs and fibroblasts [57, 58] (Figure 1.1). However, the exact mechanisms underlying pulmonary vascular remodeling remain to be completely understood. Different stimuli can activate several transcriptional factors such as hypoxia inducible factors (HIFs) [59], nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [60], fork head box protein O1 (FOXO1) [61], and peroxisome proliferator-activated receptor (PPARγ) [62]. Activation of these transcriptional factors alter various cellular pathways that lead to pulmonary vascular remodeling and PH. Additionally an altered inflammatory response, imbalance between vasodilators and vasoconstrictors, an alteration in $[Ca^{2+}]i$ homeostasis and cellular metabolism have been suggested as key cellular mechanisms implicated in pulmonary vascular remodeling. In particular, remodeling initiated by pro-proliferation and anti-apoptosis of PASMC in the medial layer (Figure 1.1) is mediated by different mechanisms such as alterations in ion channel function and expression [63], changes in cellular metabolism and mitochondrial functions [64], somatic and genetic mutations [65-67], growth factor alterations [68], an imbalance between vasodilators and vasoconstrictors originating from PECs [69], and altered chemokine and cytokine release from immune cells [70].

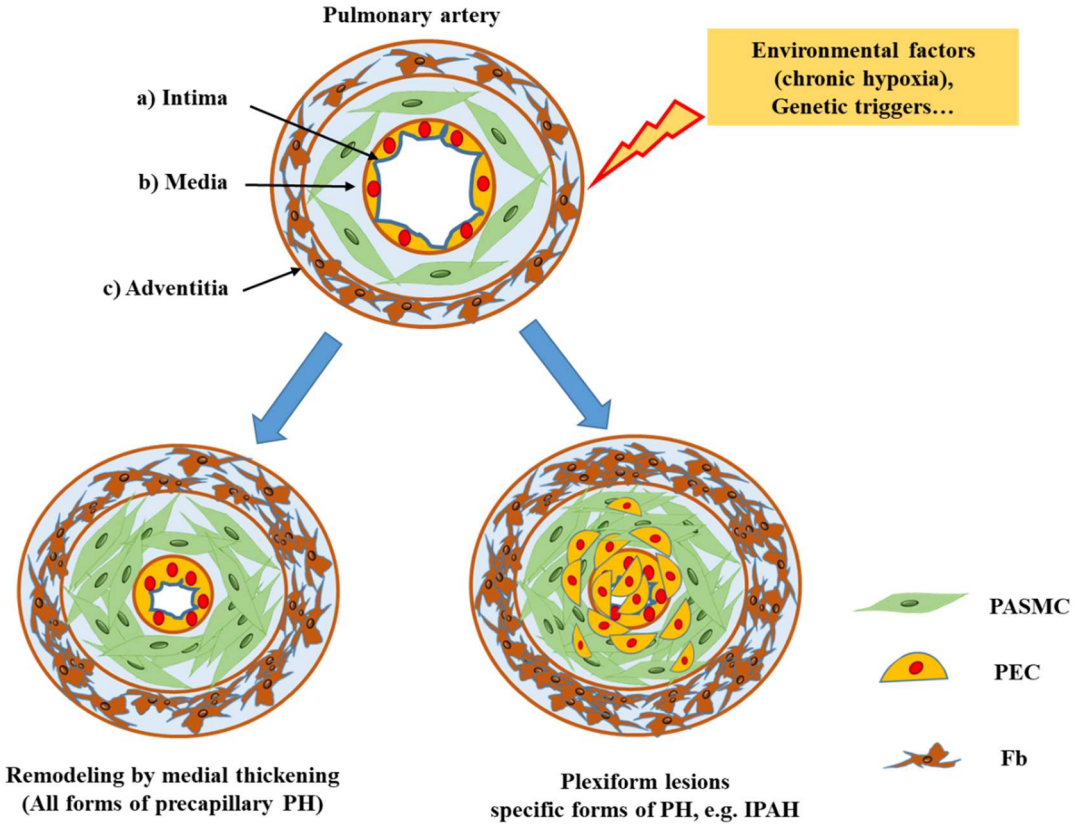


Figure 1.1. Proposed model of pulmonary vascular remodeling in PH and IPAH

Vascular remodeling is a key factor in precapillary forms of PH [55, 56] which affects all three layers of pulmonary blood vessels including the adventitia, media and intima and their major cell types including, PASMC, PECs and fibroblasts [57, 58]. Chronic hypoxia, genetic and environmental factors can trigger pulmonary vascular remodeling in PH and IPAH. Pulmonary vascular remodeling is characterized by thickening of the adventitial, medial and intimal layer. In contrast to chronic hypoxia-induced PH, IPAH also shows prominent narrowing of the vascular lumen by the formation of plexiform lesions (for details see text).

Abbreviations: PASMC - pulmonary arterial smooth muscle cells; PECs - pulmonary endothelial cells; Fb - fibroblasts.

1.1.4.3. Cellular regulators of pulmonary vascular remodeling

HIF-1α and HIF-2α are the main regulators of the cellular response to hypoxia [71]. HIF-1α has been shown to play an important role in short-term hypoxia [72]. In contrast, HIF-2α is more important for chronic hypoxia and right heart adaptation in animal studies [73]. In normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) and degraded by the E3 ubiquitin ligase [74]. Under hypoxic conditions, oxygen-dependent degradation of HIF-1α is

inhibited leading to HIF-1α stabilization, dimerization with HIF-1β and subsequently, nuclear localization of HIF-1α. HIF-1α activates the expression of numerous hypoxia-specific genes under the hypoxia response element (HRE) promoter [75]. Hypoxia decreases PHD activity since oxygen (O_2) is a substrate for PHDs [76, 77]. Besides regulation by PHDs, it has been suggested that mitochondrial reactive oxygen species (ROS) influence the stabilization of HIF-1α [78]. Silencing of *HIF-1α* by siRNA attenuates hypoxia-induced proliferation of human PASMC [79] and cell-type specific knockout of *Hif-1α* (homozygous conditional deletion of *Hif-1α* with tamoxifen-inducible smooth muscle–specific Cre recombinase) attenuates the development of chronic hypoxia-induced PH in murine model [56]. Moreover, non-hypoxic stabilization of HIF-1α in MCT-induced PH was also described [48]. Recently, the emerging role of HIF-2α for development of PH was described. It was shown that deletion of PHD2 in endothelial cells induced spontaneous PH in experimental animals, and deletion of *Hif-2α* in endothelial cells prevented the development of chronic hypoxia-induced PH [80].

One of the most investigated regulators of vascular remodeling in IPAH is BMPR-2. It was reported that almost 70% of hereditary PAH patients and 20% of IPAH patients carry a mutation leading to dysfunction of BMPR2 signaling [81]. BMPR2 as a receptor binds the bone morphogenic proteins [members of the multifunctional transforming growth factor of the beta superfamily (TGF-β)]. It was shown that BMPR2 plays a role in proliferation and apoptosis of human fibroblasts [82], PASMC [83] and PECs [84, 85] in IPAH patients and in animal models of PH [86, 87]. Homozygous mice with conditional knockout of BMPR-2 in PECs predisposed for developing a mild PH [88]. Defective BMPR2 signaling may affect development of PH by different mechanism. For example, downregulation of BMPR2 expression in PASMC resulted in an increase in ET-1 at the protein level [89, 90]. Besides mutations of the *BMPR2* gene, other genes are involved in the development of the disease, for example, the activin receptor-like kinase-1 (ALK-1) [91] and the potassium two pore domain channel subfamily K member 3 (KCNK3) [92].

Another concept in vascular remodeling in human disease and animal models is the contribution of inflammation [93]. Several investigations have reported elevated levels of tumor necrosis factor (TNFα), IL-1β and IL-6 in IPAH patients [57, 94]. Accordingly, mice with IL-6 deficiency are reported to be protected against chronic hypoxia-induced PH [93]. Chemokines such as the CC chemokine ligand 2 (CCL2) could also contribute to IPAH development by increasing PASMC proliferation and migration [95]. Furthermore, the interaction between inflammation and BMPR2 has been fairly described [96, 97]. Administration of lipopolysaccharide (LPS) to BMPR2 mutated PASMC from humans and mice showed

increased levels of IL-6 and IL-8, and an exaggerated inflammatory response in BMPR2 deficient mice which developed PH in response to chronic LPS treatment [98].

Cytosolic ion channels and transporters also play a critical role in PH development. $[Ca^{2+}]i$ concentration is considered as a key regulator of PASMC proliferation and migration in PH, and therefore, an imbalance in $[Ca^{2+}]i$ concentration will influence the development of the disease [64, 99].

In the last decades, mitochondria have taken center stage as an important organelle in the regulation of the pathogenesis of lung diseases, including PH [100-102]. Mitochondrial functions such as the control of cellular metabolism, ROS production, maintenance of $[Ca^{2+}]i$ homeostasis and regulation of apoptosis have been reported as important triggers of PH development [103-105].

1.2. Mitochondria and PH

1.2.1. Mitochondria

Mitochondria are double-membrane-bound organelles that exist almost in all eukaryotes [106-109]. They serve to maintain energy supply for cells by producing ATP and are therefore also called, "*the powerhouse of the cell*" [110, 111]. Mitochondria also control several critical cellular functions such as $[Ca^{2+}]i$ homeostasis [112], heme and phospholipid synthesis [113], apoptosis [114] cellular metabolism and production of ROS [111, 115]. Mitochondria are an important O_2 sensor of the cell, and they consume about 90% of cellular O_2 [100, 116, 117]. Recently, it was reported by Sommer *et al*. that specialized pulmonary mitochondria are essential for acute oxygen sensing underlying the HPV response [118]. The mitochondrial electron transport chain (ETC or respiratory chain) which consists of several multi-subunit complexes located in the inner mitochondrial membrane (IMM), is responsible for ATP synthesis by generating a proton (H^+) gradient across the IMM [111, 119]. Limited supply of O_2 affects the rate of ATP production, and can affect mitochondrial functions including, Ca^{2+} metabolism [120], ROS production [121, 122] and the cytosolic redox state of [NADH]/[NAD^+] (reduced form of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide) [123-125]. Moreover, HIFs are master regulators of mitochondrial genes to induce metabolic alterations in hypoxia [126, 127].

In the last decades, mitochondria have been targeted by numerous scientific/clinical investigators to determine whether alterations in the structure or function of the organelle could promote a variety of diseases and disorders including metabolic dysfunctions [128], neurodegenerative diseases [129], cancer, abnormalities in the immune system [130, 131] and development of PH [102, 116, 132].

1.2.2. The role of mitochondria in PH

Emerging knowledge regarding the pathogenesis and underlying signaling mechanisms of PH indicate an essential role of mitochondrial dysfunction in the disease process [133]. However, the exact role of mitochondria for development of PH is still under debate. Alterations in mitochondrial functions, including mitochondrial ROS release, ATP production, substrate metabolism, $[Ca^{2+}]i$ homeostasis and apoptosis [103] could play a role in both altered vascular tone [134, 135] and vascular remodeling in chronic hypoxia-induced PH [125, 136-138] as well as IPAH [133, 139, 140]. In several studies, mitochondrial ROS have been suggested as

candidates for initiation of HPV [141] and pulmonary vascular remodeling [102, 142-144]. However, the exact cellular mechanisms underlying altered mitochondrial ROS release under these conditions are still unclear [142]. There are two opposite opinions regarding the role of mitochondrial ROS in PH. One group showed a reduction of mitochondrial ROS [121], while another group suggested an increase of mitochondrial ROS in acute but not chronic hypoxia-induced PH [145]. Regardless of the direction of the net cellular ROS levels, both theories suggest that alterations in mitochondrial ROS release could modify the activity/structure of ROS sensitive proteins and various intracellular signaling pathways including the stabilization of HIF-1α [124, 146] and intracellular $[Ca^{2+}]i$ homeostasis [46, 147].

ROS production in PH could be affected by modification of mitochondrial membrane potential ($\Delta\psi_{m}$). Mitochondrial hyperpolarization, along with decreased respiration has been described since long time ago [104, 148]. $\Delta\psi_m$ was shown to be increased (i.e. mitochondrial hyperpolarization) in human PASMC during *in vitro* chronic hypoxia exposure, leading to decreased apoptosis via attenuation of the opening of the mitochondrial permeability transition pore [59, 149]. Pak *et al.* showed that deletion of the uncoupling protein (UCP) 2 increased $\Delta\psi_m$ and thus, promoted vascular remodeling and development of PH via increased ROS release in PASMC [102]. One study from Michelakis *et al.* showed that UPC2 knockout promoted development of PH in mice through the regulation of mitochondrial calcium ($Ca^{2+}m$), however, $\Delta\psi_m$ was also increased in this study [137]. Besides modifying apoptosis, $\Delta\psi_m$ may modulate the influx of Ca^{2+} into the mitochondria [150, 151].

The role of mitochondrial calcium ($Ca^{2+}m$) regulation for development of PH has been addressed in the past in detail in animal studies. Mitochondria serve as a reservoir for $[Ca^{2+}]i$ concentration [64] and maintain $[Ca^{2+}]i$ homeostasis via different Ca^{2+} exchangers and channels, including Ca^{2+} uniporter (MCU) and mitochondrial voltage-dependent anion channel-1 (VDAC1) [152, 153].

Furthermore, there exists a tight interaction between mitochondria and the ER which seems to play a significant role in Ca^{2+} buffering and homeostasis. It has been shown that alterations in the mitochondria-ER communication by Nogo-B (reticulon-4B) induction, inhibit apoptosis of PASMC in the chronic hypoxia-induced mouse model via reduced $Ca^{2+}m$ influx and phospholipid transfer from the ER to mitochondria [154]. Changes in $[Ca^{2+}]i$ concentration can induce remodeling of the pulmonary vessels [155]. In this regard, studies have shown that the elevation of $[Ca^{2+}]i$ promotes PASMC proliferation [156]. There are strong evidences that

showed the possible effect of mitochondrial ROS on Ca^{2+} regulation via ROS/redox status regulation of Ca^{2+} channels e.g. transient receptor potential cation channel 3 (TRPC3) [157].

One of the crucial tasks of mitochondria in the cell is the regulation of apoptosis [158]. Mitochondrial pro-apoptotic signaling is directly linked with $\Delta\psi_m$ [103]. It has been shown that mitochondrial hyperpolarization blocks the cells from entering into anti-apoptotic conditions whereas mitochondrial depolarization induces apoptosis [103]. Similarly, in cancer cells, preserved $\Delta\psi_m$ correlates directly with pro-proliferative and anti-apoptosis characteristics [159]. Along these lines, it was suggested that alterations in mitochondrial metabolic functions are connected to attenuated voltage-gated K^+ channel 1.5 ($K_v1.5$) expression and inhibition of PASMC apoptosis. In this study mitochondrial dysfunction and downstream effects were connected by decreased mitochondrial ROS release, which is however, under debate as outlined above [160]. In contrast to decreased apoptosis in PASMC, increased apoptosis of PEC may occur as initial trigger of vascular remodeling in the intimal media of the pulmonary vessels in PH [103, 160, 161]. Apoptosis in PECs may initiate either degeneration of pre-capillary arterioles or selection of anti-apoptotic and hyper-proliferative PECs which form plexiform lesions [161].

Mitochondrial respiration plays a central role for regulation of ROS, $\Delta\psi_m$ and calcium. Changes in metabolism (i.e. a metabolic switch) characterized by an increase of anaerobic glycolysis and decrease of mitochondrial glucose oxidation are linked to cellular proliferation and reduced apoptosis in cancer [162-164]. In PH, the mitochondria in pulmonary vascular cells exhibit similar alterations, showing decreased oxidative phosphorylation and increased glycolysis [142]. One key factor for the metabolic switch is upregulation of PDK1. Michelakis and McMurtry *et al.* showed that promotion of mitochondrial glucose oxidation by the PDK1 inhibitor dichloroacetate (DCA) could inhibit PH development in animal models of PH [165, 166]. The metabolic switch may also affect substrate availability and epigenetic alterations which promote PH [167]. Other factors contributing to the metabolic switch are mitochondrial biogenesis [142]. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) acts as a coactivator of peroxisome proliferator-activated receptor gamma (PPARγ) to increase the mitochondrial fatty acid ß-oxidation and mitochondrial biogenesis. Moreover, , PGC1α interacts directly with nuclear respiratory factor 1 (NRF1) which regulates the mitochondrial transcription factor A (TFAM) to promote mtDNA synthesis and replication [168]. Several studies showed that, different mediators of mitochondrial biogenesis can affect PH and, in some cases, they have been therapeutically targeted with beneficial effects on PH development[169-172]. Taken together, the restoration of mitochondrial functions seems to be

very important in order to attenuate the PH progression [102, 173], however, the exact role of mitochondria and its function in PH development remains unknown.

1.3 PINK1-dependent mitophagy in PH

1.3.1. General aspects of mitophagy

Lewis *et al.* were one of the first to describe the process of mitophagy [174]. However, the term mitophagy was first used by Lemaster *et al.* a century later, when they described that damaged mitochondria are selectively removed from the mitochondrial network by autophagy [175]. Thus, mitophagy serves for mitochondrial quality control and it is a selective process to eliminate damaged or malfunctional mitochondria from the network [176, 177], and to maintain mitochondrial function [142]. The selective engulfment and elimination of damaged mitochondria is mediated by interaction with autophagy pathways as well as fission and fusion (see below for detailed description) [178-180]. Mitochondria can be damage by different stress conditions, such as hypoxia, oxidative stress, mitochondrial toxins and metabolic disorders and further contribute to cellular damage via excessive ROS production [181-183].

Several mitophagy pathways have been identified which play a crucial role in the process of mitochondrial clearance [184]:

- **PINK1 (PTEN-induced putative kinase 1)-dependent mitophagy:** PINK1-dependent mitophagy, also known as PINK1 (Parkinson protein 2, E3 ubiquitin protein ligase (PARKIN)–mediated mitophagy, is the most common mitophagy pathway which was first described in Parkinson's diseases [185, 186].
- **PINK1-independent mitophagy:** PINK1-independent mitophagy includes BCL2/Adenovirus E1B 19 KDa protein-interacting protein 3-like (BNIP3L/Nix) and FUN14 domain containing 1 (FUNDC1) dependent mitophagy [184].

1.3.2. Proteins involved in PINK1-dependent mitophagy

PINK1-dependent mitophagy is the most well-studied mitophagy pathway [187, 188]. PINK1-dependent mitophagy normally requires three stages [189] namely: 1) recruitment of PARKIN by PINK1 to the OMM of damaged mitochondria [190, 191], 2) separation of the damaged mitochondria via fission mediated by dynamin-related protein 1 (DRP1) [192, 193] and 3) elimination of damaged mitochondria by autophagy [194]. There are three key proteins in PINK1-dependent mitophagy, PINK1, PARKIN and presenilins-associated rhomboid-like protein (PARL).

PINK1 or PARK6 is a 63 KDa serine/threonine protein kinase, which is mostly expressed in the brain, endocrine tissue, gastrointestinal tract, muscles and lung [195]. The *PINK1* gene is located on the short arm of chromosome 1 at position 36.12 [196] and was first reported in Parkinson's disease [197-199]. PINK1 consists of 581 amino acids and has two domains namely, the mitochondrial localization sequence (transmembrane domain) and the serine/threonine kinase domain [195]. The detailed gene of *PINK1* is depicted in figure 1.2.

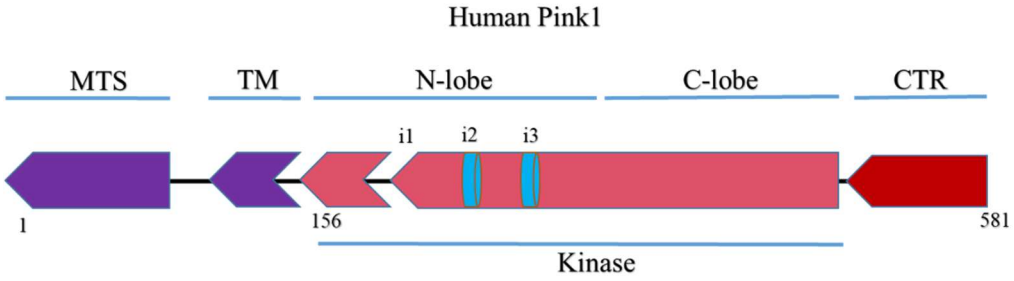


Figure 1.2. Schematic presentation of the human *PINK1* gene

The human *PINK1* gene consists of the mitochondrial targeting sequence (MTS) followed by a transmembrane domain (TM), kinase N-lobe with 3 insertions, kinase C-lobe and finally a C-terminal region (CTR).

Abbreviations: CTR - the C-terminal region; C-lobe - kinase C-terminal lobe; N-lobe - kinase N-terminal lobe; TM- transmembrane domain; MTS - Mitochondrial targeting sequence; i1-i3 - insertions 1-3.

Modified and adapted from Alexander F. Schubert *et al.*, Nature, 2017.

PARKIN or PARK2 is another key protein in the PINK1-dependent mitophagy pathway. The PARKIN protein, which is encoded by the *PRKN* gene on chromosome number 6, is an E3 ubiquitin ligase protein with 465-amino acid residues and has an amino-terminal ubiquitin-like (Ubl) domain and a C-terminal ubiquitin ligase domain [200]. PARKIN protein is highly expressed in human brain, skeletal muscle, kidney except blood, skin and eye tissue [201][Human Protein Atlas available from V. 20.0. proteinatlas.org/ENSG00000185345-PRKN/tissue]. *PARKIN* was first reported in 1997 by Matsumine *et al.* who described the loss of function mutation of *PARKIN* in a familial form of Parkinson's disease [202].

PARL is an inner mitochondrial membrane protein which is encoded by the *PARL* gene on chromosome number 3 and is also known as "mitochondrial intramembrane cleaving protease" [203]. PARL belongs to the mitochondrial rhomboid protease family [204] and is widely expressed in most human tissues except the adipose tissues [201][Human Protein Atlas available from V. 20.0. proteinatlas.org/ENSG00000175193-PARL/tissue].

1.3.3. Mechanism of PINK1-dependent mitophagy

1.3.3.1. PINK1 in healthy mitochondria

As shown in figure 1.3, in healthy mitochondria with sustained mitochondrial membrane potential ($\Delta\Psi_{m}$), the 63-KDa full length PINK1 is continuously translocated into the IMM via the translocase outer membrane (TOM)/translocase inner membrane (TIM) complex [205]. Within the IMM, the mitochondrial processing peptidase (MPP) cleaves the N-terminal MTS of the 63-kDa PINK1. Afterwards, the adjacent protease, PARL, cleaves PINK1, resulting in the 52-kDa form of PINK1 [206, 207]. Finally, the 52-kDa PINK1 is degraded by proteases. The elimination of PINK1 therefore protects healthy mitochondria against mitophagy [177].

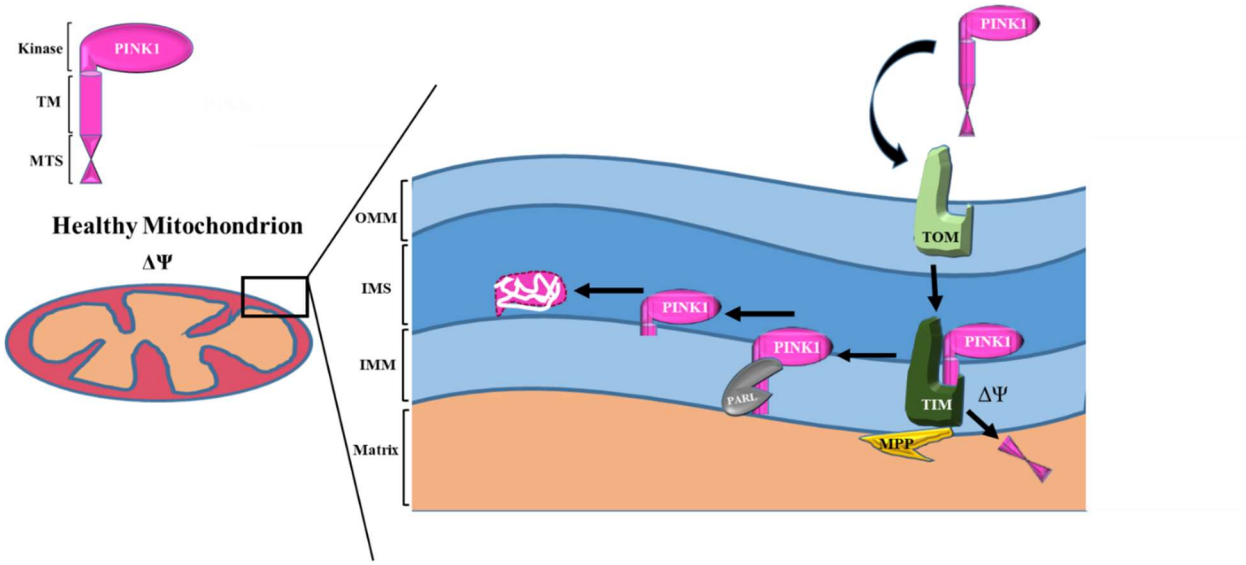


Figure 1.3 Degradation of PINK1 in healthy mitochondria

The 63-kDa full length PINK1 is translocated into the IMM via the TOM/TIM complex by attaching its MTS domain to the IMM [145]. In the IMM, PINK1 is subsequently cleaved by MPP to remove the MTS part and by the adjacent protease PARL [146, 147]. Finally, the cleaved 52-kDa PINK1 is degraded by proteases.

Abbreviations: TOM -Translocase outer membrane; TIM - Translocase inner membrane; TM - transmembrane domain of PINK1; MTS - Mitochondrial targeting sequence of PINK1; OMM- Outer mitochondrial membrane; IMS - Intermembrane space of mitochondria; IMM - Inner mitochondrial membrane; PINK1 - phosphatase and tensin homolog-induced putative kinase 1; PARKIN - Park2 E3 ubiquitin ligase protein; PARL - Presenilins-associated rhomboid-like protein; MPP-mitochondrial processing peptidase; $\Delta\Psi_m$ – mitochondrial membrane potential.

Modified from Ashrafi G *et al.*, Cell Death Differ, 2013.

1.3.3.2. PINK1 in damaged mitochondria

As summarized in figure 1.4, mitochondrial stress such as severe hypoxia, oxidative stress, and toxins results in the decrease of $\Delta\Psi_{m,}$ which is a sign of damaged mitochondria [208, 209]. Mitochondrial depolarization in damaged mitochondria prevents the translocation of PINK1 into the IMM and cleavage by PARL, since the function of TOM/TIM depends on the mitochondrial membrane potential. As a result, the full length PINK1 accumulates on the OMM with the kinase domain facing the cytosol [192, 207]. Continuous accumulation of PINK1 on the OMM recruits PARKIN from the cytosol to the OMM, a mechanism which is not completely understood yet [192]. In this regard, Kim Y *et al.* proposed that PINK1 recruits

PARKIN to the OMM by the phosphorylation of its threonine in the positions 175 and 217 [210]. PARKIN recruits ubiquitin along with autophagy mediators, microtubule-associated protein 1A/1B-light chain 3 (LC3BII)-binding autophagic adaptor P62 (SQSTM1) on the OMM [199] in order to gather all the damaged mitochondria [200] and lead them to autophagosomes [201] in order to initiates autophagosomal degradation of damaged mitochondria [211, 212]. Before that, damaged mitochondria are removed from the network by translocation of DRP1 from the cytosol to the OMM for initiation of mitochondrial fission.

Nevertheless, PINK1 may also have other molecular functions independent of its role in mitophagy. These may include modulation of complex I activity to regulate mitochondrial bioenergetics [213], regulation of $[Ca^{2+}]i$ hemostasis to protect against cell death, and the promotion of cell survival in response to cell stress conditions [214, 215].

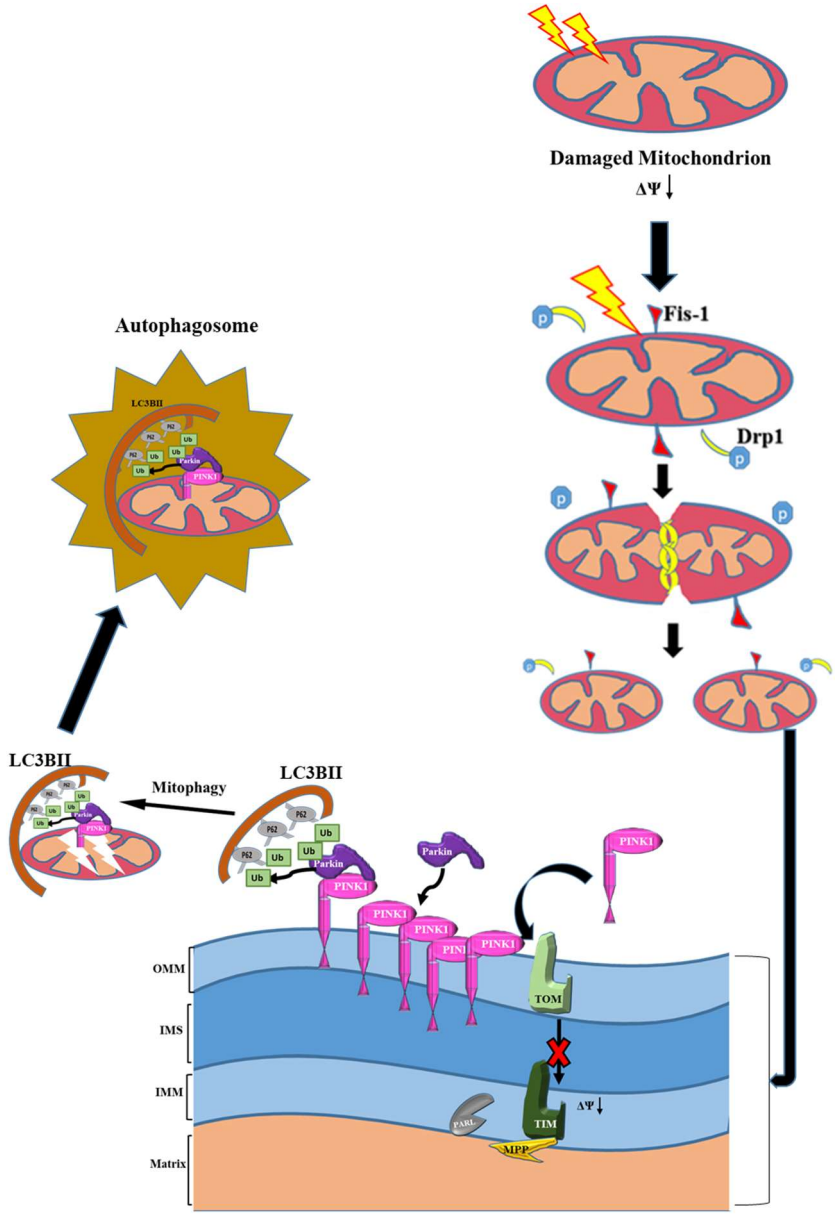


Figure 1.4 PINK1-mediated initiation of mitophagy

Continuous accumulation of PINK1 on the OMM recruits PARKIN from the cytosol to the OMM. Subsequently, damaged mitochondria due fission mediated by DRP1 will be separated. PARKIN recruits ubiquitin along with autophagy mediators (LC3B-II-binding autophagic adaptor P62) on the OMM and triggering the autophagosomal degradation of damaged mitochondria.

Abbreviations: TOM - Translocase outer membrane; TIM - Translocase inner membrane; TM - transmembrane domain of PINK1; MTS - Mitochondrial targeting sequence of PINK1; OMM - Outer mitochondrial membrane; IMS - Inter mitochondrial membrane space; IMM- Inner mitochondrial membrane; PINK1 - phosphatase and tensin homolog-induced putative kinase 1; PARKIN - Park2 E3 ubiquitin ligase protein; PARL - Presenilins-associated rhomboid-like protein; MPP - mitochondrial processing peptidase; Ub – Ubiquitin; P62 - ubiquitin-binding protein; $\Delta\Psi_m$ - mitochondrial membrane potential.

Modified from Ashrafi *et al.*, Cell Death Differ, 2013.

1.3.4. Regulation of mitochondrial fission and fusion

Mitochondria are dynamic organelles. They undergo a sequence of fusion and fission within a network to maintain and swap lipids, proteins and other required materials between them, as well as to ensure exchanges with other organelles in the cells [178]. Additionally, these dynamical fusion and fission cycles support the elimination of damaged mitochondria from the network by mitophagy. Moreover, fission serves to distribute mitochondria to the daughter cells after mitosis [216, 217]. Mitochondrial fusion is necessary for their normal function and maintenance of mitochondrial DNA (mtDNA) [218]. Mitochondrial fusion and fission are complex processes. In mammals, different GTPases of the dynamin family proteins mediate the process of mitochondrial networking. DRP1 mediates the process of mitochondrial fission [219, 220], while membrane-anchored dynamin family members (MFN2 and MFN1) and optic atrophy protein-1 (OPA-1) mediate the fusion process [221, 222]. All of these machineries are controlled by post-translational modification and proteolysis [222]. Figure 1.5 summarized the processes involved in mitochondrial fission and fusion.

During mitochondrial fission in mammals, DRP1 [223] is translocated from the cytosol to the mitochondrial outer membrane (OMM), where it binds to its receptors which include the mitochondrial fission factor (MFF) [224], mitochondrial dynamic proteins (MID49 and MID51) and the mitochondrial fission 1 protein (FIS1) [152, 225, 226] to initiate the fission process (Figure 1.5 A). One study revealed that PINK1 signals to DRP1 for initiation of fission via dislocation of protein kinase A (PKA) on the OMM on the damaged mitochondria [189]. Based on the above-mentioned studies one can conclude that PINK1 can affect on mitochondrial fission/fusion via downregulation of MFN2 and inhibition of DRP1 [176, 189]. During mitochondrial fusion, MFN1, which is anchored to the OMM via its C-terminal region, tethers two adjacent mitochondria, resulting in the fusion of their OMM. Subsequently, OPA-1 which is located on the IMM, connects both mitochondrial IMMs to complete the process of mitochondrial fusion [152] (Figure 1.5 B). There are several publications that have reported the involvement of mitochondrial dynamics in the regulation of cellular metabolism, proliferation and apoptosis [227]. For example, one comprehensive study has shown that in embryonic fibroblasts of Drp-1 knockout mice proliferation was decreased, suggesting that DRP1 is needed for normal cell proliferation [228]. Thus, the imbalance between mitochondrial fusion and fission can lead to various diseases such as neurodegenerative diseases, muscular dystrophy [229], cardiopulmonary diseases [116] and cancer [230].

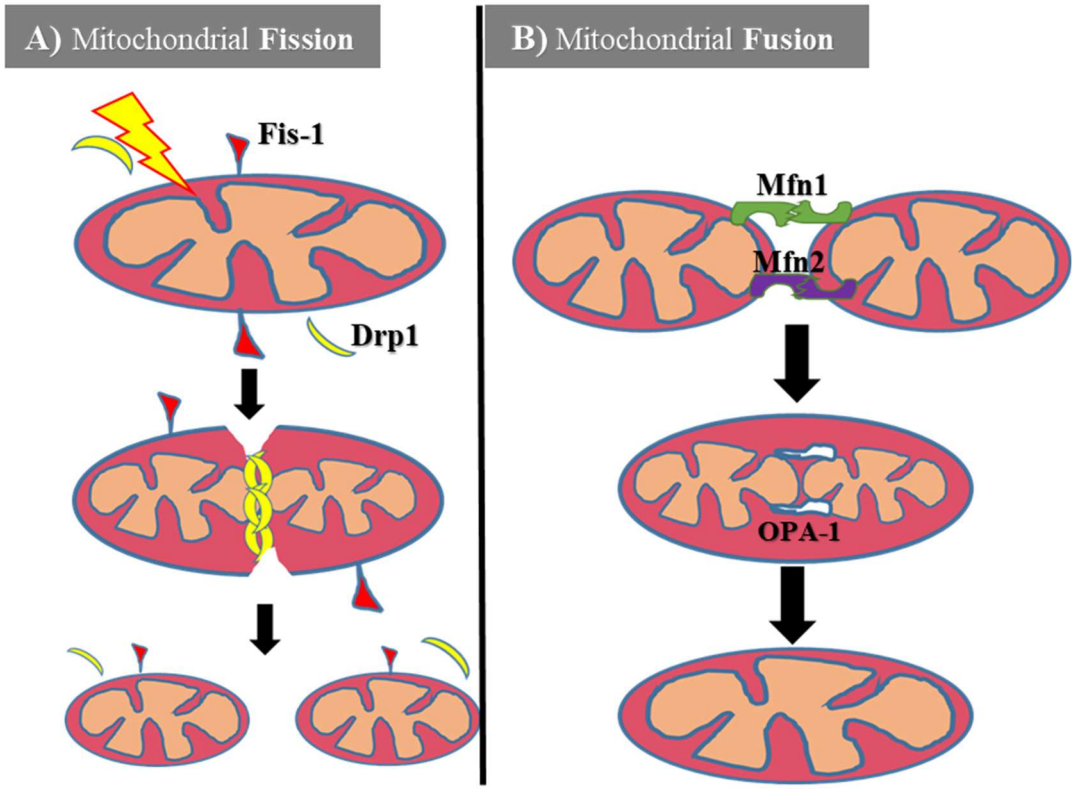


Figure 1.5. Proposed model of mitochondrial fission and fusion

A. During mitochondrial fission, DRP1 translocates from the cytosol to the OMM (by PINK1 signaling via PKA) and binds to its receptors e.g. FIS1 to initiate the fission by inserting a ring/spiral-like formation at the division site of mitochondria. **B**. During mitochondrial fusion, MFN1, which is located on the OMM starts tethering two adjacent mitochondria and after fusion of the MOM, OPA-1 on the IMM participates in the connection of both mitochondrial IMMs to complete the fusion process.

Abbreviations: DRP1 - Dynamin-related protein 1; Fis-1 - Fission protein-1; MFN1 and MFN2 - Mitofusion 1 and 2; OPA-1 - Optic atrophy protein-1; OMM - Outer mitochondrial membrane; IMM - Inner mitochondrial membrane.

Modified from Chiong *et al.*, Front Cell Dev Biol., 2014

Although the role of mitochondrial dynamics (fission/fusion) in PH development has been studied, the exact mechanism still remains unknown [231]. Bonnet and Marsboom *et al.* in different studies reported that in PAH -due to an increased rate of mitochondrial fission- the mitochondrial fragmentation increased and resulted in the elevation of anti-apoptotic and pro-proliferation properties of PASMC [105, 232]. Moreover, inhibition of mitochondrial fission via attenuation of DRP1 activity by the mitochondrial division inhibitor 1 (Mdivi-1) reduces PASMC proliferation in PAH patients and improves hemodynamics in animal models of PH [105, 233].

Accordingly, in PAH the mitochondrial fusion mediator MFN2 was downregulated [105] and *Mfn2* deficiency increased mitochondrial fragmentation as well as proliferation and anti-apoptosis in PASMC in PAH [234]. Therefore, the impact of mitochondrial fission and fusion on PH development is imminent, however the complete role of fission and fusion and in particular its connection to mitophagy in PH remains uncovered [234].

1.3.5. The role of autophagy in mitophagy

PINK1/PARKIN interact with LC3B-II during mitophagy to eliminate mitochondria by autophagosomes [235]. LC3B is one of the main cellular autophagy markers in mammalian cells. LC3B-I is a soluble protein in almost all cells and tissues [236, 237]. LC3B-II (the phosphatidylethanolamine conjugated form of LC3B-I) is recruited to the autophagosomal membrane in order to start engulfment of targeted organelles or cytoplasmic components [236]. Therefore, LC3B-II has been reported as an important and reliable autophagy marker in many experiments related to autophagy and autophagosomal studies [236, 237]. The importance of LC3B-II is highlighted for quantification of autophagy in a way that, corresponding amount of LC3B-II indicated the amount of autophagosomes during autophagy process [238].

Previously, LC3B-II was shown to be upregulated in the lung of IPAH patients and hypoxic mouse lungs [239]. However, it has been recently revealed that PINK1-dependent mitophagy can be executed independently of LC3B, whereas BNIP3L/Nix and FUNDC1 (PINK1-independent mitophagy pathways) interact with LC3B-II via their LC3B-interacting motifs [240]. According to the latest proposed model for autophagy [241], PARKIN recruits ubiquitin along with LC3B-binding autophagic adaptor P62 (SQSTM1) on the OMM [242] in order to gather all the damaged mitochondria [243]. Therefore, all the damaged mitochondria which ubiquitinated will be clustered by SQSTM1 and clamped to the LC3B-II in order to lead them for degradation by autophagosomes [192].

1.3.6. PINK1-independent mitophagy and PH

BNIP3L/Nix, a homolog of BNIP3 [244], was first reported by Boyed *et al.* in 1994 [245]. The interaction of BNIP3L/Nix with LC3B-II eliminates damaged ER and mitochondria, selectively [246-248]. In one study, Sowter *et al.* showed that, BNIP3L/Nix are induced by HIF-1α in hypoxic conditions to prevent ROS accumulation and to abolish damaged mitochondria in human brain tumors [249]. The complete signaling mechanism of BNIP3L/Nix is still not

known, though they may act as complementary molecules for mitophagy induction during hypoxia [250]. Currently, one interesting study has shown that, BNIP3L/Nix may serve as an initiator substrate of PARKIN for PINK1-dependent mitophagy induction, which may interact with PINK1 to support its accumulation on the outer mitochondrial membrane [251, 252]. Currently, the role of BNIP3L/Nix mitophagy for development of PH is unclear, however, Deng *et al.* showed that, BNIP3 plays a phase-dependent role in the pro-autophagy signaling pathway, to promote RV remodeling in MCT-induced PH model [253].

FUNDC1 is also an outer mitochondrial membrane protein which is involved in hypoxia-induced mitophagy in mammalian cells and also interacts with LC3B [254]. Currently, the role of FUNDC1 in PH has not been investigated, yet.

1.3.7. PINK1 and cellular proliferation/apoptosis

Little is known about the role of PINK1-dependent mitophagy for proliferation and apoptosis of PASMC in pulmonary vascular remodeling in PH. Most studies concerning the effects of PINK1-dependent mitophagy on cellular proliferation/apoptosis have been performed in the field of neurodegenerative diseases [255] and cancer [256]. For example, Choi *et al.* showed that *Pink1*$^{-/-}$ inhibited the proliferation of astrocytes by reducing Protein kinase B (Akt) signaling, and increasing mitogen-activated protein kinase P38 (MAPK) activity, leading to the downregulation of epidermal growth factor (EGF) receptor expression [257]. Another study proposed that the absence of PINK1 inhibits apelin-13-induced proliferation in vascular SMCs by activation of adenosine monophosphate-activated protein kinase (phosphorylated at Thr-172) (p-AMPKα) [258]. In contrast, another group showed that the absence of PINK1 in mouse embryonic fibroblasts (MEF) and primary cortical neurons increased cellular proliferation via the reprogramming of glucose metabolism by HIF-1α activity [259]. With regard to apoptosis, one study on lung tumor cells showed that by silencing *Pink1*, *in vivo* elevation of apoptosis was observed, which was caused by a sequence of processes involving Bcl-2-associated X (Bax/Bcl-2) proteins [260]. Deficiency of *PINK1* in SH-SY5Y cells impaired cell survival via the elevation of apoptosis [261]. A recent study of Sarraf *et al*. showed that, translocation and degradation of Tank Binding Kinase 1 (TBK1) was dependent on the regulation by PINK1/PARKIN. As TBK1 can act on the centrosome, they suggested that PINK1/PARKIN signaling has an indirect effect on the cell cycle progression [262]. Taken together, PINK1 may play an important role in cellular proliferation and apoptosis and thus affect vascular remodeling and the development of PH.

1.3.8. Mitophagy and autophagy in PH

There are several studies that have shown that mitophagy plays an important role in physiological processes such as embryonic development [263], the innate immunity [264, 265] and in the pathophysiology of different diseases such as cancer [266, 267], neurodegenerative diseases [197, 268, 269], and tissue injury and repair [270, 271]. Although there are a few investigations on the role of mitophagy in pulmonary diseases especially idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) [272] , little is known for PH. In one study by Haslip *et al.,* the absence of UCP2 in PECs increased PINK1-dependent mitophagy and resulted in elevated apoptosis in PECs and PH elevation [169]. In one study in mice model of pulmonary arterial banding, the importance protective role of mitophagy against right ventricular failure (RVF) was shown, interestingly, an elevation of LC3A/B protein was observed [273]. The possible role of autophagy in PH was suggested by a study showing that LC3B-II was elevated in lung tissue of IPAH patients. They proposed that increased autophagy (elevation of LC3B-II) can be considered as a protective mechanism in the lung vascular cells as well as chronic hypoxia mouse model of PH [239]. However, regulation of general autophagy mediators such as LC3B-II cannot indicate mitophagy specifically and it may show false positive results due to the volatile nature of the autophagosomes and their interaction with LC3B [272].

1.4. Aim of the study

Altered mitochondrial function plays an important role in the increased proliferation and decreased apoptosis of PASMC leading to pulmonary vascular remodeling in PH. PINK1-dependent mitophagy is an essential mechanism for mitochondrial quality control and elimination of damaged mitochondria. Although the relevance of mitophagy and its mediators for cellular proliferation and apoptosis has been described previously [257, 260, 274], the role of PINK1-dependent mitophagy in PH remains unknown.

This study thus investigates the role of PINK1 in development of hypoxia-induced PH in mice and human IPAH

The major aims of this study are as follows:

1) To study the expression levels of proteins involved in PINK1-dependent mitophagy in IPAH and in chronic hypoxia-induced PH.
2) To investigate the effect of *Pink1* deficiency on apoptosis and proliferation of PASMC after *in vitro* hypoxic exposure.
3) To investigate the effect of *Pink1* deficiency on the development of chronic hypoxia-induced PH.

Based on the above-mentioned aims, the following investigations were performed (see figure 1.6):

- ✓ Determination of mRNA and protein levels of PINK1, PARKIN and PARL in mouse PASMC after *in vitro* hypoxic exposure, lung homogenate from mice after *in vivo* chronic hypoxic exposure and lung homogenate of patients with IPAH.
- ✓ Investigations of the protein expression of DRP1 and MFN2 in precapillary PASMC after *in vitro* hypoxic exposure.
- ✓ Measurement of proliferation and apoptosis of WT and *Pink1*$^{-/-}$ PASMC after *in vitro* exposure to hypoxia.
- ✓ Determination of the effect of *Pink1* deletion on the development of chronic hypoxia-induced PH *in vivo*.
- ✓ Investigation of protein expression of PINK1-independent mitophagy

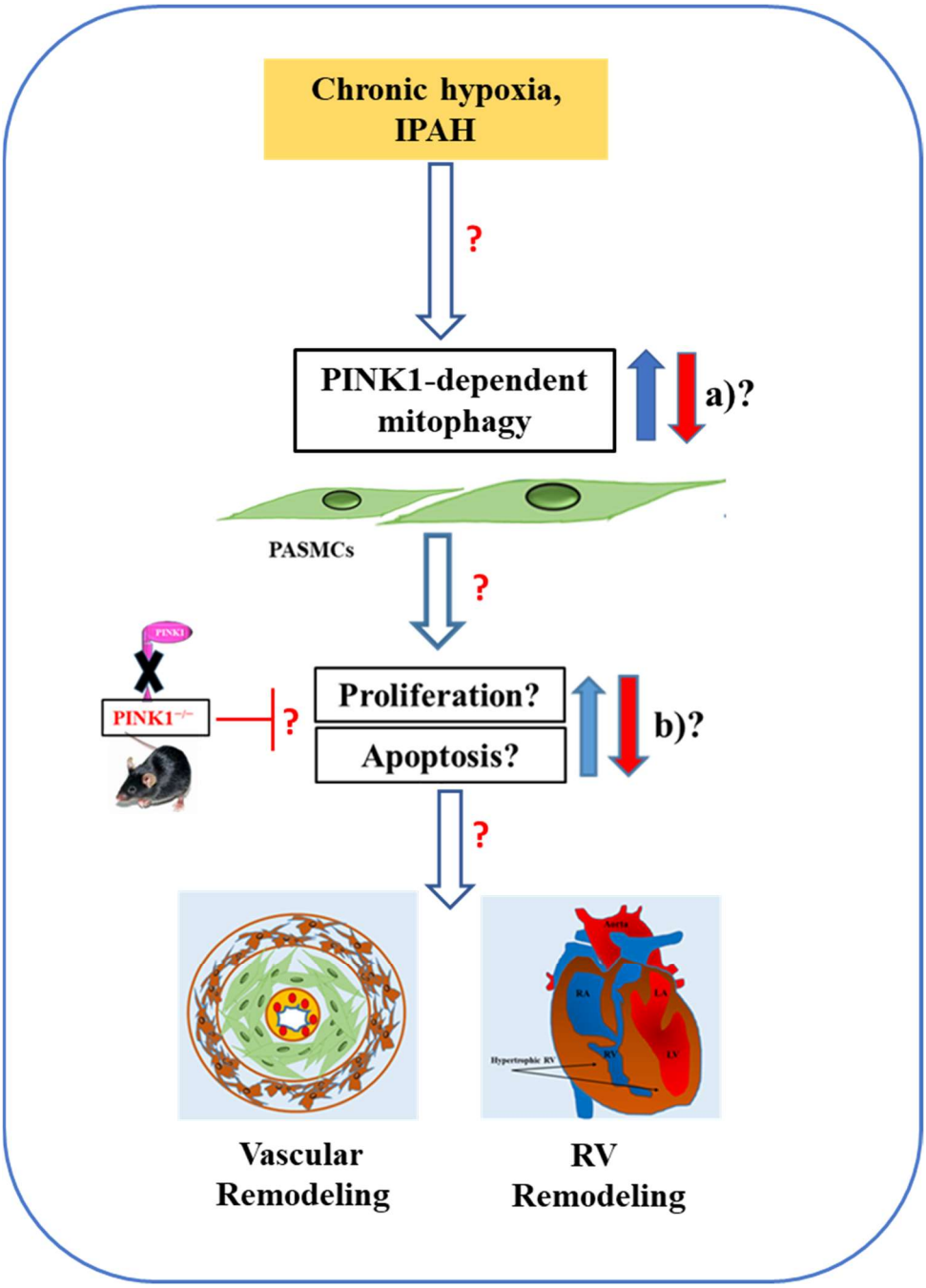


Figure 1.6 Aim of the study

The main aims of the study were:

a) To investigate the expression level of proteins that regulate PINK1-dependent mitophagy.
b) To elucidate the effects of *Pink1* deletion on proliferation and apoptosis of PASMC during i*n vitro* hypoxic exposure
c) To determine of the effect of *Pink1* deletion on the development of chronic hypoxia-induced PH *in vivo.*

Abbreviations: PASMC - pulmonary smooth muscle cells; PECs - pulmonary endothelium cells; RA - Right atrium; RV -Right ventricle; LA - Left atrium; LV - Left ventricle; PINK1 - phosphatase and tensin homolog-induced putative kinase

2. Materials

2.1 List of reagents and chemical (Table 3)

Reagent/ Chemical	Company/Supplier
2- Mercapto-ethanol	Sigma-Aldrich, St. Louis, USA
Acetic Acid, Glacial 99%	Sigma-Aldrich, St. Louis, USA
Acetone 99,5%	Sigma-Aldrich, St. Louis, USA
Albumin, Bovine serum	Sigma-Aldrich, St. Louis, USA
CaCl2	Sigma-Aldrich, St. Louis, USA
Collagenase type IV	Sigma-Aldrich, St. Louis, USA
Complete	Roche Applied Science, Penzberg, Germany
Crystal violet	Sigma-Aldrich, St. Louis, USA
Dextran sulphate	Sigma-Aldrich, St. Louis, USA
ECL and Western Blot detection	GE Healthcare, Little Chalfont, UK
EDTA (Ethylenediamine-tetraacetic acid)	Sigma-Aldrich, St. Louis, USA
Ethanol (70%, 95%, 99,6%)	SAV LP GmbH, Flintsbach, Germany
FCS (Fetal calf serum)	Invitrogen, Carlsbad, USA
Fe3O4 (Iron particles)	Sigma-Aldrich, St. Louis, USA
Formaldehyd alcohol free 3.7%	Otto Fischar GmbH&Co, Saarbrücken, Germany
H_2O_2 30%	Merck, Darmstadt, Germany
HCL	Sigma-Aldrich, St. Louis, USA
Heparin	Rathiopharm GmbH, Ulm, Germany
Hepes (2-(-4-2-hydroxyethyl)-piperazinyl-1ethansulfonate)	Sigma-Aldrich, St. Louis, USA
iScript cDNA Synthesis Kit	Bio-Rad, Hercules, USA
Isoflurane	Forene® Abbott, Wiesbach, Germany
Isopropanol (99,8%)	Fluka Chemie, Buchs, Switzerland
iTaq SYBR Green supermix with ROX	Bio-Rad, Hercules, USA
Ketavet (Ketamine hydrochloride)	Pfizer, Karlsruhe, Germany
KH2PO4	Sigma-Aldrich, St. Louis, USA
LNA™ mRNA Detection Probes	Exiqon, Vedbaek, Denmark

Low melting agarose	Sigma-Aldrich, St. Louis, USA
M199 medium	Invitrogen, Carlsbad, USA
Medical X-Ray film	Agfa, Mortsel, Belgium
Methanol	Fluka Chemie, Buchs, Switzerland
MgCl2	Sigma-Aldrich, St. Louis, USA
Milk powder	Carl ROTH, Karlsruhe, Germany
NaCl	Sigma-Aldrich, St. Louis, USA
NaH2PO4	Sigma-Aldrich, St. Louis, USA
NaOH	Sigma-Aldrich, St. Louis, USA
Nuclear Fast Red	Sigma-Aldrich, St. Louis, USA
Paraformaldehyde, 4%	Merck, Darmstadt, Germany
PMSF	Sigma-Aldrich, St. Louis, USA
Proteinase K	Sigma-Aldrich, St. Louis, USA
PVDF membrane	Pall Corporation, Dreieich, Germany
RNeasy RNA extraction kit	Qiagen, Hilden, Germany
SDS (Sodium dodecyl sulfate)	Sigma-Aldrich, St. Louis, USA
Sodium vanadate	Sigma-Aldrich, St. Louis, USA
SYBR® Safe DNA gel stain	Invitrogen, Carlsbad, USA
TEMED (N,N,N',N'-Tetramethyl-1-, 2diaminomethane)	Sigma-Aldrich, St. Louis, USA
Trypsin	PAN, Aidenbach, Germany
Tween-20	Sigma-Aldrich, St. Louis, USA
Xylazine	Bayer Healthcare, Leverkusen, Germany

2.2 List of antibodies (Table 4)

Name	Company/Supplier
Anti-Goat anti-Mouse IgG antibody	Invitrogen, Karlsruhe, Germany
Anti-Goat anti-Rabbit IgG antibody	Invitrogen, Karlsruhe, Germany
Anti- BNIP3L/Nix antibody	Abcam, Cambridge, UK
Anti-DRP1 antibody	Cell Signaling Technology, Danvers, USA
Anti-LC3B antibody	Cell Signaling Technology, Danvers, USA
Anti- MFN2 antibody	Abcam, Cambridge, UK
Anti-Parkin antibody	Novusbio, Littleton, USA
Anti-PARL antibody	Novusbio, Littleton, USA
Anti-PINK1 antibody	Novusbio, Littleton, USA
Anti-ß-actin antibody	Sigma-Aldrich, St. Louis, USA
Anti-von Willebrand factor antibody	Dako Diagnostika, Hamburg, Germany
Anti-α-smooth muscle actin antibody	Sigma-Aldrich, St. Louis, USA

2.3 List of equipment (Table 5)

Equipment Name	Company/Supplier
Absorbance reader	BioTek, Bad Friedrichshall, Germany
Anesthesia chamber	Von Keutz Labortechnik, Reiskirchen, Germany
Autoregulatory control unit (model 4010, O_2 controller)	Labotect, Göttingen, Germany
Cannula for flushing	Hugo Sachs Elektronik Harvard Apparatus GmbH, March, Germany
Catheter for carotid artery	FST GmbH, Heidelberg, Germany
Catheter for jugular vein	NuMED Inc, Hopkinton, USA
Catheter (1.4F micromanometer)	Millar Instruments, Houston, USA
ChemiDoc Touch Imaging System	Bio-Rad, Munich, Germany
Biemer microvessel clip	Aesculap, Tuttlingen, Germany
Cell culture incubator	Thermo Fisher Scientific, Waltham, USA
Digital camera microscope DC 300F	Leica Microsystems, Wetzlar, Germany
Electrophoresis chambers	Biometra, Göttingen, Germany
Flattening bath for paraffin sections, HI 1210	Leica Microsystems, Wetzlar, Germany
Flattening table, HI 1220	Leica Microsystems, Wetzlar, Germany
Fluid-filled force transducer	B. Braun Melsungen AG, Melsungen, Germany
Homeothermic plate control unit	AD Instruments, Spechbach, Germany
Homogenplus	Schuett-biotec GmbH, Göttingen Germany
Hypoxic Glove chamber	Terra Universal Inc, Fullerton, USA
inverted microscope (DMI6000 CS)	Leica Microsystems, Manheim, Germany
Makro for muscularization degree	Leica Microsystems, Wetzlar, Germany
Medical X-Ray film processor (curix 60)	Agfa, Mortsel, Belgium
Microplate reader Infinite m200	Tecan Group Ltd, Männedorf, Switzerland
Microtome RM2165	Leica Microsystems, Nussloch, Germany
MiniVent type 845	Hugo Sachs Elektronik, March-Hugstetten, Germany

MS 100 spectrometer	Magnettech, Berlin, Germany
Mx3000P QPCR System	Agilent Technologies, Santa Clara, USA
NanoDrop	PeqLab, Erlangen, Germany
Opened heated chamber	PeCon, Erbach, Germany
Polychrome II monochromator and IMAGO CCD camera	Till photonics. Munich, Germany
Precelly®24 homogenizer	PeqLab, Erlangen., Germany
Pressure converter	B. Braun Melsungen AG, Melsungen, Germany
Pressure transducer APT300	Hugo Sachs Elektronik, March-Hugstetten, Germany
Rectal thermometer	Indus Instruments, Houston, USA
Scintillation counter, TRI-CARB 2000	Canberra-Packard, Meriden, USA
Software Q Win V3 Leica Microsystems Nussloch, Germany	Leica Microsystems, Wetzlar, Germany
Top Laboratory Animal Anesthesia System	VetEquip Inc, Pleasanton, USA
Tissue embedding machine, EG 1140H	Leica Microsystems, Wetzlar, Germany
Tissue processing automated machine, TP 1050	Leica Microsystems, Wetzlar, Germany
Ultrasound platform Vevo® 2100	VisualSonics Inc. Toronto, Canada
Ultrasound gel	Parker Laboratories Inc. Fairfield, USA
Water bath	Von Keutz Labortechnik, Reiskirchen, Germany

2.4 List of consumables (Table 6)

Consumable	Company/Supplier
Blotting papers	Bio-Rad, München, Germany
Cover slips 24x36mm	Menzel, Germany
Depilatory cream	Veet, Heidelberg, Germany
Dexpanthen ophtalmic ointment	Bayer Vital, Leverkusen, Germany
Embedding cassette	Carl Roth GmbH & Co, Karlsruhe, Germany
Falcon Tubes 15 and 50 ml	Greiner bio-one, Frickenhausen, Germany
Feather disposal scalpel	Pfmmediacl, Köln, Germany
Glass Bottles: 0.1; 0.2; 1L	Schott Duran, Germany
Glass Pipettes	Greiner bio-one, Frickenhausen, Germany
Medical adhesive bands 3M	Durapore® St. Paul, MN, USA
Microtome blade S35	A. Hartenstein GmbH, Würzburg, Germany
Microscope slide	R. Langenbrinck, Emmendingen, Germany
Napkins	Tork, Mannheim, Germany
Needle (20G, 11/2",0.9x40mm)	BD Microlance, Becton Dickinson, Germany
Needle (18G,11/2",1.20x38mm	Unolok, Horsham, UK
Petri dishes	Greiner bio-one, Frickenhausen, Germany
Plastic Syringe (1,3,5, 10ml and 20ml)	B Braun Melsungen, Germany
Single use gloves Transaflex®	Ansell, Surbiton, UK
Tissue Culture Dish 100mm, 250mm	Greiner bio-one, Frickenhausen, Germany

3. Methods

3.1. Animal experiments and housing

All animal experiments were approved by the local authority of animal research (Regierungspräsidium Giessen, reference number – GI 20/10-Nr.115/2014). Adult male and female C57BL/6J mice were obtained from Charles River WIGA GmbH (Sulzfeld, Germany). Breeder pairs of *Pink1*$^{-/-}$ mice (B6.129S4*Pink1*/J) were purchased from the Charles River Laboratory (Sulzfeld, Germany) and *Pink1*$^{-/-}$ mice bred in our animal facility. According to Kitada *et al., Pink1*$^{-/-}$ mice have a deletion of the 4-7 exon on the *Pink1* gene in all cell types [275]. The animals were kept under standard conditions in our animal facility (14/10 hours day and night cycle, 55% +/- relative humidity, 22 +/- 2°C temperature). Mice were held in individually ventilated cages (IVC) in groups of up to five mice per type II long cage. Health monitoring according to the FELASA recommendations was performed on a regular basis. Male and female mice were used for the study aged 12-16 weeks old.

3.2 Human materials

The ethics committee at the faculty of medicine, Justus-Liebig University of Giessen approved use of human material according to the national guidelines and legislation for good clinical practice/international conference on harmonization under the number of AZ 10/06 and AZ 58/15. Anonymous peripheral human lung tissue samples were selected from the explanted lungs of 10 IPAH patients. The average age of the patients was 38 years. Lung tissue was collected from donor lungs (n=10) as control. The average age of donors was 46 years (n=10). Detailed characteristics of human lung tissues are described in Table 7.

SEX	Age	Diagnostics	SEX	Age	Diagnostics
Male	31	**Donor**	Male	50	**IPAH**
Male	58	**Donor**	Male	35	**IPAH**
Male	53	**Donor**	Male	50	**IPAH**
Female	34	**Donor**	Female	29	**IPAH**
Female	54	**Donor**	Female	32	**IPAH**
Female	44	**Donor**	Female	32	**IPAH**
Female	48	**Donor**	Female	33	**IPAH**
Female	41	**Donor**	Female	42	**IPAH**
Female	44	**Donor**	Female	39	**IPAH**
Female	54	**Donor**	Female	25	**IPAH**

Table 7. List of the patients' characteristics of human lung tissue samples

3.3. Isolation and culture of mouse PASMC

Isolation of mouse PASMC from precapillary pulmonary arterial vessels was described previously by Weissmann *et al.* [276]. Mice were anesthetized with ketamine (100 mg/kg body weight, Pfizer, Karlsruhe, Germany) and xylazine (20 mg/kg body weight, Bayer Healthcare, Leverkusen, Germany) and then were injected with heparin (50,000 I.E./kg body weight). After 5-10 minutes incubation time, the deep anesthesia was confirmed by the pedal withdrawal reflex test. The mouse was fixed in supine position and disinfected with 50% 2-propanol and 1% povidone-iodine solution (Braunoderm®). A longitudinal incision was made on the skin from abdominal area up to the neck and after opening abdominal skin by cutting the main abdominal vein the mouse was sacrificed by exsanguination. After opening of abdomen, the diaphragm was removed from the thorax very carefully. Following pericardium was also removed and then the thorax was opened by a fine surgical scissor very carefully. The chest muscles and ribs were fixed back in order to obtain proper space and approach to the cardiopulmonary organs of the animal. Following by removing the thymus, gently a ligature was placed around the pulomoray artery and aorta. Then by placing a small incision into the RV a catheter was placed very gently in the pulmonary artery (PA) and the pulmonary vasculature was rinsed with cold phosphate-buffered saline (PBS) followed by infusion of growth medium 199 (Invitrogen, Carlsbad, USA) containing 1% penicillin/streptomycin (10000 U/ml penicillin, 10 mg/ml streptomycin, PAN Biotech GmbH Aidenbach, Germany), 0.5% low-melting-point agarose (type VII, Sigma-Aldrich, Munich, Germany) and 5mg/ml Fe_3O_4 particles (Sigma-Aldrich, Munich, Germany). Furthermore, after making a small incision in the trachea, with the helping of another catheter the lung was filled with a mixture of 0.5% low-melting-point agarose (type VII, Sigma-Aldrich, Munich, Germany) and growth medium 199 (Invitrogen, Carlsbad, USA) containing 1% penicillin/streptomycin (10000 U/ml penicillin, 10 mg/ml streptomycin, PAN Biotech GmbH Aidenbach, Germany). After dissection, the lung was placed in cold PBS until the agarose solidified. Further, the lung was mechanically chopped with scissors, and minced tissue were washed 3 times with PBS. Pieces of precapillary pulmonary arteries filled with the iron particles were collected with a magnetic holder. The pulmonary arteries were incubated in 10 ml of M199 medium containing collagenase (80 U/ml) at 37°C for 1 hour for removing the fibroblasts. The collagenase digestion was stopped by washing 3 times with M199 medium containing 1% penicillin/streptomycin and 10% fetal calf serum (FCS, PromoCell, Heidelberg, Germany) and finally, the digested precapillary pulmonary artery pieces were disrupted by drawing them through 15- and 18-gauge needles 5-6 times. The pulmonary artery pieces including medial layer of pulmonary arteries were collected by a magnetic holder. The tissue was re-suspended

in smooth muscle cell growth medium 2 (PromoCell, Heidelberg, Germany) supplemented with normocin (1%, Invitrogen, De Schelp, Netherlands) and 15% FCS, and transferred to the cell culture dishes (60 mm). After 5-7 days of incubation at 37°C in an incubator with 21% O_2, 5% CO_2, (balanced with N_2), the cells reached about 80% of confluency. Then the grown cells were trypsinized and divided into the fresh culture flasks. The isolated PASMC in passage 0 or 2 were used for the experiments.

3.4. Invasive quantification of hypoxia-induced PH by echocardiography, *in vivo* hemodynamics and right ventricular morphometry

WT and *Pink1*$^{-/-}$ mice were exposed to normobaric hypoxia conditions (10% O_2) for 28 days along with control mice in normobaric normoxia chamber (21% O_2) (Figure 3.1). After exposure, transthoracic echocardiography was performed in the mice with the Vevo2100 high-resolution imaging system equipped with a 40-MHz transducer (VisualSonics, Toronto, Canada). Anesthesia was induced with 3% isoflurane in O_2 and maintained via a nose cone with 1.5% isoflurane (balanced with O_2). The mice were laid in a supine position on a heating platform while all four limbs were connected to electrocardiography (ECG) electrodes in order to monitor heart rate. The body temperature was monitored using a rectal thermometer (Indus Instruments, Houston, US). After shaving the chest area and spreading pre-warmed ultrasound gel over the chest, echocardiographic studies were performed and cardiac parameters measured as described previously [277].

Invasive quantification of PH was performed as described previously [2]. Anesthesia was induced with 3% isoflurane in O_2 and maintained via nose cone with 1.5% isoflurane which was balanced with O_2. Mice were laid in the supine position on a heating pad and connected to a small animal ventilator MiniVent type 845 (Hugo Sachs Elektronik, March-Hugstetten, Germany). The right jugular vein was cannulated by a fluid-filled Hyman mouse pressure catheter (Numed. Inc, Hopkinton, USA) for measurement of RVSP and systemic arterial pressure (SAP). The digital signals were recorded using LabTech Pro software. After hemodynamic measurement, mice were exsanguinated and lungs were flushed with saline and fixed by perfusion with 3.5-3.7% formaldehyde (Otto Fischar GmbH&Co KG, Saarbruecken, Germany) with a constant pressure of 22 cm H_2O in the pulmonary artery and 11 cm H_2O in the trachea. The lung and the heart were removed. Then the lung lobes were embedded in paraffin, and sections of 3 μm were cut from all lobes. The RV was dissected from the left ventricle and septum (LV+S). Finally, dissected samples were dried for 3 days at 50°C and

weighed to obtain the ratio of the weight of the right ventricle to the weight of the left ventricle plus septum (RV/LV+S).

Echocardiography and invasive hemodynamic measurements were performed with the help of Dr. A. Sydykov.

Figure 3.1. Normobaric hypoxic and normoxic chambers for exposure of mice

a) IVC cages (Type II long); **b)** Normobaric normoxic chamber (21% O_2, 5% CO_2 and rest N_2); **c)** Normobaric hypoxic chamber (10% O_2, 5% CO_2 and rest N_2); **d)** Monitoring of the O_2 pressure and conditions of the chambers.

Abbreviations: IVC - individually ventilated cage

3.5. Assessment of pulmonary vascular remodeling

The degree of muscularization of small peripheral pulmonary vessels in *Pink1*$^{-/-}$ and WT mice were assessed by performing double staining with an anti-α-smooth muscle actin antibody (1:900 dilution, clone 1A4, Sigma-Aldrich, Saint Louis, USA) and polyclonal rabbit von Willebrand factor antibody (1:900 dilution, Dako, Hamburg, Germany). The mice lung sections were counterstained with methyl green and examined by light microscopy with the use of a computerized morphometric system (Qwin, Leica, Wetzlar, Germany). At 40x magnification,

all intra-acinar vessels (20-70 µm in diameter) were blindly analyzed. Each vessel was categorized as non-muscularized (<5% vessels circumference α-smooth muscle actin positive) with no apparent smooth muscle, partially muscularized (5% to 70% vessels circumference α-smooth muscle actin positive) with a partial smooth muscle layer and fully muscularized (>70% vessels circumference α-smooth muscle actin positive) with a complete smooth muscle layer. The percentage of pulmonary vessels in each muscularization category was determined by dividing the number of vessels in that category by the total number of counted vessels (and multiplying by 100). The method was described previously [278].

3.6. Immunohistochemistry

Immunostaining was performed in 3-µm-thick sections of paraffin-embedded lung tissues from IPAH patients and donors. Paraffin-embedded lung tissue sections were deparaffinized in xylol and then rehydrated in a graded ethanol dilution series before incubation in PBS (pH 7.4). Antigen retrieval was performed by pressure-cooking in citrate buffer (pH 6.0). Following blocking with 10% bovine serum albumin (BSA,1-gram powdered BSA in 10 mL of distilled water) for 1 hour and then with blocking serum (PostBlock, ZytoMed) for 5 minutes, the sections were incubated overnight at 4°C with the primary antibody. Mouse monoclonal antibody against human anti-PINK1 (1:1000 dilution, Novus Biologicals, Littleton, USA), anti-PARL (1:1000 dilution, Novus Biologicals, Littleton, USA), and anti-PARKIN (1:1000 dilution, Abnova, Taipei, Taiwan) were used as the primary antibodies. Anti-Rabbit Ig peroxidase (ImmPRESS® REAGENT) was used as secondary antibody. Development of the dye was carried out with alkaline phosphatase and substrate according to the manufacturer's (Chromogen) instructions. Finally, sections were counterstained with hematoxylin, and coverslipped using mounting medium [279].

3.7. Protein isolation and Western blot analysis

Proteins were isolated from mouse and human lung homogenate and isolated precapillary mouse PASMC. PASMC were lysed with a scraper and tissue was disrupted by grinding in liquid nitrogen and Precelly®24 Homogenizer (Peqlab, Erlangen, Germany). For tissue 150 µl self-made Radioimmunoprecipitation assay (Ripa) buffer [for 1000 µl of Ripa buffer: cOmplete 40 µl, PMSF (100 mM) 1 µl and Sodium-Orthovanadate (200 mM)] with ceramic beads were used. For PASMC, 300 µl 1x cell lysis buffer (Cell Signaling, Danvers, USA), containing 2 mM Na_3VO_4 (pH 10) and 1x Complete (Roche, Basel, Switzerland) were added. After incubation on ice for 15 minutes, samples (PASMC or tissue) were centrifuged (20,000 x g, 5

minutes, 4°C). The protein concentration in the supernatant was determined by a spectrophotometric assay (DC Protein-Assay; Bio-Rad, Munich, Germany). For each western blot, 2.8 µg of total protein per line were used.

Proteins isolated from tissue and PASMC were separated in 10% and 12 % SDS (sodium dodecyl sulfate) polyacrylamide gel, followed by electrotransfer to a 0.45µm polyvinylidene fluoride (PVDF) membrane (ImmobilonTM-P, Millipore Corporation, Bedford, US). The membranes were blocked with 5% non-fat dry milk in TBS-T buffer (Tris Buffer Saline + 0.1% Tween20). Thereafter, membranes were incubated overnight in a cool room with one of the following antibodies: anti-PINK1 (1:1000 dilution, Novus Biologicals, Littleton, USA), anti-PARL (1:1000 dilution, Novus Biologicals, Littleton, USA), anti-PARKIN (1:1000 dilution, Abnova, Taipei, Taiwan), anti- MFN2 (1:1000 dilution, Abcam, Cambridge, UK), anti- DRP1 (1:500 dilution, Abcam, Cambridge, UK), Anti-LC3B (1:1000 dilution, Cell Signaling Technology Inc., Danvers, USA), anti- BNIP3L/Nix (1:1000 dilution, Cell Signaling Technology Inc., Danvers,USA), and anti-β-actin (dilution 1:50000, Sigma-Aldrich, St. Louis, USA), as primary antibodies. The membranes were washed 5 times with TBS-T buffer and using a secondary antibody (anti-rabbit or anti-mouse IgG) coupled to horseradish-peroxidase (dilution 1:5000, W4011; Promega, USA), the specific immune-reactive signals were detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK).

3.8. RNA Isolation, cDNA synthesis and real-time PCR

RNA was isolated by using the total RNA Kit (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. For cDNA synthesis, the iScriptTMcDNA synthesis kit (Bio-Rad Laboratories GmbH, Munich, Germany) was used. Quantitative real-time PCR was performed using iQTM SYBR® Green supermix (Bio-Rad Laboratories GmbH) on a Stratagen Mx3000PTMQPCR Thermocycler (Stratagene, Kirkland, USA). Quantitative real-time PCR was performed with the cycle details: 10 minutes at 95°C, [10 seconds at 95°C, 10 seconds at 59°C, and 10 seconds at 72°C] x 40, 1 minute at 95°C, 3 minutes at 55-95°C. As the SYBR® Green I dye binds non-selectively to the double stranded DNA, melting curve analysis and gel electrophoresis were performed to confirm the exclusive amplification of the expected PCR product. The ΔCt values for each target gene were calculated by $\Delta Ct = Ct_{\text{reference gene}} - Ct_{\text{target gene}}$. The ΔΔCT was calculated by subtracting the experimental from the control samples, and the fold change 2^ΔΔCt was calculated. β 2 microglobulin (*β2M*) and hypoxanthine phosphoribosyl transferase *(HPRT)* were used as the housekeeping genes for mouse and human respectively, after their evaluation.

The primer sequences used are shown in table 8.

Table 8. List of the primer sequences for qPCR

Species	Primer	Orientation	Sequences	Accession No.
Mouse	*Pink1*	Sense	TTGGCTGGAGAGTATGGAGCA	NM_026880.2
		Antisense	AGGGGCACAGATGAGGTGAA	
Mouse	*Parkin*	Sense	ACCTGACAGAAACGCTGGTG	NM_016694.4
		Antisense	ACCTCCACTGGGAAGCCATA	
Mouse	*Parl*	Sense	GGGAGCTCTCTGCGACCC	NM_001005767.4
		Antisense	TGCTTCTCCACTCGACCCTG	
Mouse	*β2M*	Sense	AGCCCAAGACCGTCTACTGG	NM_009735.3
		Antisense	TTCTTTCTGCGTGCATAAATTG	
Human	*PINK1*	Sense	TCTTTCTGGCCTTCGGGCTA	NM_032409.2
		Antisense	TCTCGTGTCCAACGGGTCAG	
Human	*PARKIN*	Sense	AGGAGGTGGTTGCTAAGCGA	NM_013987.3
		Antisense	TCTCCACGGTCTCTGCACAA	
Human	*PARL*	Sense	ATCATGCGGCACATCTTGGG	NM_018622.7
		Antisense	TGCCAGATTTTCACTAGCGGC	
Human	*HPRT*	Sense	GGCGTCGTGATTAGTGATGATGAA	NM_000194.2
		Antisense	ACATCTCGAGCAAGACGTTCAGT	

All primers were purchased from Metabion (Martinsried, Germany).

3.9. EdU- incorporation proliferation assay of PASMC

PASMC from passage 1 isolated from *Pink1*$^{-/-}$ and WT mice were cultured on 24-well plates in SMC growth medium 2 (PromoCell, Heidelberg, Germany) supplemented with normocin (1%, Invitrogen, De Schelp, Netherlands) and 15% FCS, for 5 days at hypoxia (5% O_2) and normoxia (21% O_2), followed by the addition of EdU (10 μM) for 24 hours with prior 6-8 hrs starvation. The 24-well plates were washed with 3% BSA (Sigma-Aldrich, Seelze, Germany) in PBS and fixed with methanol-acetone (1:1) for 5 minutes at room temperature. Then EdU-labeled cells were visualized using the Click-iT reaction cocktail (Invitrogen, Carlsbad, USA) for 30 minutes at room temperature. Further, the cell nuclei were stained with the Hoechst 33342 dye (Invitrogen; 1:1000 diluted). Subsequently, 6-8 representative pictures per sample and condition (normoxic/hypoxic) were taken. Finally, proliferation of PASMC was quantified by counting EdU positive cells (Excitation/Emission: 495/519 nm) and total cell number (Hoechst positive cells; Excitation/Emission: 358/461 nm) by the use of a fluorescence Leica DMIL microscope (Leica Microsystems, Manheim, Germany).

3.10. PASMC apoptosis assay

The commercial kits Caspase-Glo® 3/7 assay (Promega Corporation, Madison, USA) were purchased and used, according to the manufacturer's instructions. 20.000 PASMC per well were plated into a 96-well plate and incubated overnight. The cells were then treated with 8-hydroxy-ar-turmerone. After incubation with 8-hydroxy-ar-turmerone, the plates were kept at room temperature, and then, 50 µL of Caspase-Glo® reagent was added to the wells. The cells were then incubated at room temperature on a plate shaker for 20 min. Luminescent intensity was quantified using a luminescence microplate reader (Infinite M200PRO, Tecan, Männedorf, Switzerland).

3.11. Statistics

For comparison of two samples student's two-tailed *t*-test was used, for comparison of more than two samples *1- way-ANOVA* with Tukey post hoc test. For multiple comparisons *2- way-ANOVA* with Tukey or Bonferroni post hoc test was used. A $p<0.05$ was considered as statistically significant. All data values are shown as means ± SEM. For statistical analysis and data presentation, GraphPad 6 (GraphPad Software Inc., San Diego, USA) was used. Whenever possible, data analysis was performed blinded. For *in vivo* experiments, mice were randomly assigned for hypoxic or normoxic incubation. N-numbers are provided for each experiment and may vary for different parameters of the *in vivo* experiments, if measurement parameters could not be obtained from specific mice due to technical reasons.

4. Results

4.1. Expression of PINK1-dependent mitophagy mediators in IPAH and chronic hypoxia

4.1.1. Histological expression pattern of PINK1, PARKIN and PARL in the lung of IPAH patients

The histological expression pattern of the key proteins of PINK1-dependent mitophagy that is, PINK1, PARKIN, and PARL, were investigated in lung sections of IPAH patients and healthy donors (Figure 4.1). PINK1 and PARKIN was expressed in the medial layer of pulmonary vessel's wall in the lung of IPAH patients. Visually, PARL was expressed in almost all area of the pulmonary vessel's wall in the lung of IPAH patients.

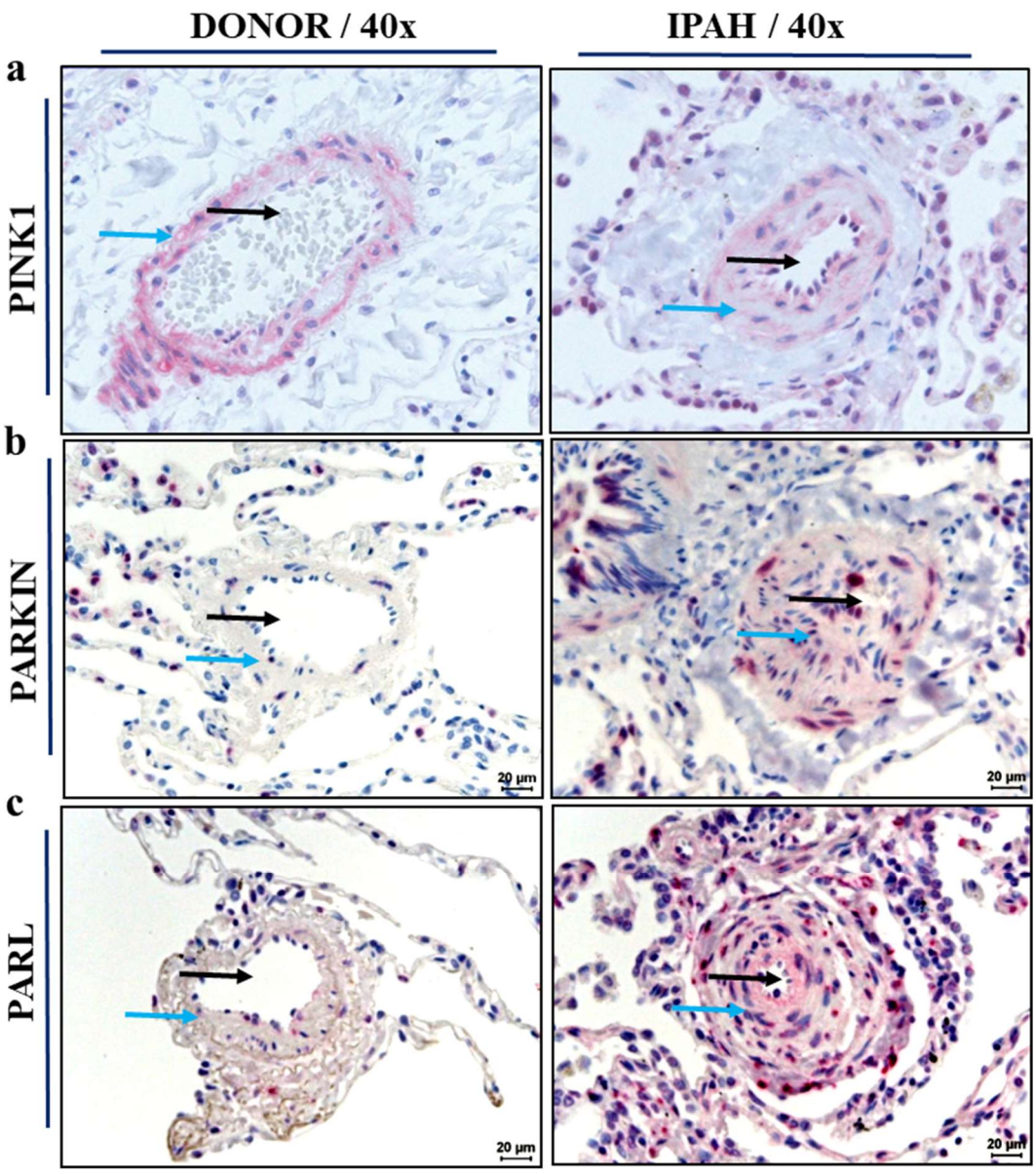


Figure 4.1 Histological expression pattern of PINK1, PARKIN and PARL in lung sections of IPAH patients

Representative immunostaining shows the expression of PINK1 **(a),** PARKIN **(b)** and PARL **(c)** in the lung sections. Blue arrows indicate pulmonary vessels and black arrows indicate vascular lumen.

4.1.2. mRNA and protein expression of PINK1, PARKIN and PARL in the lung homogenate of IPAH patients

To quantify expression of proteins involved in PINK1-dependent mitophagy, WB and qPCR were performed in lung homogenates of IPAH patients and donors.

mRNA level of *PINK1* was significantly upregulated in lung homogenates of IPAH patients (Figures 4.2a). Neither *PARKIN* mRNA nor *PARL* mRNA were altered in IPAH (Figure 4.2 b-c).

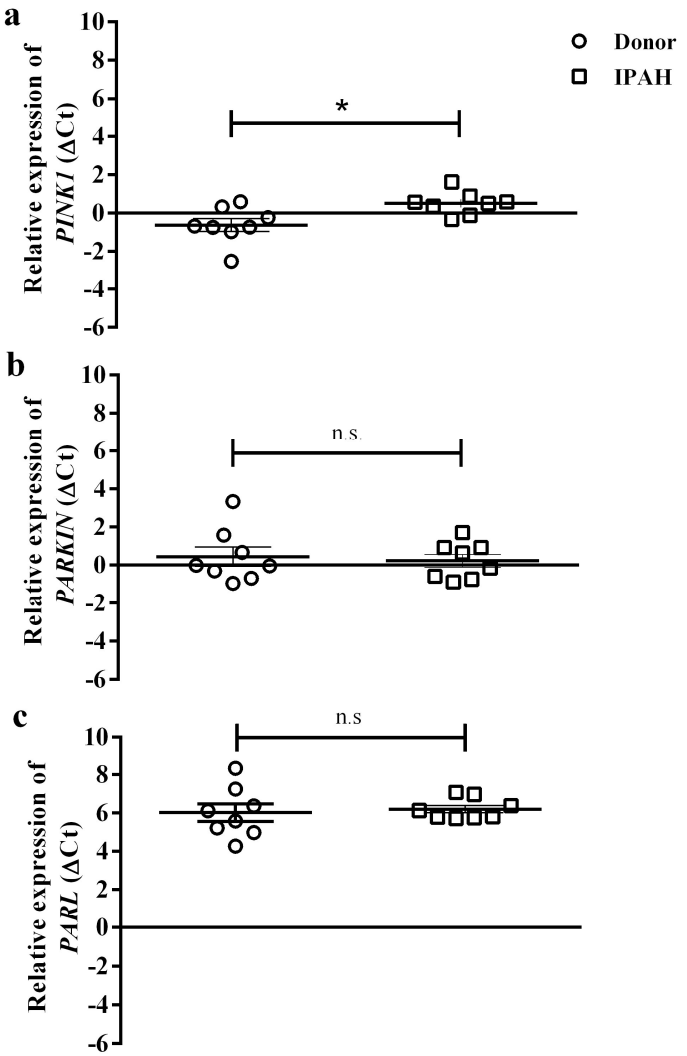


Figure 4.2 mRNA expression of *PINK1, PARKIN* and *PARL* in lung homogenate of IPAH patients

(a-c) mRNA expression of *PINK1*, *PARKIN* and *PARL* in lung homogenate of IPAH patients using *HPRT* as housekeeping gene. n=8 in each group. * $p<0.05$ compared to donor. All data were analyzed by *Student's t test* and shown as mean ± SEM.

Protein expression of PINK1 and PARL was significantly upregulated in lung homogenate of IPAH (Figures 4.3a, d,c,f).

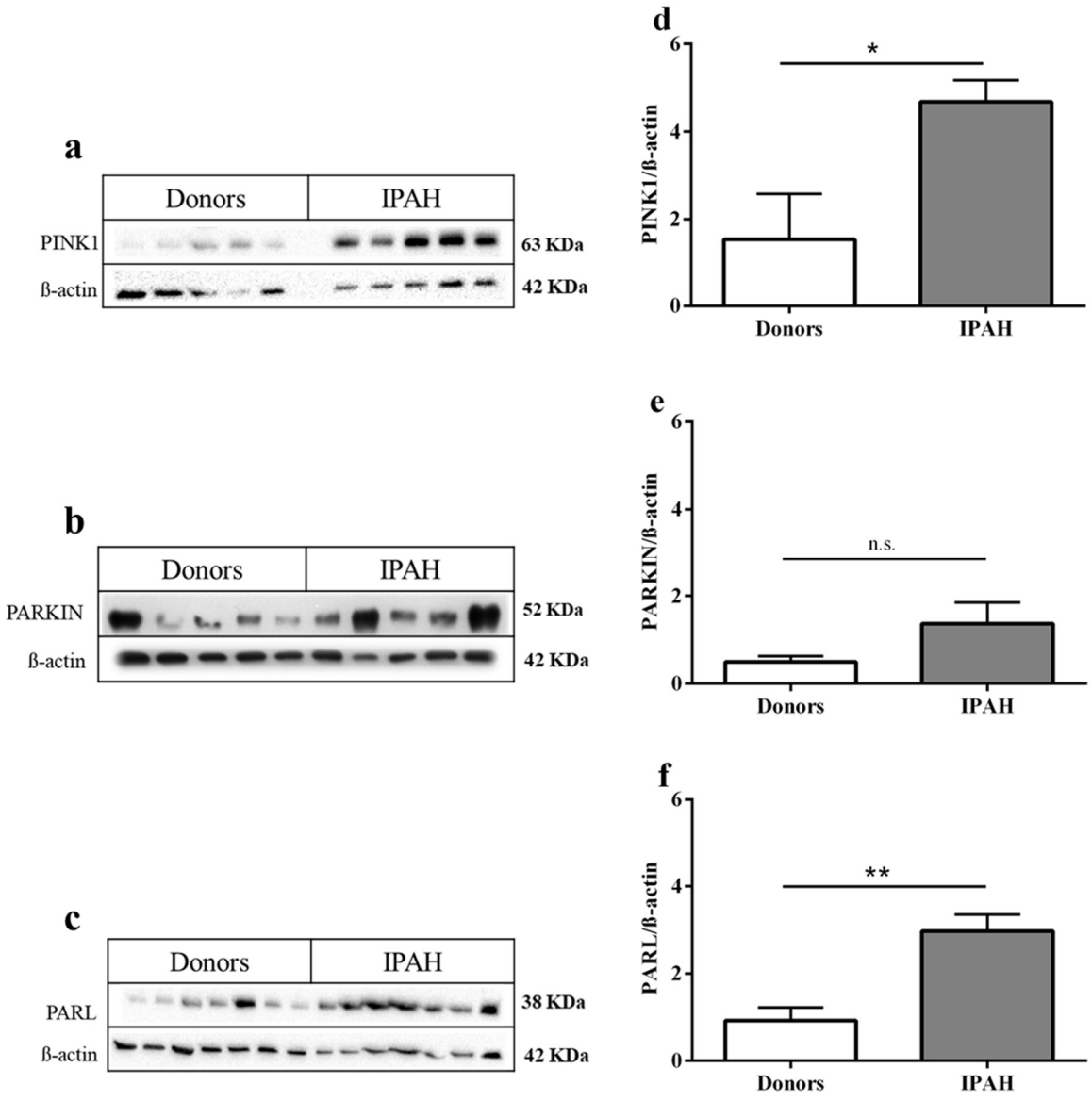


Figure 4.3 Protein expression of PINK1, PARKIN and PARL in lung homogenate of IPAH patients

(a-c) Representative WB and **(d-f)** quantification of PINK1, PARKIN and PARL expression in lung homogenate of IPAH patients and donors using β-actin as control. n=5 for PINK1 and PARKIN, n=7 for PARL in each group. * $p<0.05$ and ** $p<0.001$ compared to donor. All data analyzed by *Student's t test* and shown as means ± SEM.

4.1.3. mRNA and protein expression of PINK1, PARKIN and PARL in lung homogenate of mice after *in vivo* chronic hypoxic exposure

To examine the regulation of proteins involved in PINK1-dependent mitophagy in chronic hypoxia-induced PH, WB and qPCR using the lung homogenate from WT mice after *in vivo* exposure to 10% O_2 for 28 days were performed.

There were no significant changes in the mRNA expression for all three targets (Figure 4.4a-c).

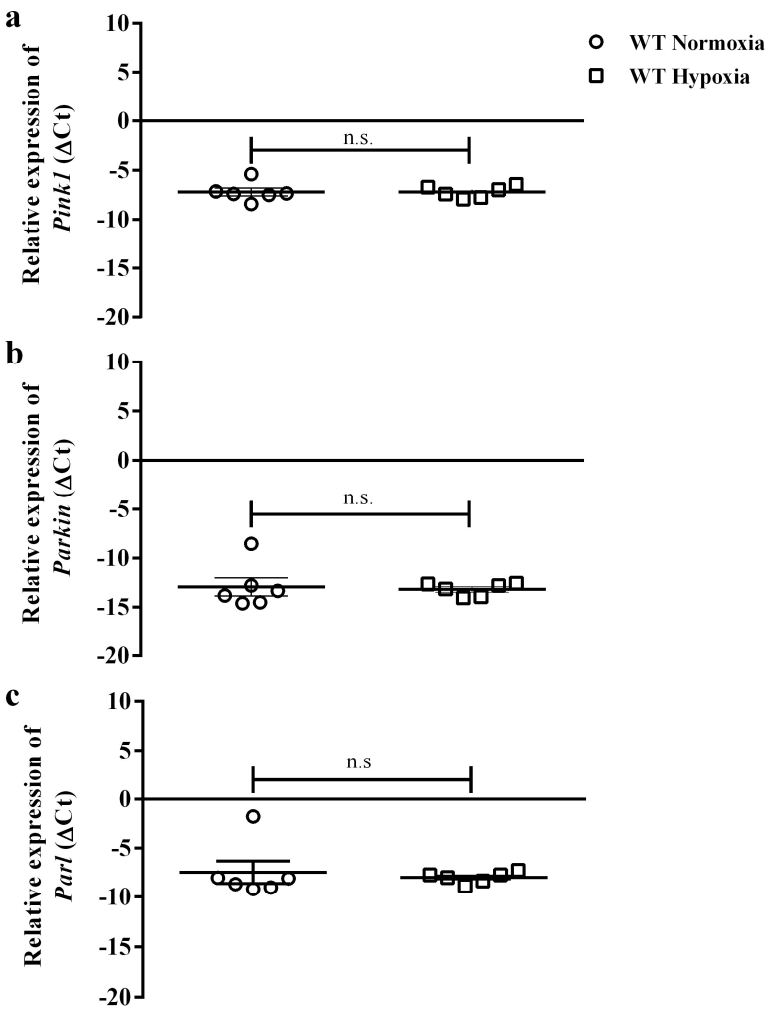


Figure 4.4 mRNA expression of *Pink1, Parkin* and *Parl* in lung homogenate of mice after *in vivo* chronic hypoxic exposure

(a-c) mRNA expression of *Pink1*, *Parkin* and *Parl*, using *B2M* as housekeeping. n=6 *in* each group. All data were analyzed by *Student's t test* and shown as single values and mean ± SEM.

Protein levels of PINK1 and PARKIN were increased after chronic hypoxic exposure (Figure 4.5a-b, d-e). However, there was no regulation in the PARL protein level after chronic hypoxic exposure (Figure 4.5c, f).

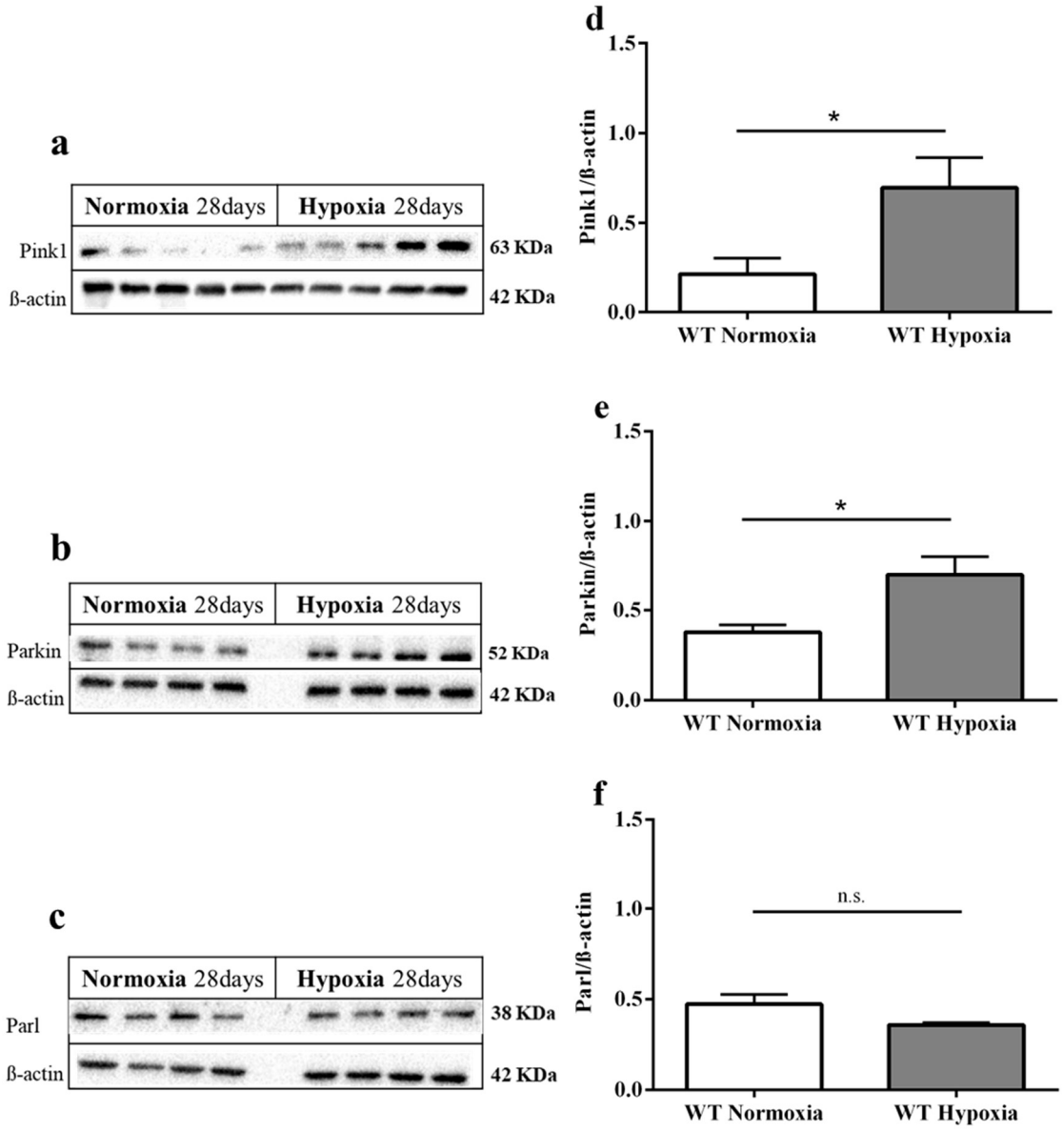


Figure 4.5 Protein expression of PINK1, PARKIN and PARL in lung homogenate of mice after *in vivo* chronic hypoxic exposure

(a-c) Representative WB and **(d-f)** quantification of PINK1, PARKIN and PARL expression in lung homogenate from WT mice exposed to 10% O_2 (hypoxia) and 21% O_2 (normoxia) for 28 days, using β-actin as control. n=5 for PINK1 and n=4 for PARKIN and PARL in each group (see appendix) * $p<0.05$ compared to donor. All data analyzed by *Student's t test* and shown as means ± SEM.

4.1.4. mRNA and protein expression of PINK1, PARKIN and PARL in mouse precapillary PASMC of WT mice *after in vitro exposure* to 1% O_2 for 5 days

Further, primary PASMC were isolated from WT mouse lungs and exposed to 1% O_2 for 5 days. There was no change observed at mRNA level for *Pink1, Parkin* and *Parl* (Figure 4.6 a-c).

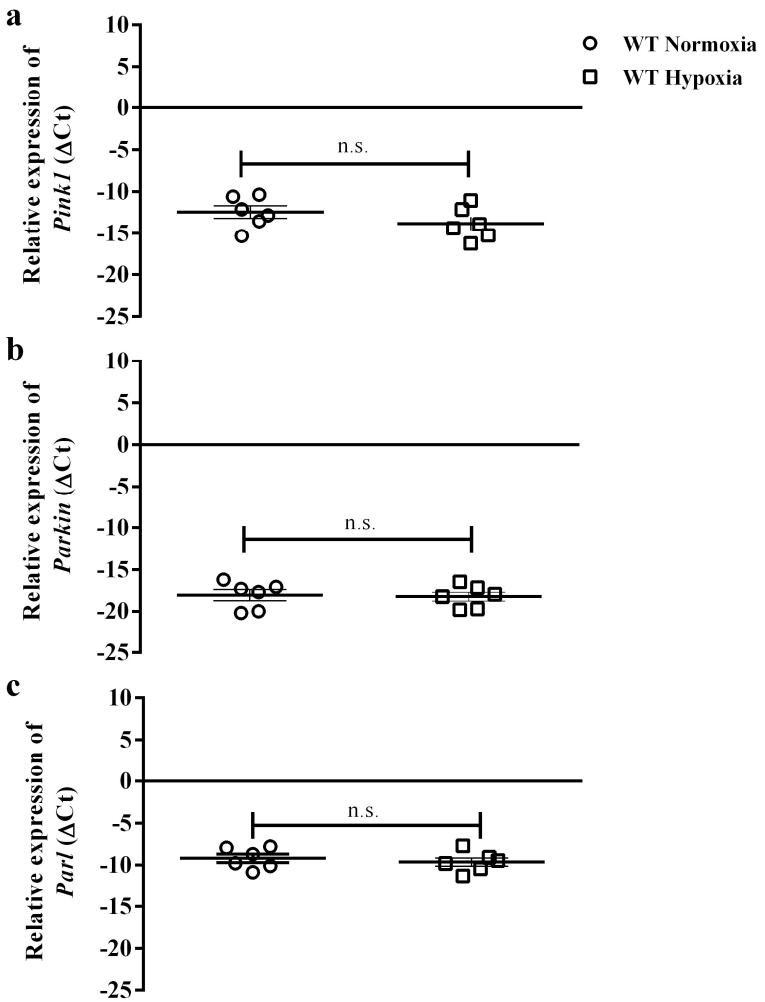


Figure 4.6 mRNA expression of *Pink1, Parkin* and *Parl* in mouse precapillary PASMC after *in vitro* exposure to hypoxia for 5 days

(a-c) mRNA expression of *Pink1*, *Parkin* and *Parl* using *B2M* as housekeeping gene in PASMC isolated from WT mice after exposure to 1% O_2 and 21% O_2 for 5 days. n= 6 in each group. All data were analyzed by *Student's t test* and are shown as mean ± SEM.

Only the protein expression of PINK1 was significantly increased after exposure to hypoxia for 5 days (Figure 4.7 a, d).

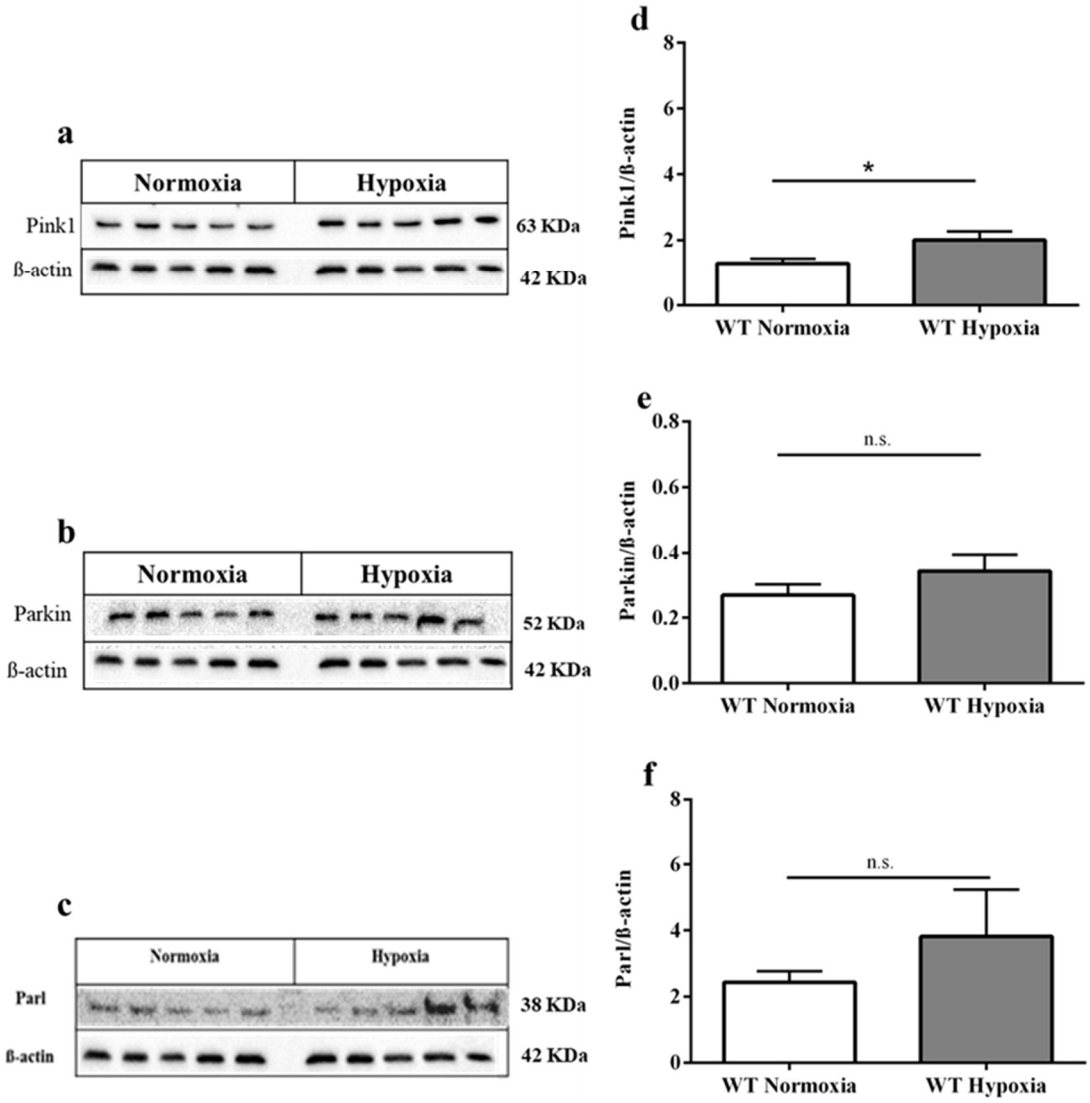


Figure 4.7 Protein expression of PINK1, PARKIN and PARL in mouse precapillary PASMC after *in vitro* exposure to chronic hypoxia

(a-c) Representative WB and **(d-f)** quantification of PINK1, PARKIN and PARL expression in PASMC isolated from WT mice exposed to 1% O_2 (hypoxia) and 21% O_2 (normoxia) for 5 days, using β-actin as control. n=5 in each group. * $p<0.05$ compared to donor. All data analyzed by *Student's t test* and shown as means ± SEM.

4.2.Effect of chronic hypoxia on mitochondrial fission/fusion in precapillary PASMC isolated from WT mouse

4.2.1. Protein expression of DRP1 and MFN2 in mouse precapillary PASMC after *in vitro* exposure to 1% O_2 for 5 days mice

To investigate the alterations of mitochondrial fission and fusion during exposure to chronic hypoxia, the protein expression of DRP1 and MFN2 was examined in precapillary WT PASMC exposed *in vitro* to hypoxia (1% O_2 for 5 days).

Chronic hypoxia did not affect the protein level of DRP1 in PASMC (Figure 4.8a-b). In contrast, MFN2 was significantly downregulated in PASMC exposed to prolonged hypoxia (1% O_2 for 5 days) (Figure 4.8 c-d).

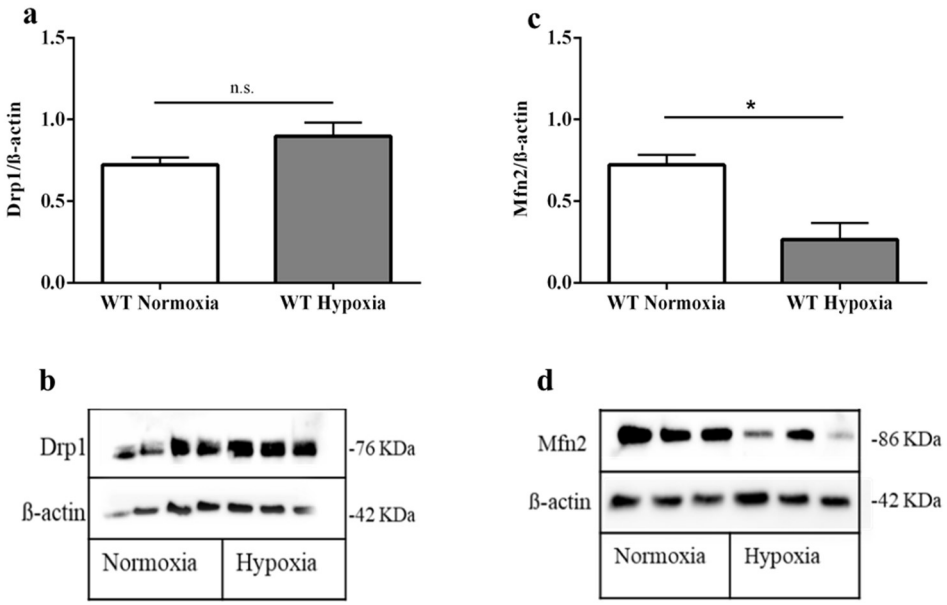


Figure 4.8 Expression of DRP1 and MFN2 in precapillary WT PASMC after exposure to 1% O_2 for 5 days

(a,c) Quantification of DRP1 and MFN2 expression and (b,d) representative WB of PASMC isolated from WT mice exposed to 1% O_2 (hypoxia) and 21% O_2 (normoxia) for 5 days, using β-actin as control. n=3 in each group. * $p<0.05$ compared to donor. All data analyzed by *Student's t test* and shown as means ± SEM.

4.3.Effect of *Pink1*$^{-/-}$ and hypoxia on general autophagy

4.3.1 Protein expression of LC3B-II in lung homogenate of *Pink1*$^{-/-}$ and WT mice after *in vivo* chronic hypoxic exposure

To investigate the effect of *Pink1*$^{-/-}$ on LC3B-II regulation as general autophagy marker during chronic hypoxia, the LC3B-II was investigated in lung homogenate of mice after exposure to 10% O_2 and 21% O_2 for 28 days. The expression level of LC3B-II was not altered in chronic hypoxia-induced PH and in *Pink1*$^{-/-}$ mice (Figure 4.9a-b).

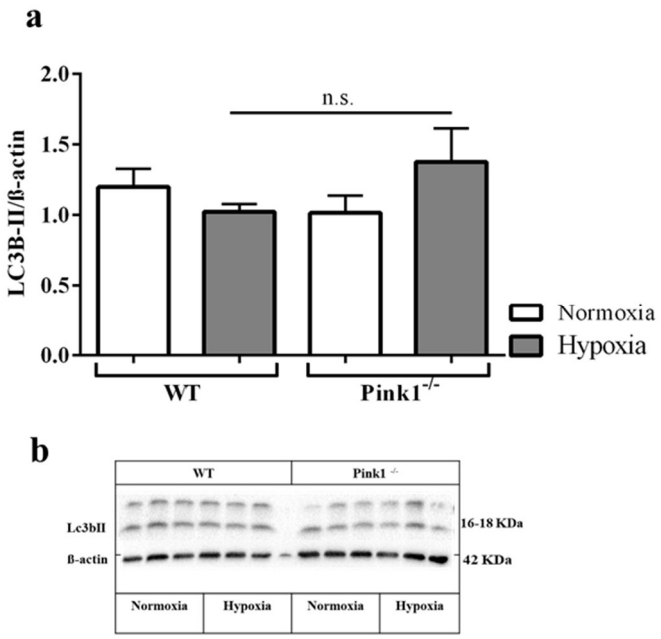


Figure 4.9 Protein expression of LC3B-II in lung homogenate of *Pink1*$^{-/-}$ and WT mice after *in vivo* chronic hypoxia exposure

(a) Quantification and (b) representative WB of expression of LC3B-II compared to the expression of β-actin in lung homogenate of *Pink1*$^{-/-}$ and WT mice after normoxic or hypoxic exposure. n=3 in each group. Data analyzed by *2-way ANOVA* with bonferroni post hoc test and shown as means ± SEM.

4.4.The effect of *Pink1*$^{-/-}$ on proliferation/apoptosis in PASMC after hypoxic *in vitro* exposure

The effect of *Pink1*$^{-/-}$ on PASMC proliferation and apoptosis was studied in isolated PASMC after exposure to hypoxia (1% O_2, 5 days). Exposure of mouse WT PASMC to hypoxia resulted in increased proliferation and decreased apoptosis (Figure 4.10 a-b). *Pink1*$^{-/-}$ inhibited the hypoxia-induced increase of PASMC proliferation (Fig. 4.10a) and reversed the hypoxia-induced decrease of PASMC apoptosis (Fig. 4.10b).

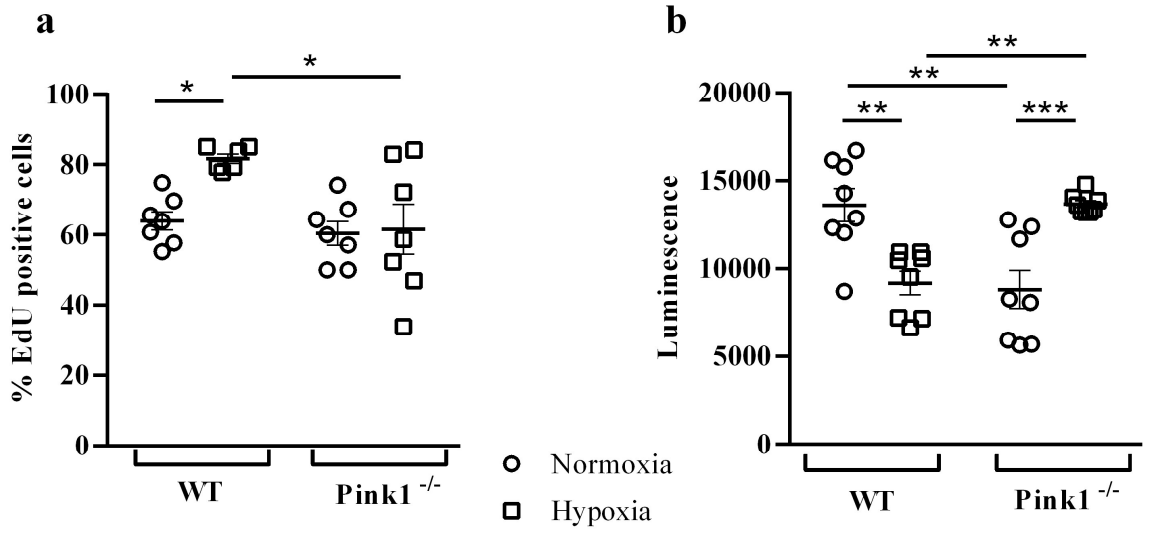


Figure 4.10 Effect of *Pink1*$^{-/-}$ on proliferation and apoptosis in precapillary PASMC after *in vitro* exposure to 1% O_2 for 5 days

(a) Proliferation of precapillary PASMC isolated from WT and *Pink1*$^{-/-}$ mice after exposure to hypoxia. **(b)** Apoptosis of precapillary PASMC isolated from WT and *Pink1*$^{-/-}$ mice after exposure to 1% O_2 as hypoxia and 21% O_2 as normoxia**.** $n \geq 7$, data were obtained from at least 8 independents precapillary PASMC cell isolations. * $p<0.05$, ** $p< 0.01$ and **** $p< 0.0001$. All data analyzed by *2-way ANOVA* with Tukey post hoc test and shown as means ± SEM.

4.5.Effects of *Pink1*$^{-/-}$ on chronic hypoxia-induced PH

4.5.1. Effect of *Pink1*$^{-/-}$ on hemodynamics

To investigate the effects of *Pink1* on the development of chronic hypoxia-induced PH, WT and *Pink1*$^{-/-}$ mice were exposed to chronic hypoxia (10% O_2) for 28 days.

Chronic hypoxia induced an increase in RVSP to a similar level in both WT and *Pink1*$^{-/-}$ mice (Figure 4.11a). Systemic blood pressure was not affected by chronic hypoxic exposure (Figure 4.11 b).

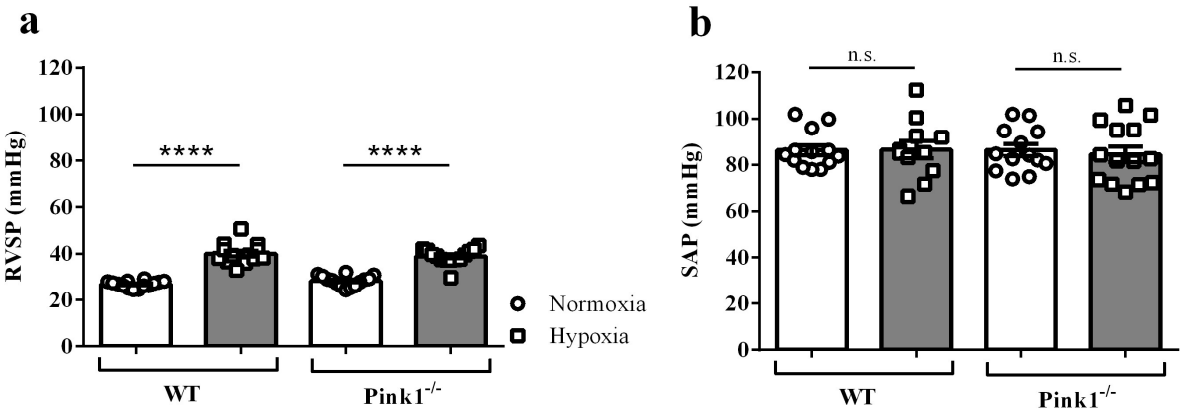


Figure 4.11 Effect of *Pink1*$^{-/-}$ on the hemodynamic alterations in mice after *in vivo* chronic hypoxic exposure

(a) Right ventricular systolic pressure (RVSP) and **(b)** SAP quantified by heart catheterization in WT and *Pink1*$^{-/-}$ mice after exposure to 10% O_2 and 21% O_2 as normoxia for 28 days.

**** $p<0.0001$ compared to the respective controls, n= 12-14 per each group. Data analyzed by *2-way ANOVA* with bonferroni post hoc test and shown as means ± SEM.

4.5.2 Effect of *Pink1*$^{-/-}$ on chronic hypoxia-induced pulmonary vasculature remodeling

In contrast to the hemodynamic data, the degree of pulmonary vascular remodeling was significantly reduced in the *Pink1*$^{-/-}$ mice compared with the WT mice (Figure 4.12a-b).

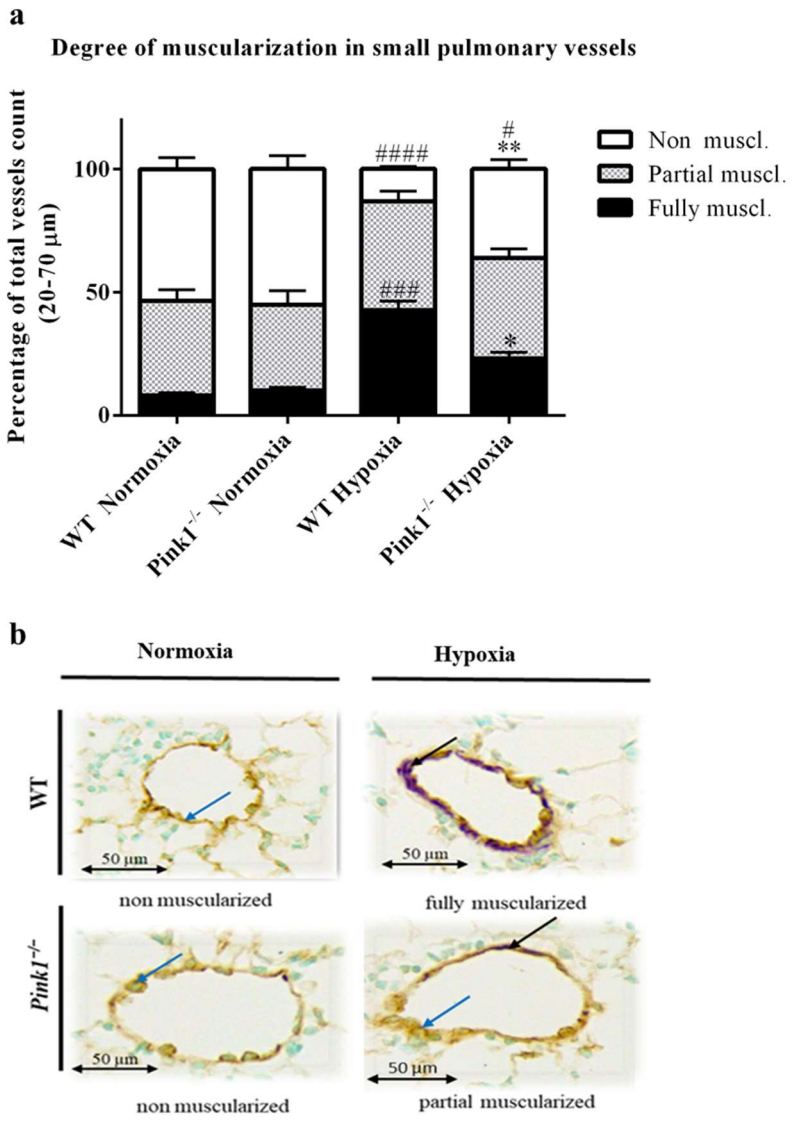


Figure 4.12 Effect of *Pink1*$^{-/-}$ on pulmonary vascular remodeling in mice after *in vivo* chronic hypoxic exposure

(a) Degree of muscularization of small pulmonary arteries (diameter 20–70 µm). Data are given as percentages of total vessel count for fully muscularized (Fully muscl.), partial muscularized (Partial muscl.), and non-muscularized (Non muscl.) vessels. * $p<0.05$, ** $p< 0.01$, compared to the respective WT control. #$p<0.05$, ###$p<0.001$ and ####$p<0.0001$ compared to the respective normoxic values. n= 12-14 per each group. Data analyzed by *2-way ANOVA* with bonferroni post hoc test and shown as means ± SEM. **(b)** Representative pictures of pulmonary vessels in lung sections co-stained against α-smooth muscle actin (purple color, black arrows) and von Willebrand factor (brown color blue arrows).

4.5.3. Effect of *Pink1*$^{-/-}$ on echocardiographic parameters

The effect of *Pink1* deletion on RV hypertrophy and function in chronic hypoxia-induced PH was further assessed.

The mass of the RV measured as ratio of the weight of the RV to the weight of the left ventricle (LV)+septum was increased to the same degree in both WT and *Pink1*$^{-/-}$ mice after exposure to chronic hypoxia for 28 days (Figure 4.13a). Echocardiography revealed that *Pink1*$^{-/-}$ mice showed less chronic hypoxia-induced dilatation of the RV, while the changes in right ventricular wall thickness (RVWT) were comparable to the WT mice (Figure 4.13 b-c and 4.14 a-h). The function of the RV was improved in *Pink1*$^{-/-}$ mice after hypoxic exposure compared to WT mice determined by the tricuspid annular plane systolic excursion (TAPSE) (Figure 4.13d). However, cardiac output (CO) and cardiac index (CI) was not different between the two strains after normoxic or hypoxic exposure (Figure 4.13e-f). Representative echocardiographic images demonstrating the RVID (Figure 4.14a-d) and RVWT (Figure 4.14e-h) in WT and *Pink*$^{-/-}$ mice exposed to 10% O_2 and 21% O_2 for 28 days.

Echocardiography and invasive hemodynamic measurements were performed with the help of Dr. A. Sydykov.

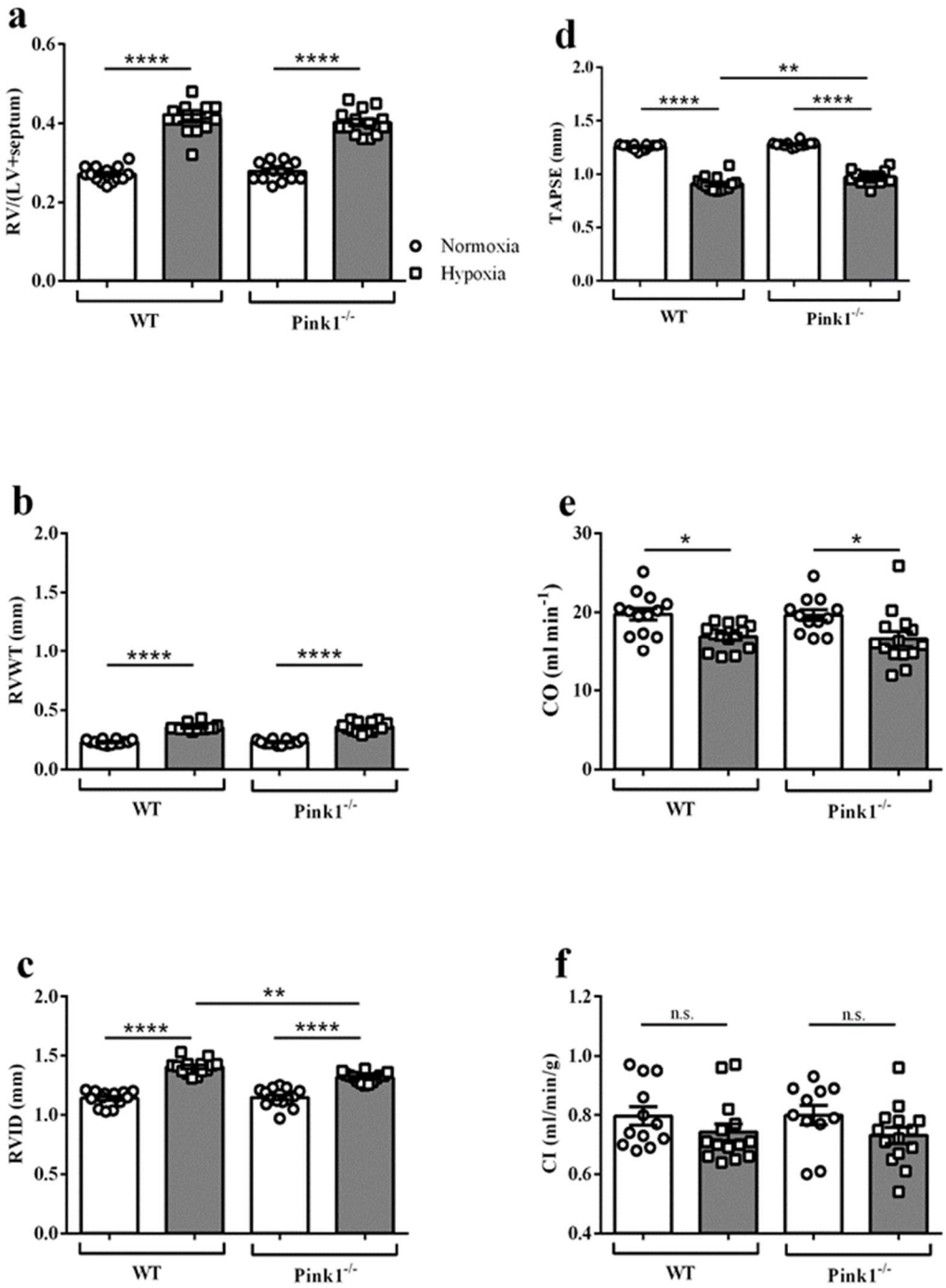
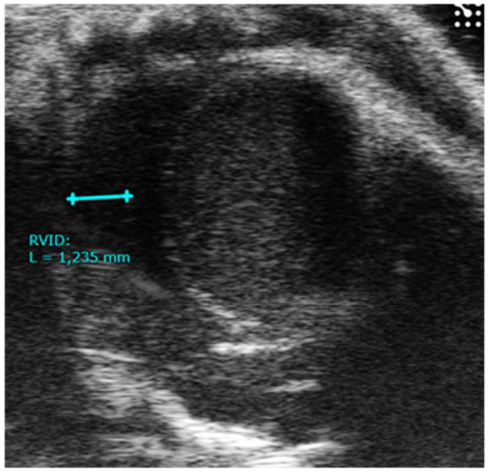


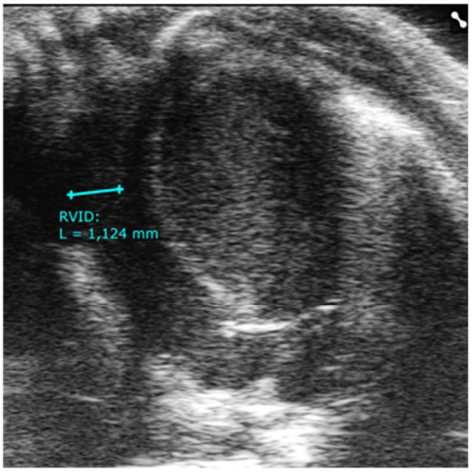
Figure 4.13 Effect of *Pink1*$^{-/-}$ on echocardiographic parameters in mice after *in vivo* chronic hypoxic exposure

(a) Fulton index presented as ratio of the RV/(LV+S), **(b)** right ventricular wall thickness (RVWT), **(c)** right ventricular internal diameter (RVID), **(d)** tricuspid annular plane systolic excursion (TAPSE)**, (e)** cardiac output (CO) and (f) cardiac index (CI) in WT and *Pink1*$^{-/-}$ mice after exposure to normoxia (21% O_2) or chronic hypoxia (10% O_2 for 28 days). (b-f) determined by echocardiography. *p< 0.05, ** p< 0.01 and **** p< 0.0001 compared to the respective controls (WT after 28 days normoxic exposure). n= 12-14 in each group. Data analyzed by *2-way ANOVA* with Bonferroni *post hoc* test and shown as means ± SEM.

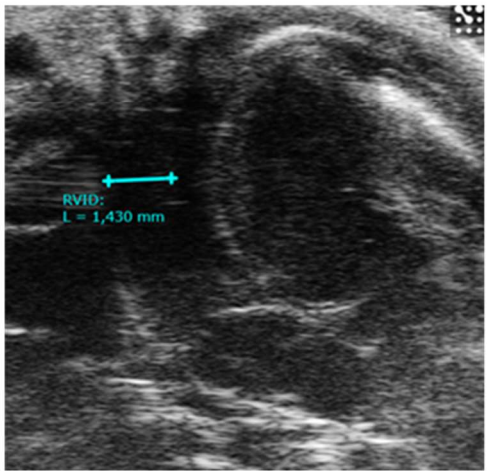
a) WT normoxia



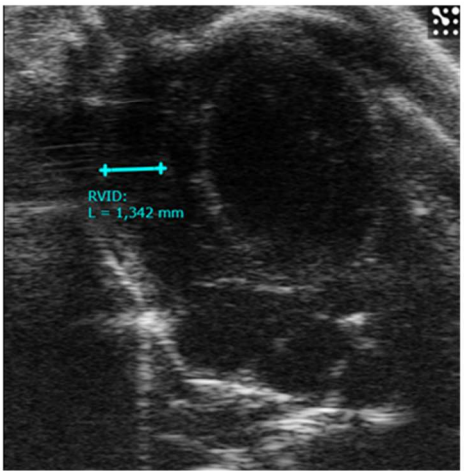
c) Pink1$^{-/-}$ normoxia



b) WT chronic hypoxia



d) Pink1$^{-/-}$ chronic hypoxia



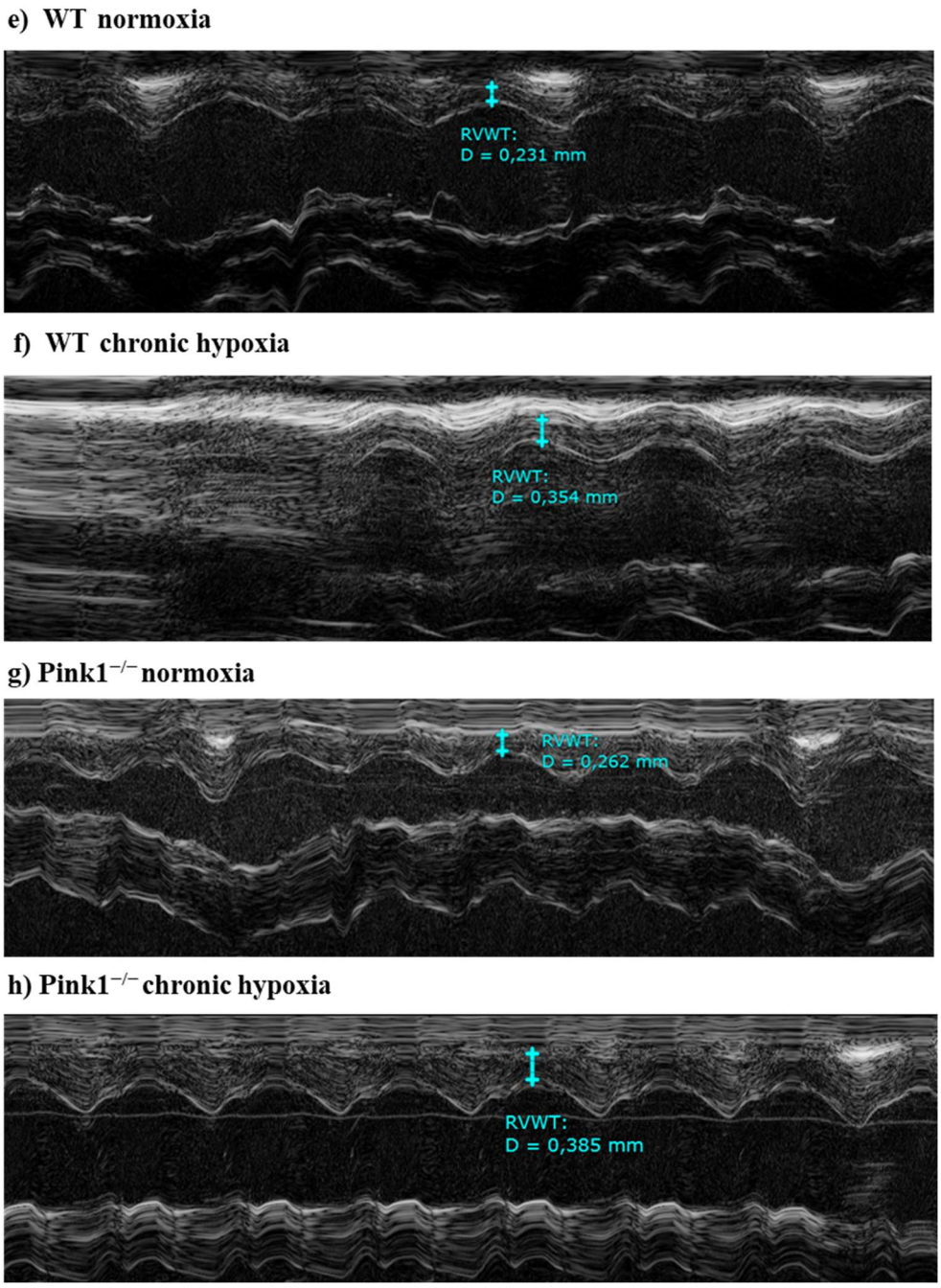


Figure 4.14 Representative echocardiographic images of the right heart ventricle in chronic hypoxia-induced PH

Echocardiographic images demonstrating the RVID in the right parasternal short axis view **(a-d)** and **(e-h)** the RVWT in the right parasternal long axis view in WT and *Pink*$^{-/-}$ mice exposed to 10% O_2 and 21% O_2 for 28 days.

4.6. Effect of *Pink1*$^{-/-}$ and hypoxia on alternative mitophagy (PINK1-independent) pathways

4.6.1. Protein expression of BNIP3L/Nix in lung homogenate of *Pink1*$^{-/-}$ and WT mice after *in vivo* chronic hypoxic exposure

Finally, in order to investigate whether *Pink1*$^{-/-}$ is able to induce PINK-independent mitophagy, the expression of BNIP3L/Nix protein were investigated in the lung homogenate of mice after exposure to 10% O_2 and 21% O_2 for 28 days.

Interestingly, the protein expression of BNIP3L/Nix was increased in chronic hypoxic *Pink1*$^{-/-}$ compared to WT mice (Figure 4.15a-b).

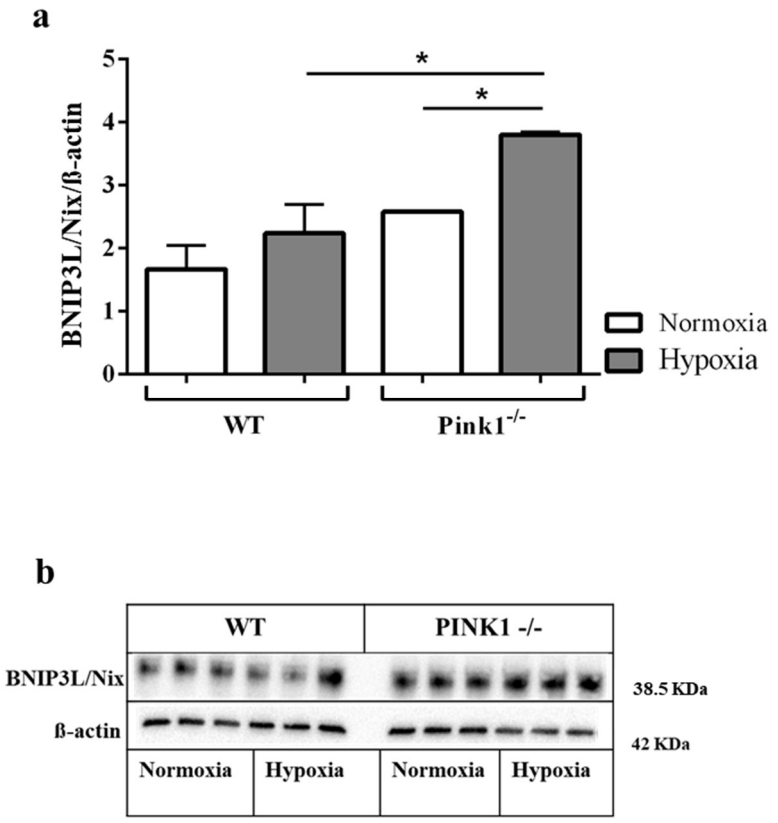


Figure 4.15 Protein expression of BNIP3L/Nix in lung homogenate of *Pink1*$^{-/-}$ and WT mice after *in vivo* chronic hypoxic exposure

Expression of BNIP3L/Nix compared to the expression of β-actin in lung homogenate of *Pink1*$^{-/-}$ and WT mice after normoxic or hypoxic exposure. n=3 in each group. *p<0.05compared to the respective control. Data analyzed by *2-way ANOVA* with Tukey post hoc test and shown as means ± SEM.

5. Discussion

PH remains a complex disease which is associated with different pathogenic mechanisms [280]. The exact cellular signaling pathways underlying PH are still under investigation. Mitochondrial dysfunction has been reported in various forms of PH [281]. Mitochondria play a critical role in the pro-proliferative and anti-apoptotic signaling pathways triggering pulmonary vascular remodeling in PH, including ROS production [144, 282], anaerobic glycolysis [283], metabolism of fatty acids [284] and Ca^{2+} signaling [285]. Mitophagy is a selective process initiated to remove damaged mitochondria which are the result of oxidative or other kinds of stress stimuli [177]. PINK1-dependent mitophagy is currently one of the most investigated pathway of mitophagy. Therefore, the present study aimed to elucidate the role of PINK1-dependent mitophagy in the development of PH. As PH model, chronic hypoxia-induced PH in mice was used. It was hypothesized that altered mitophagy mediated by PINK1 results in increased proliferation and decreased apoptosis of PASMC, leading to the development of PH.

In this study, the following results were obtained:

- In samples of IPAH patients and mice after chronic hypoxic exposure the expression of proteins involved in PINK1-dependent mitophagy including (PINK1, PARL and PARKIN) were increased, indicating the alteration of PINK1-dependent mitophagy in PH.
- *Pink1* deletion inhibited the hypoxia-induced increase in PASMC proliferation and decrease in PASMC apoptosis *in vitro.*
- *Pink1* deletion attenuated pulmonary vascular and RV remodeling but did not prevent the increase in RVSP in chronic hypoxia-induced PH.
- Upregulation of PINK1-independent mitophagy may be considered as a regulatory mechanism of *Pink1*$^{-/-}$.

The findings of this study suggest that, PINK1-dependent mitophagy is involved in pulmonary vascular remodeling in chronic hypoxia-induced PH via regulation of the proliferation and apoptosis of PASMC.

5.1. Limitation of models and methods in this study

5.1.1. Hypoxic *in vitro* and *in vivo* models

In the present study for *in vivo* investigations mice were exposed to chronic hypoxia (10% O_2, 28 days). Various triggers including exposure to chronic hypoxia have been reported to induce the increase in pulmonary artery pressure, development of pulmonary vascular remodeling and RV hypertrophy [15, 55, 286-290]. Although these models of PH have contributed a lot to the knowledge in the field, none of these models is able to mimic all pathological characteristics of human PAH, for example, plexiform lesions are absent in the hypoxic PH model [15]. With regard to the very complicated pathophysiological nature of human PH, so far, there has been not a single animal model proposed that mimics all the aspects of human PH [16]. Furthermore, the animal response to hypoxia may be different in humans as it differs among species [291]. These limitations has to be kept in mind when transferring the results from hypoxic exposure of mice or murine cells to human IPAH samples.

5.1.2. Human samples

In this study, the human samples have been collected from anonymous surgical peripheral human lung tissue of the IPAH patients and donors according to the approval by the ethics committee at the faculty of medicine, Justus-Liebig university of Giessen.

The donor's samples have been selected from unused parts of donor lungs. Notably, to work with human materials several important factors must be considered. The limitation of human material in addition of ethical issue consists of high variability of the used tissue due to the individual history, life style and background of the patient or donor, the potential presence of other undiagnosed disease, effects of different drugs and medication which the patients or donors may have taken before [292], gender and ethnicity differences [293] and quality of the exact phenotyping of the respective disease (in this case IPAH) [292]. Considering all the above-mentioned limitation factors which may affect proteins and mRNA of the selected tissue, even the location of the part of the sample which has been selected is important to represent the exact disease phenotype. [292]. Although, the samples which have been used in this study were selected very carefully from the available data provided by the biobank with regard to the above-mentioned limitations heterogeneity in the samples may exist as detected also in the high variability of the WBs.

5.2. Expression of proteins involved in PINK1-dependent mitophagy in IPAH and in chronic hypoxia-induced PH in mice

Alteration of mitochondrial functions are the key players in development of PH [133, 294-297]. Mitophagy which serves to eliminate damaged mitochondria from the mitochondrial network [298], may contribute to development of PH. The best studied pathway of mitophagy, PINK1-dependent mitophagy, is mediated mainly by "three 'P's" [299] namely, PINK1, PARKIN and PARL [171, 300-302]. Thus, in the present study the role of PINK1-dependent mitophagy and expression of PINK1, PARKIN and PARL in PH was investigated.

Immunohistological staining of the lung tissue sections of IPAH patients showed expression of PINK1 and PARKIN in the medial layer of pulmonary vessel's wall. However, based on the visual observation, PARL was expressed in almost all area of the pulmonary vessel's wall in the lung of IPAH patients and it may suggest higher expression of PARL in IPAH (despite the limitation of IHC, see chapter 5.1.3). With regard to mRNA expression only *PINK1* mRNA was increased in lung homogenate from IPAH patients. Protein expression of PINK1 and PARL was upregulated in lung homogenate of IPAH, whereas in chronic hypoxic mice and PASMC exposed to hypoxia for 5 days, PINK1 and PARKIN or only PINK1 protein expression was increased, respectively.

Alteration in PINK1 has been reported in different diseases, especially in neurodegenerative diseases such as Parkinson's disease [303], Huntington's disease [304], Alzheimer's disease [305] as well as type 2 diabetic [306], cancer [307, 308] and heart failure [309]. The alteration of PINK1 expression in response to hypoxia could be triggered by several transcription factors such as HIF [59], NF-κB [60] and FOXO1 [61]. One of the most important transcription factor implicated in the cellular hypoxic response belongs to the HIF family. HIF-1α plays a critical role for the response to short-term hypoxia [71, 72] and HIF-2α for the response to long-term hypoxia, as described above [73]. In Parkinson's disease Lin *et al.* proposed that HIF-1α regulated PINK1 by showing decreased PINK1 in MEF cells under hypoxic condition [310]. In one recent study which used hepatocellular carcinoma cells (HCC), a connection between HIF1, the enhancer-of-split related with YRPW motif protein 1 (HEY1) and PINK1 was found and they showed the reverse correlation between *PINK1* and *HEY1* mRNA expression in hypoxia. In this study they showed how HEY1 is induced due to hypoxia as a transcriptional target of HIFs family and attenuated *PINK1* mRNA level in HCC to reduce the mitochondrial ROS production [311]. However, in contrast to these findings the presented study found that PINK1 was upregulated in mouse PASMC during chronic hypoxia exposure. In a study by Mei *et al.* the importance of the "PI3K/Akt/FOXO/PINK1" axis in response to oxidative stress was

explored. The authors showed that PINK1 was upregulated as a response to oxidative stress in MEF cells in a mechanism which is modulated by transcription factors of the FOXO family to attenuate the PI3K/Akt signaling pathway [312]. In this study the importance of PINK1 regulation by FOXO3a under physiological oxidative stress has been highlighted [312]. FOXO family with their important functions such as anti-oxidative stress, playing role in the protection of the cell from oxidative stress (hypoxia)-mediated apoptosis by enhancing of magnesium superoxide dismutase (MnSOD) and peroxisomal catalase in the cell [313]. However, according to a recent study of Wang *et al*. in bone marrow mesenchymal stem cells (BMSCs), was shown that in hypoxia, due to decreased proteasomal activity, PGC-1α, SIRT3 and HIF-1α protein levels were increased and resulted in the increase of PINK1/PARKIN at protein level [314]. Therefore, one can speculate that upregulation of PINK1 which was shown in this study, could be as a result of decreased proteasomal activity and enhancing of PGC-1α, SIRT3 and HIF-1α axis accordingly (see discussion below).

5.2.1. The discrepancy between mRNA and protein levels for PINK1-dependent mitophagy mediators (PINK1, PARKIN and PARL)

According to the results of this study, protein expression of PINK1 was increased in precapillary PASMC exposed to 1% O_2 for 5 days *in vitro* and in lung homogenate of mice exposed to 10% O_2 for 28 days *in vivo.* In lung homogenate of IPAH patients PINK1 as well as PARL protein expression was increased. However, at mRNA level only *PINK1* in the lung homogenate of IPAH patients was increased. The discrepancy between mRNA and protein level expression could be explained either by different mRNA translation, post-translational modification or protein degradation. For example, in hypoxic exposure [310] the cell type and culture conditions can affect protein translation [315]. Protein degradation may be affected by proteasomal activity. The increase of PINK1 in protein level can be described according to Wang *et al.* which in hypoxia, due to the decreased proteasomal activity and enhancing "PGC-1α/SIRT3/HIF-1α" axis [314]. According to the findings of the present study, the protein expression of PARKIN and PARL was different in IPAH and hypoxic exposure. PARKIN protein expression was upregulated in lung homogenates from mice exposed to chronic hypoxia for 28 days, whereas PARL but not PARKIN was increased in the lungs of IPAH patients. Alterations of PARKIN expression and its involvement in several diseases has been reported previously, for example in Parkinson's diseases [316] and cancer [317]. As described above, during mitophagy PARKIN is recruited by PINK1 to the OMM and then, by phosphorylation

of PARKIN, the E3 ligase activity of PARKIN enhances mitochondrial ubiquitination [318]. While increased expression of PARKIN in hypoxia is in accordance with an increase in PINK1-dependent mitophagy, the concomitant increase of PINK1 and PARL expression in IPAH seems counterintuitive. Regulation of PARL, which is required for the cleavage of PINK1 in mitochondria is depended on mitochondrial membrane potential [207]. Thus, upregulation of PARL would indicate increased of PINK1 degradation and inhibition of mitophagy, which contrasts with the hypothesis of increased mitophagy in hypoxia-induced PH. One could speculate that PARL is involved in development of PH independently of PINK1. One study showed that PARL can enhance apoptosis by mediating the proteolytic maturation of 1) mitochondrial pro-apoptotic proteins and 2) the mitochondria-derived activator of caspases (Smac) [319]. Another study showed that *Parl* deficiency can impair complex III function, leading to mitochondrial dysfunction [320]. Despite its function to degrade PINK1, it has also been reported that PARL can promote PINK1-dependent mitophagy via the activation of PARL- prohibiting 2 (PHB2)- phosphoglycerate mutase family member 5 (PGAM5) signaling [321]. Moreover, it has been explained that *Parl* deficiency decrease the recruitment of PARKIN to the mitochondria [322, 323]. Taken together, alteration in expression levels of PINK1, PARKIN and PARL indicates the elevation of mitophagy in hypoxia-induced PH, while the net effect of upregulation of PINK and PARL in IPAH on mitophagy remains less clear. One speculation could be that PARL may upregulated as a response to increased mitophagy. Nevertheless, since the human samples utilized in this study were obtained from the end stage of IPAH, which may reflect a later stage of PH, compensatory mechanisms are more pronounced than in the murine hypoxic model (rather the short-term hypoxic exposure). Furthermore, mechanisms underlying chronic hypoxia-induced PH and IPAH may differ as discussed above.

5.3.Effect of chronic hypoxic exposure on mitochondrial fission and fusion

Mitochondrial fission process is involved in mitophagy [324]. In mammals, different GTPases of the dynamin family proteins mediate the process of mitochondrial fission and fusion. DRP1 mediates the process of mitochondrial fission [223, 225] while MFN2 [325] and Opa1 [326] mediate fusion processes.

Surprisingly, DRP1 was not changed in PASMC after chronic hypoxia exposure. In contrast, MFN2 protein level was significantly downregulated in PASMC exposed to chronic hypoxia. These data thus suggest, that mitophagy and mitochondrial network formation may interact in PH via different MFN2 but not DRP1 expression levels. In contrast to the current data on DRP1, a previous study of Ryan *et al.* [234] demonstrated increased DRP1 protein expression in the lung homogenate of IPAH patients, human PAH PASMCs as well as in the lung homogenate of the animal models of chronic hypoxia plus Sugen-5416 (CH+SU) and MCT-induced PH. One reason for the discrepancy may be different treatment protocols of hypoxia, since the duration of hypoxic exposure in the current experiments was 5 days, while it was in the cited study only one day.

With regard to MFN2, Ryan *et al.* demonstrated in line with the current study that MFN2 was downregulated in the lung homogenate of IPAH patients as well as in the lung homogenate of the mouse model of CH+SU and the MCT rat model of PH [234]. The transcriptional factor, HIF-1α, has been suggested to trigger these changes in PH [116, 234, 327]. PGC1α, was shown as a transcriptional coactivator of MFN2 to increase mitochondrial fusion [328]. Later a bidirectional relation (positive feedback) between MFN2 and PGC-1α has been also found [234]. They showed that an increase of MFN2 increased the PGC-1α in PAH PASMC as well as *in vivo* and attenuation of MFN2 decreased PGC-1α [234]. Downregulation of MFN2 could affect the apoptosis and proliferation of PASMC via the phosphoinositide 3-kinases (PI3K)/Akt signaling pathway [329]. All these results suggest that MFN2-mediated fusion is downregulated in PH and may contribute to disease development. As impaired mitochondrial network formation can activate the process of PINK1-dependent mitophagy, these results are in line with increased PINK1-dependent mitophagy in PH [192]. Conversely, the PINK1/PARKIN pathway can also catalyze a rapid MFN2 ubiquitination, triggering detachment of the mitochondria from ER, thereby triggering mitophagy [330]. Thus, MFN2 plays an important role in PINK-dependent mitophagy pathway [331-333].

5.4. Effect of *Pink1*$^{-/-}$ and hypoxia on general autophagy

During mitophagy LC3B-I is transformed to LC3B-II, which are linked to the vesicles of autophagosomes [323]. LC3B-II is a general marker of autophagy and interacts with PINK1 and PARKIN at their LC3B-interacting (LIR) motifs to induce autophagy [240]. During that process PARKIN employs ubiquitin along with the LC3B adaptor protein (P62) on the OMM in order to gather all damaged mitochondria and lead them to degradation by autophagosomes [242, 243]. LC3B-II has been reported to be elevated in the pulmonary artery constriction (PAC) mouse model of RV remodelling [273]. In the present study, no significant regulation of LC3B-II was found in WT or *Pink1*$^{-/-}$ mice after chronic hypoxia-induced PH for 28 days. This finding may be explained by the time-dependent adaptation of LC3B-II regulation in response to hypoxia [334]. It was shown that LC3B-II was upregulated in a time-dependent manner in PECs but not in PASMCs. The authors claimed that the absence of regulation of LC3B-II in PASMC could be related to the time-dependent dissociation of LC3B-II by interact with caveoline-1in response to hypoxia after localization on caveolae [239].

5.5. Effect of *Pink1*$^{-/-}$ on proliferation/apoptosis of precapillary PASMC of mouse

Increased proliferation and decreased apoptosis are key characteristics underlying pulmonary vasculature remodeling in PH [335-338]. Previously, it was proven that mitochondria play a crucial role in these alterations in PH [105, 234]. It has been described that accumulation of hyperpolarized mitochondria that show decreased glucose oxidation and increased fission could contribute to the increased proliferation and decreased apoptosis of PASMC in various forms of PH via altered mitochondrial ROS production [281, 339], mitochondrial Ca^{2+} metabolism and other different factors [134, 142, 340]. Furthermore, in a recent study in *Drosophila*, it was suggested that PINK1/PARKIN signalling can downregulate cellular apoptotic signalling by mono-ubiquitination of VDAC1 [341]. PINK1-dependent mitophagy could be connected to this mitochondrial phenotype by degradation of mitochondria with low membrane potential and selection of hyperpolarized mitochondria. Furthermore, increased fission may be a sign of mitophagy or lead to mitophagy. In line with this hypothesis, the present study shows that *Pink1* deletion prevented the hypoxia-induced increase in proliferation and reversed the hypoxia-induced decrease in apoptosis in PASMC.

Based on the evidence from previous studies, various triggers of PH (IPAH and chronic hypoxia) could stimulate the accumulation of dysfunctional mitochondria and thus, initiate the activation of PINK1-dependent mitophagy. Moreover, increased expression of PINK1 could be caused by hypoxia or inflammation-dependent mechanisms as outlined above. The increased rate of PINK1-dependent mitophagy could result in a selection and accumulation of hyperpolarized mitochondria [342] in PASMC leading to an increase in PASMC proliferation and decreased apoptosis [343, 344]. Another study in atherosclerosis proposed that PINK1-dependent mitophagy enhanced apelin-13-induced proliferation in vascular SMC by activating of p-AMPKα and this leads to exacerbation of atherosclerotic lesions formation [345]. Contrary to the above-mentioned studies, another study showed that *Pink1*$^{-/-}$ in MEF and primary cortical neurons increased cell proliferation via the reprogramming of glucose metabolism by enhancing HIF-1α activity [259].However, it remains unclear by which mechanisms PINK1-dependent mitophagy regulates proliferation and apoptosis in PASMC. PINK1-dependent mitophagy may directly or indirectly enhance HIF-1α signaling and enhance mitochondrial ROS [346, 347]. Therefore, in the present study PINK1 deficiency during hypoxia may directly or indirectly inhibit HIF-1α stabilization and therefore block hypoxia-induced proliferation and anti-apoptosis in PASMC. Further studies are necessary to explain the communication between PINK1-dependent mitophagy and HIF-1α during hypoxia in PASMC.

Independently from HIF-1α, current literature suggests that PINK1 regulates cellular proliferation in different cell types including cancer cell lines [257, 259, 348-350]. PINK1 can promote proliferation via the activation of inflammatory pathways e.g. NF-κB pathway, which was shown in non-small cell lung cancer (NSCLC) [350]. Another study revealed that *Pink1*$^{-/-}$ inhibits proliferation in astrocytes via attenuation of AKt activation and EGFR expression[257]. Recent studies demonstrated that enhanced PINK1-dependent mitophagy translocated the activated tank binding kinase 1 (TBK1) from the centrosome to the damaged mitochondria which resulted in TBK1 deactivation [351, 352]. Translocation and deactivation of TBK1 from the centrosome will result into arrest of the cell cycle in the G2/M phase. Therefore PINK1/PARKIN have been suggested to play an indirect role in the progression of cellular mitosis [262].

Going beyond proliferation, in the present study, it was found that *Pink1* deletion reversed the hypoxia-induced decrease in apoptosis of PASMC. The role of PINK1 in regulation of apoptosis has been investigated in different cell lines and experimental conditions [260, 353, 354]. In accordance with the present study on PASMC, it was found that in lung tumour cells, silencing of *PINK1* resulted in cell cycle inhibition, attenuation of proliferation and increase of apoptosis [260]. Another group showed that inhibition of PINK1-dependent mitophagy in rat cardiomyocytes induced apoptosis and overexpression of PINK1-dependent mitophagy transferred protection against cardiac hypoxia/reoxygenation injury [355]. With regard to hypoxia, it was shown in a study on porcine granulosa cells (GCs) that activation of PINK1-dependent mitophagy could attenuate hypoxia-induced apoptosis of GCs and protect them against hypoxia damage [356]. Accordingly, Arena *et al*. showed that, PINK1 in human embryonic kidney 293 cells (HEK293) and the SH-SY5Y cell line is protective against carbonylcyanide m-chlorophenylhydrazone (CCCP)-induced apoptosis [357]. Contrary to the present study in PASMC, Carrol *et al.* demonstrated that PINK1 induced apoptosis. They showed that after recruitment and phosphorylation of PARKIN by PINK1, PARKIN ubiquitinated the anti-apoptotic agent Mcl-1 (a Bcl-2 family member) to activate the caspase cascade to induce apoptosis [318, 358].

In summary, the results from the present study support the hypothesis that PINK1-dependent mitophagy plays an important role in hypoxia-induced pulmonary vascular remodeling via inhibition of hypoxia-induced proliferation and reversal of hypoxia-induced anti-apoptosis of PASMC.

5.6. Effect of *Pink1*$^{-/-}$ on chronic hypoxia-induced PH

To examine the hypothesis that PINK1-dependent mitophagy plays an important role for *in vivo* pulmonary vascular remodeling, mice were exposed to 4 weeks of hypoxia (10% O_2) and the study compared pulmonary vascular remodeling, development of PH and RV alterations between WT and *Pink1*$^{-/-}$ mice. Exposure to chronic hypoxia induced an increase in RVSP to the same degree in both mouse strains. However, pulmonary vascular remodeling quantified by the degree of muscularization of the small pulmonary arterial vessels was significantly lower in *Pink1*$^{-/-}$ mice after chronic hypoxic exposure compared to WT mice. Different reasons may explain the discrepancy between RVSP and pulmonary vascular remodeling.

First, pulmonary vasoconstriction could be enhanced in *Pink1*$^{-/-}$ and thus lead to increased PVR despite decreased remodeling. In this regard, *Pink1*$^{-/-}$ may interfere with intracellular Ca^{2+} metabolism via alterations of the $\Delta\Psi_m$ [215]. Moreover, PINK1 directly phosphorylates the mitochondrial proton/Ca^{2+} antiporter (LETM1) at Thr^{192} position [359] thereby possibly affecting mitochondrial calcium uptake. In this regard, it was shown that *Pink1* deletion reduced the uptake of Ca^{2+} by mitochondria after physiological stimulation [215]. Subsequently increased cytosolic ($[Ca^{2+}]_{cyt}$) concentration (which is a major trigger of PASMC contraction and pulmonary vasoconstriction [360]) may enhance pulmonary vasoconstriction. Furthermore, *Pink1*$^{-/-}$ may affect PEC and therefore vasoconstriction, which needs further investigations.

Secondly, it cannot be excluded that *Pink1* deletion affects other compartments of the pulmonary vasculature than only small precapillary vessels. It is known that pulmonary vascular remodeling in PH is characterized by increased vascular wall thickness of precapillary vessels but also peripheral vascular pruning (accompanied by a loss of capillaries) and increased stiffness of larger pulmonary arteries [361]. *Pink1* deletion could increase vascular pruning and stiffness of pulmonary arteries in chronic hypoxia, thereby counteracting decreased remodeling of precapillary vessels. In this regard it has been shown that PINK1 protects murine pulmonary PECs against hypoxic injury [362] and thus may affect different compartments of the pulmonary vascular tree differently. However, more investigations are necessary to elucidate the role of PINK1 on PECs, vascular stiffness and pruning.

Third, and most importantly, RVSP can be affected by heart function. Although, the investigations on global heart function did not detect a difference of CO in WT or *Pink1*$^{-/-}$ mice after normoxic or hypoxic exposure, it is likely that better RV function maintained RVSP despite decreased PVR and vascular remodeling. The reason for this uncertainty lies in the technical limitation that RVSP and CO are not measured simultaneously by the same technique, but CO

is measured by echocardiography prior to RVSP which is determined by invasive hemodynamics. Both parameters are measured under normoxic conditions and thus may not reflect hemodynamic conditions in hypoxia. In this regard, the better TAPSE and lower RVID in hypoxic *Pink1*$^{-/-}$ mice indicate that difference in RV load in hypoxia may contribute to improved RV remodeling and that increased heart function may contribute to the discrepant finding of the effect of *Pink1*$^{-/-}$ on RVSP and pulmonary vascular remodeling. Possibly, *Pink1*$^{-/-}$ has also a direct effect on cardiomyocytes during RV remodeling. In this regard, numerous studies have demonstrated that PINK1 affects on cardiomyocytes apoptosis in response to oxidative stress (hypoxia) and different stress injuries [355, 363, 364]. However, it is not clear if the beneficial effect of PINK1 on RV remodeling is due to its effect on the mechanical stress (due to increased afterload) or on the effect of chronic hypoxia on the RV *per se*.

Taken together, *Pink1* deletion attenuated chronic hypoxia-induced vascular remodeling as well as RV remodeling and suggesting overall beneficial effects of Pink1$^{-/-}$ on hypoxia-induced PH by improving RV function.

5.7. Alternative mitophagy (PINK1-independent) pathways

Besides the classical activation of mitophagy by PINK1, there are alternative mitophagy pathways such as the BNIP3L/Nix and FUNDC1 mitophagy pathways which may act independently of PINK1 or interact with PINK1-dependent mitophagy [184]. BNIP3L/Nix interacts with its LIR motifs with LC3B-II as the main protein involved in autophagy [365]. Finding of this study showed that BNIP3L/Nix protein expression was increased in *Pink1*$^{-/-}$ lung homogenate after exposure to chronic hypoxia *in vivo*. It has been reported that BNIP3L/Nix can compensate the absence of PINK1 to maintain mitochondrial quality control [252]. In accordance with the finding of this study, the increase of BNIP3L/Nix in *Pink1*$^{-/-}$ mice has been reported in a very recent study by Livinston *et al.* [366]. They showed that in the presence of chemical stress and absence of *Pink1*, BNIP3L/Nix was accumulated on mitochondria to compensate the PINK1-induced mitophagy abolishment in renal ischemic preconditioning (IPC) [366]. Furthermore, there is recent evidence that BNIP3L/Nix can influence PH development by showing its role on RV remodeling in the MCT-induced PH model [253]. Thus, the mild phenotype of *Pink1* deletion may be at least partially be explained by the compensatory increase of PINK1-independent mitophagy (BNIP3L/Nix) in *Pink1*$^{-/-}$ mice after exposure to chronic hypoxia *in vivo.*

5.8. Conclusion

In conclusion, this study provides experimental evidence that PINK1-dependent mitophagy promotes pulmonary vascular remodeling in hypoxia-induced PH. The protein level of PINK1 was increased in PASMC exposed to hypoxia for 5 days, in the lung homogenate of mice with chronic hypoxia-induced PH and in lung homogenate of IPAH patients, however, in the latter also the expression of PARL which degrades PINK1 was increased. Congenital deletion of *Pink1* attenuated the chronic hypoxia-induced increase of PASMC proliferation and decrease of PASMC apoptosis *in vitro*. Chronic hypoxia-induced remodeling was attenuated *in vivo* in *Pink1*$^{-/-}$ mice, but not the hypoxia-induced increase of RVSP. Moreover, *Pink1* deletion attenuated hypoxia-induced RV remodeling thus suggesting overall beneficial effects of Pink knockout on hypoxia-induced PH. Regulatory upregulation of PINK1 -independent mitophagy may attenuate the phenotype of *Pink1*$^{-/-}$.

This finding may provide a novel therapeutic target in the treatment of PH. Further studies on the role of mitophagy in human IPAH are necessary. A summary of the mechanisms defined in this study is shown in figure 5.1.

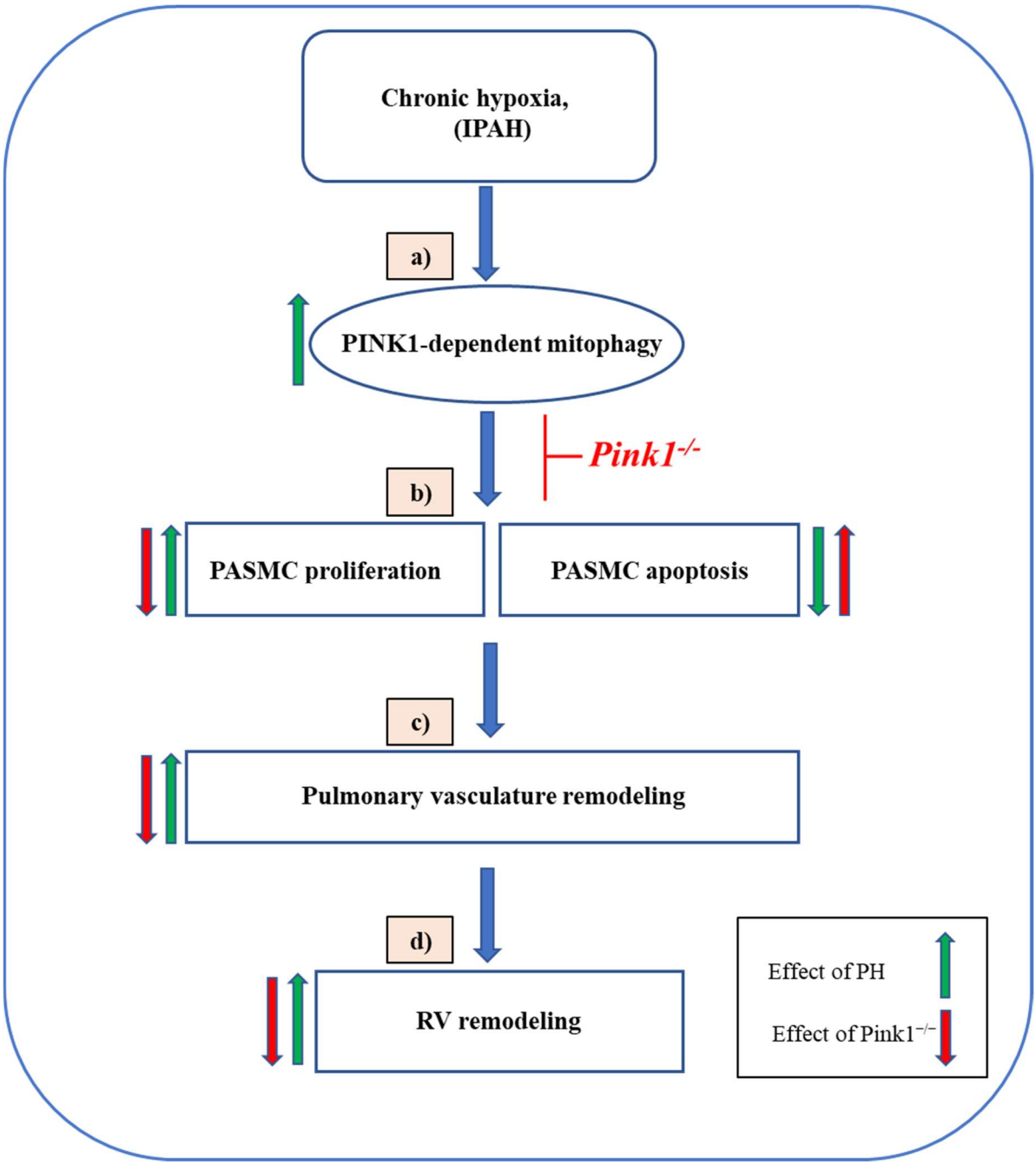


Figure 5.1. Summary of the proposed role of PINK1-dependent mitophagy in chronic hypoxia-induced PH

a) PINK1 protein expression is increased in chronic hypoxia-induced PH and IPAH.
b) *Pink1* deletion attenuates the hypoxia-induced increase in PASMC proliferation as well as the decrease in PASMC apoptosis *in vitro*.
c) *Pink1*$^{-/-}$ attenuates hypoxia-induced pulmonary vascular remodeling without affecting RVSP.
d) *Pink1*$^{-/-}$ attenuates chronic hypoxia-induced RV remodeling and improves RV function.

6. Summary

Pulmonary hypertension (PH) is a severe multifactorial disease characterized by increased pulmonary vascular resistance and pulmonary vascular remodeling with subsequent right heart remodeling and ultimately right heart failure. Mitochondrial dysfunction is suggested to play a key role in PH development. Mitophagy serves for mitochondrial quality control to remove dysfunctional mitochondria. Mitophagy is induced by accumulation of PTEN-induced putative kinase 1 (PINK1) and Parkinson protein 2, E3 ubiquitin protein ligase (PARKIN) at the outer mitochondrial membrane (OMM) of dysfunctional, depolarized mitochondria, while the Presenilins-associated rhomboid-like protein (PARL) degrades PINK1 in healthy mitochondria. Currently, the role of PINK1-dependent mitophagy for development of PH remains unknown. This study thus investigated the role of PINK1/PARKIN-dependent mitophagy in the development of chronic hypoxia-induced PH.

Protein expression of PINK1 was increased in precapillary pulmonary arterial smooth muscle cells (PASMC) exposed to 1% O_2 for 5 days *in vitro* and in lung homogenate of mice exposed to 10% O_2 for 28 days *in vivo.* In lung homogenate of patients with idiopathic pulmonary arterial hypertension (IPAH) PINK1 as well as PARL protein expression was increased compared to donor controls. Hypoxia-induced proliferation of *Pink1*$^{-/-}$ PASMC was attenuated compared to WT PASMC, while their apoptosis was enhanced after *in vitro* hypoxic incubation compared to WT PASMC. Accordingly, pulmonary vascular remodeling was lower in *Pink1*$^{-/-}$ mice exposed to 10% O_2 for 28 days compared to hypoxic WT mice. In contrast, the chronic hypoxia-induced increase in right ventricular systolic pressure (RVSP) was similar in *Pink1*$^{-/-}$ and WT mice, however, hypoxia-induced right ventricle (RV) remodeling was also attenuated in *Pink1*$^{-/-}$ after chronic hypoxic exposure compared to hypoxic WT mice. The PINK1-independent mitophagy pathway regulated by BCL2/Adenovirus E1B 19 KDa protein-interacting protein 3-like (BNIP3L/Nix) was increased in *Pink1*$^{-/-}$ mice compared to WT mice, possibly attenuating the phenotype of *Pink1*$^{-/-}$ *mice*.

The data presented in this thesis suggests that PINK1-dependent mitophagy promotes hypoxia-induced pulmonary vascular remodeling by affecting PASMC proliferation and apoptosis. Furthermore, PINK1-dependent mitophagy participates in RV remodeling during chronic hypoxia-induced PH. This finding provides insight into a novel mechanism of hypoxia-induced PH and RV remodeling. Further studies on the role of mitophagy in IPAH and the potential as therapeutic target are necessary.

7. Zusammenfassung

Die pulmonale Hypertonie (PH) ist eine schwere multifaktorielle Erkrankung, die durch einen erhöhten Lungengefäßwiderstand und Lungengefäßumbau und daraus folgenden Umbau des rechten Herzens und letztendlich Rechtsherzinsuffizienz gekennzeichnet ist. Es wird vermutet, dass die mitochondrialen Dysfunktionen eine Schlüsselrolle bei der PH-Entwicklung spielt. Die Mitophagie dient zur mitochondrialen Qualitätskontrolle, um dysfunktionelle Mitochondrien aus dem mitochondrialen Netzwerk zu entfernen. Die Mitophagie wird durch Akkumulation von den Proteinen PTEN-induced putative kinase 1 (PINK1) und Parkinson protein 2, E3 ubiquitin protein ligase (PARKIN) an der äußeren Mitochondrienmembran von dysfunktionellen, depolarisierten Mitochondrien eingeleitet, während das Presenilins-associated rhomboid-like protein (PARL) in gesunden Mitochondrien abbaut. Derzeit ist die Rolle der PINK1-abhängigen Mitophagie für die Entwicklung der PH unbekannt. Diese Studie untersuchte daher die Rolle der PINK1/PARKIN-abhängigen Mitophagie bei der Entwicklung der Hypoxie-induzierten PH.

Die Proteinexpression von PINK1 war in präkapillären pulmonalen glatten Gefäßmuskelzellen (PASMC), die 5 Tage *in vitro* 1% O_2 ausgesetzt waren, und in Lungenhomogenat von Mäusen, die 28 Tage *in vivo* 10% O_2 ausgesetzt waren, erhöht. Im Lungenhomogenat von Patienten mit idiopathischer pulmonaler Hypertonie (IPAH) war die PINK1- sowie PARL-Proteinexpression im Vergleich zu Spenderkontrollen erhöht. Die Hypoxie-induzierte Proliferation war in *Pink1*$^{-/-}$ PASMC im Vgl. zu Wildtyp (WT) PASMC vermindert, während ihre Apoptose nach hypoxischer *in vitro* Inkubation im Vergleich zu WT PASMC verstärkt war. Dementsprechend war der pulmonale Gefäßumbau bei *Pink1*$^{-/-}$ Mäusen, die 28 Tage lang 10% O_2 ausgesetzt waren, im Vergleich zu WT-Mäusen geringer. Im Gegensatz dazu war der durch chronische Hypoxie induzierte Anstieg des RVSP bei *Pink1*$^{-/-}$ und WT-Mäusen ähnlich, jedoch war der durch Hypoxie induzierte rechtsventrikuläre Umbau bei *Pink1*$^{-/-}$ nach chronischer hypoxischer Exposition im Vergleich zu WT-Mäusen ebenfalls abgeschwächt. Der durch BNIP3L/Nix regulierte PINK1-unabhängige Mitophagie-Signalweg war bei *Pink1*$^{-/-}$ Mäusen im Vergleich zu WT-Mäusen erhöht, wodurch möglicherweise der Phänotyp von *Pink1*$^{-/-}$ Mäusen abgeschwächt wurde.

Die in dieser Arbeit präsentierten Daten legen nahe, dass die PINK1-abhängige Mitophagie den Hypoxie-induzierten Lungengefäßumbau fördert, indem sie die PASMC-Proliferation und Apoptose beeinflusst. Darüber hinaus ist die PINK1-abhängige Mitophagie am

rechtsventrikulären Umbau während einer durch chronische Hypoxie induzierten PH beteiligt. Dieser Befund liefert einen Einblick in einen neuartigen Mechanismus der Hypoxie-induzierten PH- und des rechtsventrikulären Umbaus. Weitere Studien zur Rolle der Mitophagie bei IPAH und zum Potenzial als therapeutisches Ziel sind erforderlich.

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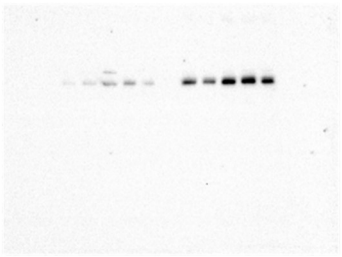
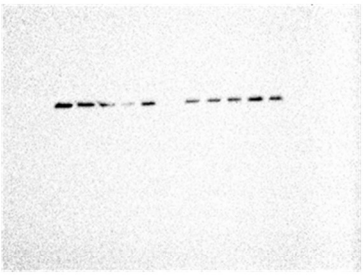
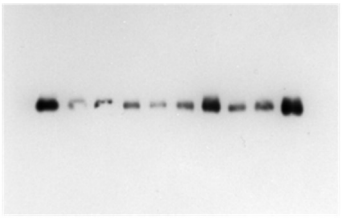
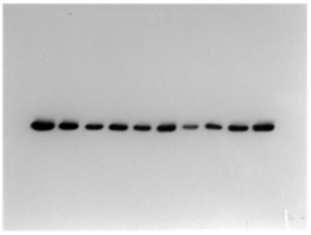
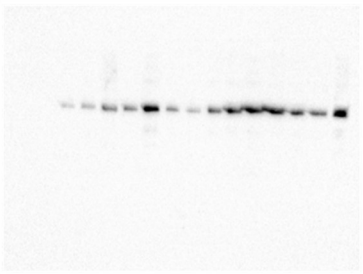
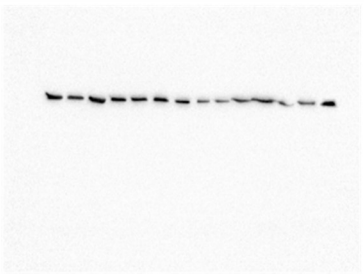
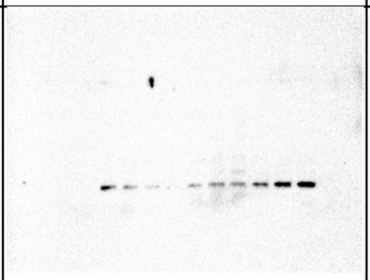
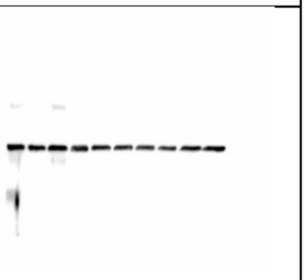
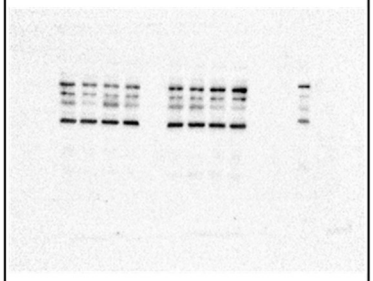
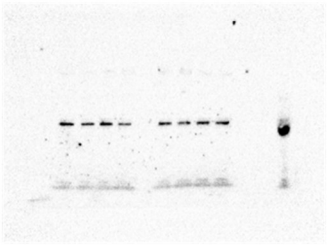
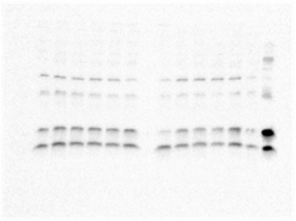
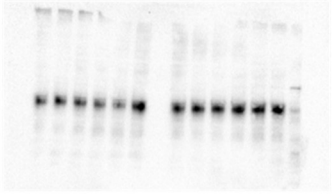
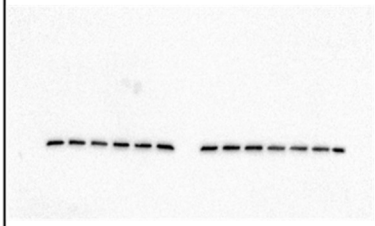
9. Appendix

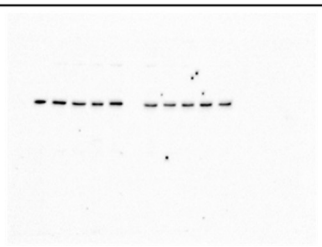
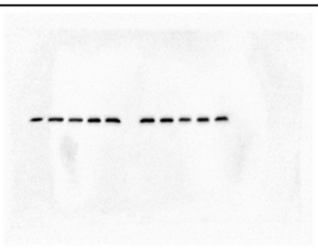
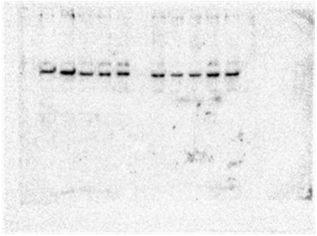
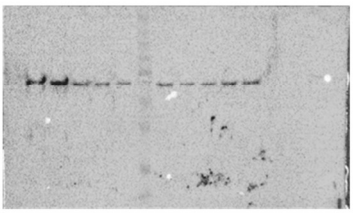
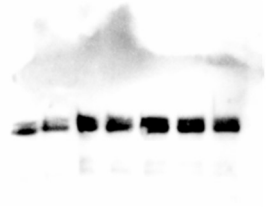
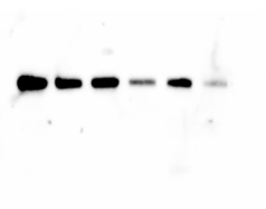
9.1. Full-length blots for figure 4.3a-c (human lung homogenate)

Reference Figure	sample	Primary Ab	Original blot	ß-actin
Fig. 4.3a	Human Lung	PINK1		
Fig. 4.3b	Human Lung	PARKIN		
Fig. 4.3c	Human Lung	PARL		

9.2. Full-length blots for figures 4.5a-c, 4.9b and 4.15b (mouse lung homogenate WB)

Reference Figure	sample	Primary Ab	Original blot	ß-actin
Fig. 4.5a	Mouse Lung	PINK1		
Fig. 4.5b	Mouse Lung	PARKIN		
Fig. 4.5c	Mouse Lung	PARL		
Fig. 4.9b	Mouse Lung	LC3B-II		
Fig. 4.15b	Mouse Lung	BNIP3L/ Nix		

9.3. Full-length blots for figures 4.7a-c, 4.8b, d (mouse PASMC WB)

Reference Figure	sample	Primary Ab	Original blot	ß-actin
Fig. 4.7a	Mouse PASMC	PINK1		
Fig. 4.7b	Mouse PASMC	PARKIN		
Fig. 4.7c	Mouse PASMC	PARL		
Fig. 4.8b	Mouse PASMC	DRP1		
Fig. 4.8d	Mouse PASMC	MFN2		

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