Investigating Sex-related Differences in Renal Tissues from Prohibitin-1 Knock-in Mouse Models

By

Yeshika Bhatia

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Department of Physiology & Pathophysiology

Max Rady College of Medicine

Rady Faculty of Health Sciences

University of Manitoba

Winnipeg

Abstract

Biological sex-related differences are present in many aspects of renal biology and pathobiology. However, our knowledge of the underlying mechanisms is currently limited. Prohibitin-1 (PHB1) is an evolutionarily conserved pleiotropic protein. Transgenic mouse models of PHB1 have revealed its role in sex differences in adipose and immune functions. However, it is not known whether PHB1 plays a role in sex differences in other cell and tissue types. To explore this at the systemic level, we focused on two key conserved post-translational modification sites in PHB1 (i.e., the Cys^{69} and Tyr^{114} residues) and developed *Phb1*^{C69A} and *Phb1*^{Y114F} knock-in mouse models (together referred to as the *Phb1-Ki* mice, unless specified separately). The *Phb1-Ki* mice displayed both similarities and dissimilarities in sex differences in their immunometabolic phenotypes, signifying the importance of Cys^{69}- and Tyr^{114}-linked functions in the sexually dimorphic features of PHB1. Interestingly, an increase in kidney mass was found in the *Phb1-Ki* mice. The *Phb1-Ki*^{Y114F} mice had significantly larger kidneys than the *Phb1-Ki*^{C69A} mice and wild-type mice. Further analysis of kidney samples from the *Phb1-Ki* mice showed structural differences in renal tubules and glomeruli. Particularly, a dysregulation of mitochondrial structure in tubular epithelial cells and increase in glomerular size was observed, which were more pronounced in the male *Phb1-Ki*^{Y114F} mice. Moreover, lipid droplets were frequently encountered in tubular epithelial cells from the male *Phb1-Ki*^{C69A} mice, whereas an accumulation of damaged mitochondria and immune cell infiltration were more apparent in the male *Phb1-Ki*^{Y114F} mice. Notably, sex differences in ubiquitin proteasome activity was apparent between the wild-type male and female mice, which was differently affected in renal tissues from the male and female *Phb1-Ki* mice and inversely correlated with the accumulation of the damaged mitochondria in tubular epithelial cells. In addition, sex-related differences in IGF1 receptor-mTOR singling and in the markers of mitochondrial dynamics were found in the *Phb1-Ki* mice, which were differently altered in the *Phb1-Ki*^{C69A} mice and *Phb1-Ki*^{Y114F} mice. These cellular and molecular changes correlated with the observed changes at the organ level, including sex-related differences. Collectively, the research findings herein suggest that PHB1's Cys^{69}- and Tyr^{114}-linked functions play a role in sex differences in renal biology, which was previously unknown. The *Phb1-Ki* mouse models have created new opportunities to advance our understanding in this field. A better understanding of sex differences in renal biology and pathobiology may open opportunities for better management and treatment of renal diseases, which is a prevalent health problem worldwide.

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Abbreviations

℃	Degree Celsius
%	Percent
AKT/Protein kinase B	Serine/threonine protein kinase
AMPK	Adenosine 5'-monophosphate-activated protein kinase
BAP	B-cell membrane associated protein
BSA	Bovine Serum Albumin
DRP1	Dynamin Related Protein
IGF-1	Insulin growth factor
IGF1-R	Insulin growth factor 1 receptor
LC3	Light chain 3
MEC	Mechanosensory complex
m-PHB-Tg	Mutant PHB Transgenic
mRNA	Messenger Ribonucleic acid
mTOR	Mechanistic target of rapamycin
OPA	Ocular atrophy
PAS	Periodic acid Schiff's
p-AMPK	Phosphorylated AMP-activated protein kinase
p-AKT	Phospho- serine/threonine protein kinase
PBS	Phosphate buffered saline
PHB1	Prohibitin-1

PHB2	Prohibitin-2
REA	Repressor of estrogen activity
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RTEC	Renal tubular epithelial cells
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SPFH	Stomatin, Prohibitin, Flotillin and HflC/K
S6RP	S6 Ribosomal protein
TBS-T	Tris-buffered saline, 0.1% Tween 20
TCA	Tri carboxylic acid
TEM	Transmission electron microscopy
WT	Wild type
µl	Microliter
µm	Micrometer

CHAPTER I

Literature Reviews

1.1 Identification of Prohibitin

The ability to negatively regulate cell proliferation is a necessity for all living organisms. Unicellular organisms must limit their replication to the times when adequate nutrients are present, and multicellular organisms must maintain homeostasis of their component tissues. Failure to provide adequate negative growth control in the developmental period may result in a deformity in tissues and organs, which may be lethal; in the post-developmental period, such a failure may result in abnormal growth of tissues and organs, such as tumour development [McClung et al. 1989; Nuell et al. 1991]. Because negative control is so critical, specific genes have evolved whose role is actively antiproliferative. Prohibitin (PHB) was identified in the search to identify antiproliferative genes, hence its name [McClung et al, 1989]. Subsequently, a homologous protein with almost 50% sequence homology with PHB was identified as a repressor of estrogen activity (REA, also known as PHB2) [Montano et al. 1999]. After the discovery of PHB2, PHB was renamed to PHB1 [Mishra et al. 2006; Osman et al. 2009]. Early on in a separate study, both proteins were also identified as B cell membrane-associated proteins (BAP)-32 and -37, based on their molecular masses [Terashima et al. 1994]. However, the B cell-specific function of BAP32 (PHB1) and BAP37 (PHB2) remained largely unexplored. Subsequently, using c-Myc protein-tagged PHB1 and baby hamster kidney cells, PHB1 was demonstrated to primarily localize to the mitochondria [Ikonen et al. 1995]. Since PHB1 was discovered as an anti-proliferative gene, initial scientific works primarily focused on the mechanisms involved in its anti-proliferative function, and its potential link in cancer development [Manjeshwar et al. 2003; Sato et al. 1992; Zhu et al. 2010]. Subsequently, many studies have reported alterations in the expression of PHB1 at the gene and protein levels in different cell and tissue types in various biological and pathological states beyond the scope of its anti-proliferative functions, suggesting the existence of pleiotropic attributes of this evolutionarily conserved protein [Mishra et al. 2005].

The PHB1 gene has been mapped to the chromosome 17q12–q21 locus in humans [Sato et al. 1992]. The chromosome 17q12–q21 region has been reported to be genetically linked to the early onset of breast cancer [Sato et al. 1992]. Other genes mapped to the same region which are associated with cell proliferation and cancers are those encoding the human epidermal growth

factor receptor 2 and nerve growth factor receptor (both are receptor tyrosine kinases) [Tsai-Pflugfelder et al. 1988; Vidgren et al. 1999; Huebner et al. 1986]. Locus 17q21 has also been identified as a susceptibility locus for different autoimmune diseases, including type 1 diabetes [Fung et al. 2009]. Consistent with this view, findings from a previous study in our laboratory using transgenic mouse models of mutant-PHB1^{Y114F} are suggestive of PHB1 as a susceptibility gene for type 1 diabetes (T1D) [Nguyen et al, 2016]. Furthermore, locus 17q21.1 has been identified in a genome-wide scan among chromosomal regions harbouring genes that influence the propensity to store fat in the abdominal area [Pérusse, 2001] and is close to genes involved in the regulation of food intake and sex steroid levels [Pérusse, 2001]. Moreover, our laboratory and others have found that PHB1 plays a role in steroidogenesis [Bassi & Mishra, 2022; Chowdhury et al. 2016]. In addition, PHB1 has an opposing effect on longevity under different metabolic states (normal conditions *vs*. diapause signaling) in *Caenorhabditis elegans* [Artal-Sanz & Tavernarakis, 2009], as well as in metabolic, immune and antiproliferative phenotypes in different transgenic and tissue-specific knockout mouse models [Ande et al. 2014; Ande et al. 2016; Ko et al. 2010; Li et al. 2019; Gao et al. 2021], which are findings compatible with the location of PHB1 on the susceptibility locus 17q12–q21. Taken together, these findings have established PHB1 as a pleiotropic protein with cell type- and tissue-specific functions. However, the molecular basis of the pleiotropic attributes of PHB1 remain unclear at large.

1.2 Cellular Localization & Tissue-specific Functions of PHB1

PHB1 belongs to the SPFH domain protein family (stomatins, flotillins, and HflK/C), and is a highly conserved and ubiquitously expressed membrane-associated protein [Tvernarakis et al. 1999; Mishra et al. 2005]. Two of the most studied members of the SPFH gene family, PHB1 and its homologue protein PHB2, localize to different cellular compartments and have cell compartment- and tissue-specific functions [Chowdhury et al. 2014; Merkwirth et al. 2012]. Of note, the expression levels of both proteins are much higher in cell types that have rich mitochondrial contents and tissue-specific mitochondrial functions, such as brown adipose tissue, the kidneys, the heart, skeletal muscle, and steroidogenic tissues (The Human Protein Atlas). By coincidence, the first demonstration of mitochondrial localization of PHB1 was reported in a kidney cell line [Ikonen et al. 1995], which is rich in mitochondrial content. This correlation

between higher mitochondrial contents and PHB1 protein levels is consistent with previous demonstrations of primary localization of PHB1 in the mitochondria, and indicative of their importance in tissue-specific mitochondrial biology (e.g., thermogenesis in brown adipose tissue and steroidogenesis in the adrenal glands and gonads) apart from its housekeeping role of mitochondrial biology at large. In the mitochondria, PHB1 and PHB2 form heterodimers and assemble into mega-complexes that play an important role in the structural and functional integrity of the mitochondria [Tetsuta et al. 2005]. This appears to be mediated by their protein and lipid scaffolding function. Moreover, growing evidence suggests that both proteins play a role in mitophagy and in the regulation of mitochondrial dynamics [Caron and Bertolin, 2024; Oyang et al. 2022]. In addition, PHB1 and other members of the SPFH gene family have been reported to have roles in the regulation of mitochondrial phospholipids [Richter-Dennerlein et al. 2022; Birner et al. 2011]. Collectively, this evidence suggests that PHB1 and PHB2 play a multifaceted role in mitochondrial biology. Consistent with this view, PHB1 and PHB2 knockdown in different cell types often leads to severe damage in mitochondrial structure and functions, which also display protein-specific phenotype indicating their interdependent and independent functions [Mishra 2019]. For example, the major liver-specific phenotypes of the hepatocyte-specific *Phb1* knockout mice and the *Phb2* knockout mice are largely different, with distinctions such as increased liver weight and the development of hepatocellular carcinoma in the former, and reduced liver weight and severe hypoglycemia in the latter [Ko et al. 2010; Li et al. 2019; Gao et al. 2021]. It appears that the mitochondrial biology of PHB1 and PHB2 is more complex than a simple interdependence, as often described in current literature.

Apart from the mitochondria, PHB1 has also been consistently identified in association with plasma membranes in various cell types, where it plays a role in membrane signaling that involves their post-translational modification at various sites [Ande at al. 2009; Kim et al. 2013; Chowdhury et al. 2016]. The post-translational modification of PHB1 and PHB2 has also been implicated in their intracellular trafficking [Mishra et al. 2010, Lee et al. 2010; Chowdhury et al. 2016]. Moreover, both proteins also localize to the nucleus, where they function as transcriptional co-regulators [He et al. 2011; Chen et al. 2015]. The plasma membrane-related and nuclear functions of PHB1 and PHB2 do not appear to require their heterodimerization (such as in the mitochondria) or at least have not been reported in current literature. Initially, evidence on the pleiotropic nature of PHB1 and PHB2 came from *in vitro* studies [Mishra et al. 2005; Mishra et al.

2006; Ande at al. 2012; Chowdhury et al. 2016; Terashima et al. 1994; Kang et al. 2013] and later, *in vivo* animal models further substantiated some of the functions of both proteins [Ko et al. 2010; He et al. 2011; Ande et al. 2014; Ande et al. 2016]. Importantly, tissue-specific perturbation of the functions of PHB1 or PHB2 in mouse models have been found to trigger diseases such as diabetes, liver and mammary tumors, and neurodegenerative disease [Merkwirth et al. 2012; He et al. 2008; Mussi et al. 2006; Ko et al. 2010; Supale et al. 2013; Ising et al. 2015]. Furthermore, a number of *in vitro* and *in vivo* studies, including studies from our laboratory, have reported a role of PHB1 in adipocyte and adipose tissue biology [Ande at al. 2012; Kang et al. 2013; Liu et al. 2012; Ande et al. 2014; Gao et al. 2021]. As an application to its properties, plasma membrane-associated PHB1 has been exploited to specifically deliver small molecules to target and ablate adipose tissue in obese mouse models [Won et al. 2014; Kolonin et al. 2004].

1.3 Relationship between PHB and Renal Function and Disease

Besides their role in the above tissues, growing evidence suggests that PHB1 and PHB2 play a role in renal biology, including in renal tubular epithelial cells [Liu et al. 2014; Zhou et al. 2012], as well as in glomerular podocytes and mesangial cells [Ising et al. 2015; Ising et al.2017; Wu et al. 2020], which appears to be mediated by both their mitochondrial and plasma membrane-linked functions in a cell type-specific manner [Ising et al. 2015; Ising et al. 2016]. Podocyte-specific PHB2 knockout results in a perturbed mitochondrial structure and an increase in insulin/IGF-1 signaling lead to mTOR (mammalian target of rapamycin) activation and a detrimental metabolic switch [Ising et al. 2015]. In addition, experimental evidence suggests that PHB1 confers additional, extra-mitochondrial functions in podocytes as they localize to the slit diaphragm and thereby stabilize the unique intercellular contact between podocytes required to maintain an effective filtration barrier [Ising et al. 2017]. Moreover, PHB1 and PHB2 are implicated in oxidative damage protection and extracellular matrix accumulation [Ye at al. 2015; Zhou et al. 2013], whereas PHB2 has been shown to be a dysregulated protein in hyperglycemia-induced disruption in mitochondrial homeostasis, which involve its ubiquitination and proteasomal degradation [Liu et al. 2014]. Increasing PHB1 levels have beneficial effects in renal biology under different experimental conditions [Zhou et al. 2012; Zhou et al. 2013]. Additionally, podocin, a key member of the slit diaphragm protein complex, contains a PHB-like domain that serves as a

signaling platform, regulating podocyte functions through associated transmembrane proteins [Schurek et al. 2014]. Mutations in *NPHS2* gene, which encodes podocin, a family of PHB domain protein, are major cause of nephrotic syndrome, a severe human kidney disorder [Schurek et al. 2014]. In addition, a heterozygous *PHB2* polymorphism, c.873-3_873-2 delCA (rs111523336), has been reported in patient with minimal change nephrotic syndrome [Sugimoto et al. 2017] suggesting that the PHB2 variant may contribute to increased susceptibility towards the recurrence of this disease. In renal tubular epithelial cells, PHB2-mediated mitophagy attenuates renal injury by regulating mitochondrial dysfunction and inflammasome activation [Xu et al. 2019]. Of note, a link between PHB2 deficiency and hyperactive insulin or the insulin-like growth factor-1 (IGF-1) signaling has also been reported in response to the deletion of PHB2 in the podocytes of mice [Ising et al. 2015]. This was found to involve the hyper-phosphorylation of the S6 ribosomal protein (S6RP), a known mediator of the mTOR signaling pathway [Ising et al. 2015]. Notably, inhibition of the insulin/IGF-1 signaling system through genetic deletion of the insulin receptor, alone or in combination with the IGF-1 receptor or treatment with rapamycin (an inhibitor of mTOR), prevented the hyper-phosphorylation of S6RP without affecting the mitochondrial structural defect, alleviated renal disease, and delayed the onset of kidney failure in PHB2-deficient animals [Ising et al. 2015]. This finding suggested that the perturbation of insulin/IGF-1 receptor signaling contributes to tissue damage in mitochondrial disease [and works in a mitochondrial independent manner]. Moreover, podocyte-specific PHB2 knockout models develop proteinuria and glomerulosclerosis, and eventually experience a loss of renal function [Ising et al. 2015]. Consistent with the evolutionarily conserved extra-mitochondrial function of PHB1, the ortholog of PHB2 in *Caenorhabditis elegans* was also not restricted to mitochondria but co-localized with the mechanosensory complex that requires the podocin ortholog MEC2 (mechanosensory protein 2) for assembly [Huber et al. 2006; Rinschen et al. 2016]. The knockdown of *Phb2* partially phenocopies the loss of MEC2 in touch neurons of the nematode, resulting in impaired gentle touch sensitivity [Huber et al. 2006]. Collectively, these data indicate that, besides its established role in the mitochondria, PHB1 and PHB2 have an additional function as evolutionarily conserved protein-lipid complexes at the plasma membrane in different organisms and cell types, including tubular epithelial cells and podocytes in the kidney.

1.4 Discovery of the Sex-specific Role of PHB1 in Adipose & Immune Functions

Our laboratory has been interested in investigating the role and regulation of PHB1 and discerning its cell compartment-specific function. Previously, our laboratory has shown that the posttranslational modification of PHB1 by palmitoylation at the Cys^{69} residue facilitates its membrane anchoring and subsequent phosphorylation at the Tyr^{114} site and a few other related sites in different metabolic cell types, such as adipocytes, hepatocytes, and skeletal muscle *in vitro* [Ande et al. 2009; Ande & Mishra, 2009; Ande & Mishra, 2010]. This was also found to be the case in different immune cell types, including macrophages, mast cells, and B and T cells [Xu et al. 2022; Kim et al. 2013; Kirken et al. 2008]. In addition, using the *in vitro* cell culture system, our laboratory has shown that PHB1 plays a role in pre-adipocyte differentiation [Ande at al, 2012], which is further confirmed by other studies [Kang et al. 2013; Liu et al. 2012]. Subsequently, to explore its biological and physiological relevance at the systemic level, our laboratory developed transgenic (Tg) mouse models by overexpressing PHB1 [Ande at al. 2014] or mutant-$PHB1^{Y114F}$ [Ande et al, 2016] from the fatty acid binding protein 4 (*Fabbp4*) gene promoter to simultaneously manipulate adipocytes and immune cell-specific functions (monocytic macrophages), as well as to discern the membrane signaling and mitochondrial functions of PHB1. Both male and female PHB1-Tg mice developed obesity (independent of diet); however, only the male mice developed obesity-linked metabolic dysregulation, insulin resistance, hepatic steatosis, and liver tumours, whereas the female mice remained protected from obesity-linked metabolic abnormalities [Ande at al, 2014; Ande et al. Sci Rep, 2016]. The mutant PHB1-Tg mice shared the development of obesity and metabolic phenotype with the male and female PHB1-Tg mice; however, the male mice developed splenomegaly and lymphadenopathy (and did not develop hepatic steatosis and liver tumors), [Ande et al, 2016] suggesting the importance of the Tyr^{114} phosphorylation site in macrophage biology. Again, the female m-PHB1-Tg mice remained protected from obesity-linked metabolic dysregulation and tumour development. The female-specific metabolic protection in the PHB1 and m-PHB1 transgenic mouse models raised an obvious question about the potential role of ovarian estrogens in this phenomenon. When the female m-PHB1-Tg mice were subjected to a surgical ovariectomy, they developed metabolic dysregulation (i.e., hyperinsulinemia and insulin resistance) and lymphadenopathy (similar to the male m-PHB1-Tg mice), despite a substantial reduction in adipose tissue mass, suggesting a

relationship between sex steroids and the immunometabolic phenotype of the m-PHB-Tg mice [Ande et al, 2016].

Moreover, the findings from the gonadectomized male and female PHB1-Tg mice revealed that PHB1 requires sex steroids for the development of the obese phenotype, which differentially affects glucose homeostasis and insulin sensitivity in males and females [Ande et al, 2016; Xu et al. 2018]. It appears that PHB1 plays a sex- and adipose depot-specific role and involves additional factors in this regulation process. *In vitro* studies using primary preadipocytes from the PHB1 transgenic mice suggested that PHB1 influenced preadipocyte differentiation diversely in the presence and absence of sex steroids [Xu et al. 2018]. These *in vivo* and *in vitro* studies suggested a multifaceted relationship between PHB1 and sex steroids, which may work in a cell and tissue type- and sex-specific manner.

Interestingly, on a high-fat diet (HFD), only the male m-PHB1-Tg mice displayed marked mononuclear cell infiltration in the pancreas and developed insulitis that mimics adult-onset T1D [Nguyen et al, 2016]. The male PHB1-Tg mice that shared the metabolic phenotype of the male m-PHB1-Tg mice, and the female m-PHB1 mice that expressed m-PHB similar to the male m-PHB1 mice, did not develop insulitis [Nguyen et al, 2016]. Thus, the development of insulitis in the male m-PHB1-Tg mice in response to metabolic stress requires both obesity-related abnormalities and m-PHB1. Collectively, this finding provided a proof-of-concept that obesity-associated abnormalities coupled with environmental triggers and genetic susceptibility to manifest adult-onset T1D and revealed PHB1 as a potential susceptibility gene for T1D. In addition, recent *in vitro* studies in our laboratory suggested the potential role of PHB1 in integrating cell signaling with immunometabolism in the functional polarization of M1 and M2 macrophages [Xu et al. 2022]. In summary, findings from the PHB1-Tg and m-PHB1-Tg mouse models established the role of PHB1 in adipose and immune functions at the systemic level and revealed that PHB1 plays a role in sex-related differences in immunometabolism, and that it possesses a multifaceted relationship with sex steroids.

1.5 Challenges in the Study of the Cell Compartment-specific Function of PHB1

The multifaceted, conjoint role of PHB1 and PHB2 in mitochondrial biology (as described earlier) makes it challenging to discern their extra-mitochondrial membrane signaling and their nuclear functions using the loss of gene or protein function approaches. This is because the cell-specific PHB1 or PHB2 knockout often severely impacts mitochondrial structure and function leading to severe tissue and organ damage and even premature death [Ko et al. 2010; Merkwirth et al. 2012; Supale et al. 2013; Li et al. 2019;]. For example, the neuron-specific inactivation of *Phb2* in the mouse forebrain causes a substantial decrease in body weight, extensive neurodegeneration associated with behavioral impairments and cognitive deficiencies, as well as premature death [Merlwirth et al. 2012]. A substantial reduction in body weight and premature death have also been reported in pancreatic beta-cell specific *Phb2* knockout mice [Supale et al. 2013]. In general, a dysregulation of mitochondrial structure and function is a common feature in *Phb1* or *Phb2* knockout mouse models. These challenges also extend to the study of the relationship between the cell compartment-specific role of PHB1 and sex steroids. Of note, PHB1 has been identified as a cysteine oxidized mitochondrial protein under oxidative stress in relation to mitochondrial damage [Suh et al. 2004] indicating a potential role of the lone and conserved Cys^{69}-linked function in the mitochondrial biology, which is currently unknown to us. Thus, targeting the plasma membrane-related function of PHB1 by manipulating the Cys^{69} site may affect its mitochondrial function or prevent cysteine oxidation-linked functional alterations. In addition, recent advances in click chemistry and mass spectrometry in relation to the large-scale mapping of posttranslational modification at the cysteine residue in proteins have revealed that the Cys^{69} residue in PHB1 is subjected to multiple posttranslational modification, such as S-nitrosylation, S-sulfenylation, and S-sulfhydration [Qu et al. 2020; Yang et al. 2014; Wu et al. 2019]. However, cell compartment-specific knowledge of these modifications in PHB1 at the Cys^{69} residue is currently unknown. The posttranslational modification of PHB1 by phosphorylation at the Tyr^{114} site has also been reported in relation to its intracellular trafficking between different cellular compartments, including the plasma membrane, mitochondria and the nucleus in many cell and tissue types [Ande and Mishra, 2020; Chowdhury et al. 2016; Lee at al. 2010]. As the posttranslational modification of proteins creates enormous diversity from a limited number of genes, it is possible that the acquisition of Cys^{69} and Tyr^{114} sites during evolution have contributed to the pleiotropic attributes of mitochondrial PHB1, including its role in sex-related differences, which may work in a cell compartment- and cell type-specific manner.

1.6 Knock-in Mouse Models of PHB1

To explore the importance of the Cys^{69} and Tyr^{114}-linked function of PHB1 in its pleiotropic characteristics at the systemic levels, including its sexually dimorphic functions, as well as to delineate the link between Cys^{69} and Tyr^{114} sites in cellular functions, our laboratory developed two knock-in mouse models of PHB1, separately [Bernier, 2024; Shah, 2023; Bhatia et al. 2024; Dehkordi et al. 2024]. In the first model, the Cys^{69} site was substituted with a structurally similar, non-modifiable alanine residue (***Phb1-Ki*$^{\mathbf{C69A}}$**) [Bernier, 2024; Shah, 2023] whereas in the second model, the Tyr^{114} residue was substituted with a structurally similar, non-modifiable phenylalanine (***Phb1-Ki*$^{\mathbf{Y114F}}$**) [Bhatia et al. 2024; Dehkordi et al. 2024]; hereafter, collectively referred as the *Phb1-Ki* mice unless specified separately. Consistent with previous findings from the PHB1 transgenic mouse models relating to PHB1's role in adipose and immune function, replacing the Cys^{69} and Tyr^{114} sites influenced sex-related differences in metabolic and immune phenotypes [Bernier, 2024; Shah, 2023; Bhatia et al. 2024; Dehkordi et al. 2024]. Interestingly, during their phenotypic characterization, sex-related differences in kidney size was revealed in the *Phb1-Ki* mice [Bhatia et al. 2024]. The male *Phb1-Ki*^{Y114F} mice displayed significantly larger renal mass than the male *Phb1-Ki*^{C69A} mice and wild-type mice, as well as compared to their female counterparts [Bhatia et al. 2024]. Of note, sex-related differences are known to exist in various aspects of renal physiology and pathophysiology (**Figure 1.1**) [Bairey Merz et al. 2019; Clotet-Freixas et al. 2024].

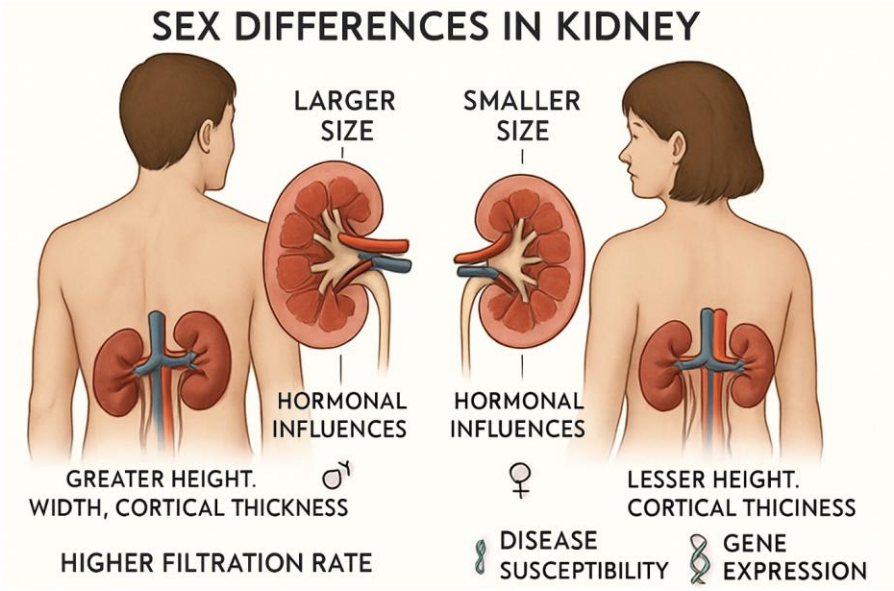


Figure 1.1. A schematic summary of sex-related differences in renal biology in human.

However, the underlying mechanisms involved in these processes remain largely unclear and the role of PHB1 in sex-related differences in renal biology and pathobiology is virtually unknown in current literature, which provided the basis and impetus for this project. In the following section, a review of existing literature will be conducted to understand basic renal structure and function as a bridge to next chapter on the Study Rationale, Hypothesis and Objectives.

1.7 Anatomy of the Kidneys

The kidneys are bean-shaped structures located on either side of the vertebral column (generally between the thoracic 12 and lumbar 3 vertebrae in human) and are referred to based on left and right position; they are encapsulated by a membrane called the renal capsule. A schematic of the basic structure of the kidney and its functional unit, nephron, are shown in **Figure 1.2**.

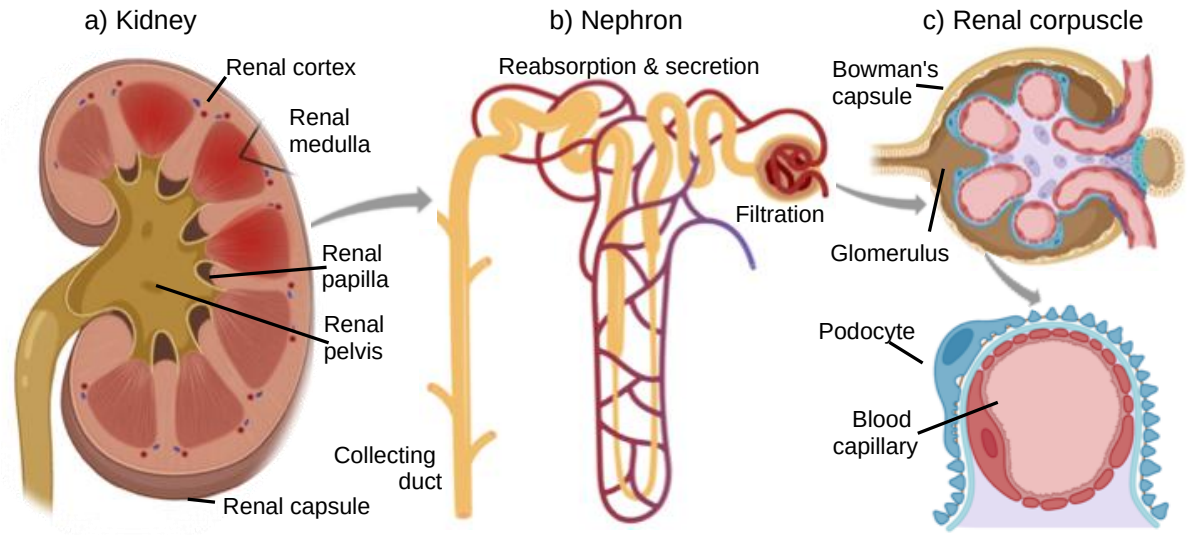


Figure 1.2. Schematic diagrams showing major structural components of kidney (a), its functional unit the nephron (b), and the renal corpuscle (c) ─ a crucial part of the nephron responsible for filtering blood. This schematic was prepared using BioRender – a scientific image and illustration software.

Histologically, the kidney consists of two major parts, the outer cortex and the inner medulla. The cortex contains renal corpuscles, proximal convoluted tubules, and distal convoluted tubules, which are involved in functions such as filtration of the blood and facilitate the reabsorption of essential substances from the filtrate to the bloodstream. The renal medulla is the inner segment and possesses striations due to the presence of proximal and distal straight tubules. The main function of the medulla is the concentration of urine and the maintenance of the body's water and salt. Nephron, the structural and functional unit of the kidney, is made of renal corpuscles and renal tubules (**Figure 1.2b**). The former comprises of glomerulus and Bowman's capsule, whereas the latter is made of proximal convoluted tubules, distal convoluted tubules, loop of Henle and collecting ducts (**Figure 1.2 b,c**). Nephrons are responsible for the major functions performed by kidneys, such as filtration, reabsorption, and excretion. The glomerulus is a network of capillaries that receives blood from afferent arterioles and drains it into the efferent arterioles, whereas the Bowman's capsule is a cup-like structure surrounding the glomerulus, where filtrate is collected after passing through the glomerulus.

1.8 Key Cell Types in the Kidneys

The renal corpuscle is a major part of the kidney, responsible for filtration and the formation of urine. It comprises of the following main cell types:

a) **Renal tubular epithelial cells**: These cells are present in the inner line of proximal and distal convoluted tubules, which is an important component for reabsorbing filtered water, ions, and amino acids. These cells are identified by the presence of many microvilli on their surface, which are meant to increase surface area for absorption. Renal tubular epithelial cells also line collecting duct cells, which play a crucial role in balancing sodium and water, as well as in the loop of Henle, and are specialized for generating a concentration gradient in the renal medulla, which is important for the kidney to concentrate urine.

b) **Endothelial cells:** These cells are present in the lining of blood vessels in the kidneys and function in filtration of blood in the glomerulus, maintaining the glomerular filtration barrier.

c) **Mesangial cells:** These cells are found in the glomerulus and are responsible for maintaining both a structural and functional role in the kidneys, including the regulation of blood flow and filtration.

d) **Podocytes:** These are specialized cells present on the outer surface of the glomerulus capillaries of kidney, forming a major part of the glomerular filtration barrier, and are responsible for filtering blood to form urine. A characteristic structure called the pedicels, also known as foot processes, are responsible for filtering blood and water products efficiently.

e) **Juxtaglomerular cells**: These cells are located on the juxtaglomerular apparatus and are responsible for the secretion of renin, which is involved in the renin-angiotensin-aldosterone system and is responsible for regulating blood pressure and fluid balance.

1.9 The Mitochondria in Renal Cells

The kidneys have distinctive anatomical and physiological characteristics, and kidney cell types have specialized metabolic traits and mitochondrial contents tailored to their specific location and functions [Bhargava and Schnellmann, 2017; Hoogstraten et al. 2023]. For example, the proximal tubule, the loop of Henle, the distal tubule, and the collecting duct all require active transport to reabsorb ions [Soltoff, 1989]. Accordingly, their component epithelial cells are enriched in mitochondrial contents. By contrast, glomerular filtration is a passive process that is dependent on the maintenance of hydrostatic pressure in the glomeruli [Holechek et al, 2003]. Consequently, the mitochondrial contents of the main glomerular cell types, such as podocytes, endothelial cells, and mesangial cells are relatively low compared to tubular epithelial cells. Moreover, the kidneys are one of the most energy-demanding organs in the human body under a basal state, along with the heart, and both organs have the highest mitochondrial contents in their main cell types (tubular epithelial cells and cardiomyocytes) [Bhargava and Schnellmann, 2017]. The resting metabolic rate for the kidneys is high because they require an abundance of mitochondria to provide sufficient energy to enable them to remove waste from the blood, reabsorb nutrients, regulate the balance of electrolytes and fluid, maintain acid–base homeostasis, and regulate blood pressure [Hoogstraten et al. 2023]. The proximal tubules require more active transport mechanisms than other renal cell types, because they reabsorb 80% of the filtrate that passes through the glomerulus, including glucose, ions, and nutrients. As such, they contain more mitochondria than any other structure in the kidney. The combination of these two attributes also make tubular epithelial cells vulnerable to mitochondrial dysregulation under metabolic dysregulation. Active transport in tubules compared to passive filtration in the glomeruli correlates with their mitochondrial contents. The disruption of mitochondrial homeostasis in the early stages of acute kidney injury is an important factor that drives tubular injury and persistent renal dysfunction in diabetic nephropathy and chronic kidney disease [Liu et al. 2022]. Chronic kidney disease, when kidneys are not working properly for $\geq$ 3 months and are beginning to lose their function, remains among the top causes of morbidity and mortality in the world affecting around 12% of the population globally with no cure. In Canada, chronic kidney disease is a major health problem affecting nearly 4 million Canadians. The economic burden of chronic and end-stage kidney disease (when the kidneys are no longer able to work at a level needed for day-to-day life) is staggering, costing the national health care system more than $40 billion per year in Canada [Kitzler and Chun, 2023]. This growing health problem highlights the need to advance our

knowledge of chronic kidney disease onset and progression to develop strategies to delay these processes while developing new therapeutics.

1.10 Sex-related Differences in Renal Biology & Pathobiology

Males have larger kidneys and a greater number of nephrons than females. Larger kidney size and comparatively more nephrons correspond to a higher glomerular filtration rate in males compared to females [Bairey Merz et al. 2019]. However, females tend to have more tubular reabsorption than males. Some of these sex-related differences are potentially due to the influence of sex steroid hormones, more specifically estrogens in females and androgens in males [Layton and Sullivan, 2019]. Moreover, estrogen is known to have protective effects over kidney injury, while testosterone may promote kidney disease progression in diseases such as chronic kidney disease and diabetic nephropathy, which can subsequently lead to hyperfiltration, causing further kidney damage [Lauretta et al. 2018]. Additionally, men retain more sodium in their kidneys than women due to higher levels of testosterone, which increases the production of aldosterone, hence increasing sodium reabsorption [McDonough et al. 2023]. In females, estrogen promotes sodium excretion, which is observed in higher levels during the menstrual cycle and pregnancy [Stachenfeld, 2008]. Moreover, differences in fluid retention also exists between males and females, where women are known to have a higher fluid retention, especially during pregnancy and the luteal phase of the menstrual cycle, while men, due to higher testosterone levels and lower body fat, have a more balanced fluid retention [Tkachenko et al. 2014].

Furthermore, many studies have shown sex differences in the risk of expressing kidney diseases. For example, diabetic nephropathy is often found to be more severe in males than females [Piani et al. 2020]. Moreover, chronic kidney disease progresses faster in males than females, and this is attributed to both hormonal and chromosomal differences and differences in kidney size [Mayne et al. 2023]. Polycystic kidney disease also shows differences in disease progression between biological sexes, where females tend to exhibit slower progression than males towards kidney failure [Conte et al. 2023]. The epidemiology of diabetic kidney disease (which is responsible for ~ 40% of chronic kidney disease) differs by sex, with the cumulative incidence of diabetic kidney disease being higher in men (4.1%) compared to women (2.5%) [Melsom et al. 2022]. However, women show a higher prevalence of chronic kidney disease and diabetic kidney disease [Francis et al. 2014]. Diabetic men are at a higher risk of progressing to end-stage kidney

disease, with hazard ratios ranging from 2.5 to 3.0 [Melsom et al. 2022; Ma et al. 2021]. Studies involving patients with type 1 diabetes and type 2 diabetes show that more men develop micro- and macro-albuminuria [Xu et al. 2019; Sgnorile et al. 2019]. The epidemiology of chronic kidney disease and diabetic kidney disease suggests that there could be differential pathogenic mechanisms in men and women.

Moreover, a recent metabolomics study has identified sex-based differences in kidney metabolism and in the blood metabolome of male and female individuals with diabetes [Clotet-Freixas et al. 2024]. Primary human proximal tubular epithelial cells (PTECs) from healthy males display increased mitochondrial respiration, oxidative stress, apoptosis, and greater injury when exposed to high glucose compared with PTECs from healthy females [Clotet-Freixas et al. 2024]. Male human PTECs showed increased glucose and glutamine fluxes to the TCA cycle, whereas female human PTECs showed increased pyruvate content [Clotet-Freixas et al. 2024]. Moreover, the male human PTEC phenotype was enhanced by dihydrotestosterone and mediated by the transcription factor HNF4A (hepatocyte nuclear factor 4A) and histone demethylase KDM6A (lysine demethylase 6A). Of note, KDM6A is an X-linked gene and is known to escape from X chromosome inactivation [Ngwa et al. 2025]. These findings suggest that differences in male and female kidney metabolism may contribute to sex-dependent outcomes in diabetic kidney disease.

1.11 Sex Differences in the Renal Ubiquitin Proteasome System

Emerging evidence suggests that the ubiquitin proteasome system plays a role in sex-related differences in renal tissues [Meyer-Schwesinger, 2019; Jenkins et al. 2020]. Mutations in genes encoding enzymes of the ubiquitin proteasome system result in renal disease, and mutations in kidney-specific genes result in ubiquitin–proteasome dysfunction that leads to renal disease [Meyer-Schwesinger, 2019], suggesting that the ubiquitin proteasome system plays an important role in kidney biology and pathobiology. In a recent study, the mapping of proteasome activity in different tissues from male and female mice have shown strong sexual dimorphism, where females have significantly higher proteasome activity in renal tissues, independent of proteasome concentration [Meyer-Schwesinger, 2019; Jenkins et al. 2020], indicating its potential importance in sex-related differences in renal physiology and pathophysiology, including in diabetic nephropathy and chronic kidney disease.

1.12 Sex Differences in Mitochondrial Functions in Renal Diseases

The mitochondria plays an important role in regulating several processes in the kidneys, such as filtration and homeostasis [Yang et al. 2023]. In kidney diseases, mitochondrial dysfunction in renal tubular epithelial cells is often a key factor. As the mitochondria are the primary source of reactive oxygen species (ROS) in cells, their dysregulation in diseases such as acute kidney injury leads to an excessive production of ROS, which further accelerates mitochondria damage and dysfunction, and leads to injury and inflammation in the renal tubes [Zhao et al. 2021]. In diabetic nephropathy, high blood sugar produces more mitochondrial ROS, which increases oxidative stress and inflammation [Jin et al. 2023]. Additionally, in diabetic nephropathy, high blood sugar leads to an imbalance in mitochondrial fusion and fission in the podocytes, which disrupts the filtration barrier [Ma et al. 2019]. The mitochondria plays an important role in cellular homeostasis and filtration, which are both high energy-dependent processes and are affected in kidney diseases [Bhatia et al. 2020]. Sex differences in mitochondrial functions are known to affect the progression of kidney diseases, as well as the severity, such as in the case of chronic kidney disease, which progresses more rapidly in males than in females due to the increased presence of testosterone, which promotes higher oxidative metabolism at the time of kidney diseases, producing more ROS and dysregulating mitochondrial dynamics [Sultanova et al. 2020]. However, in females, estrogen has a protective effect on the mitochondria by suppressing the amount of ROS when produced in kidney diseases [Wang et al. 2023]. Of note, the mitochondria are known to display sex differences, especially in tissues with higher mitochondrial contents (e.g., heart and brown adipose tissue) [Cao et al. 2022; Nadal-Casellas et al. 2013] and have a different relationship with male and female sex steroid hormones [Mauvais-Jarvis, 2024, Mishra and Singh, 2023]. However, our understanding of the relative contribution of mitochondrial biology and their relationship with sex steroids (and the factors involved) in sex-related differences in renal biology, physiology, and pathophysiology remain limited.

1.13 Factors Affecting Sex Differences in Kidney Biology

A number of factors contribute to differences in the kidneys of men and women, such as genetics, hormones, age, and environmental factors. A few of them are briefly described below.

1.13.1 Sex Chromosomes

X and Y chromosomes determine sex and gonad development, which in turn influence sex steroid levels between biological sexes. These hormones affect functions in different tissues in the body, including renal tissue. For example, estrogen is known to have a protective effect on renal functions due to its anti-inflammatory properties, and potentially modulates the renin-angiotensin-aldosterone system, which regulates fluid balance, while testosterone is known to have a contribution in higher rates of kidney disease progression in males, such as in chronic kidney diseases [Buléon et al. 2020].

1.13.2 Sex Steroid Hormones

Estrogen has been reported to have a protective effect on kidneys, wherein it helps to reduce inflammation and prevent kidney damage [Bairey Merz et al. 2019; Melamed et al. 2011]. Additionally, estrogen also promotes vasodilation, which causes blood vessels to relax and helps in the reduction of blood pressure, hence improving renal blood flow, which plays a major role in protecting the kidneys [Bairey Merz etal. 2019; Elliot et al. 2003]. On the other hand, testosterone has different impacts on kidney health as it promotes sodium retention in kidneys, which eventually leads to high blood pressure [Catanuto et al. 2009]. It is also known that high levels of testosterone contribute to the development of renal fibrosis [Grasielle et al. 2018]. Apart from estrogen in females, progesterone also plays a significant role in affecting kidney function as progesterone is known to modulate renal blood flow and glomerular filtration rates [Bairey Merz et al. 2019]. At the time of pregnancy, progesterone contributes largely to electrolyte balance and fluid retention, which increases blood volume [Andrianova et al. 2024]. At the postmenopausal stage, a decline in estrogen levels leads to a decline in estrogen-related renal protection, associated with an increased risk of developing chronic and diabetic kidney diseases and hypertension [Ahmed et al. 2008].

1.13.3 Escape from X-chromosome Inactivation (XCI) & Mitochondrial Genes

Growing evidence suggests that XCI, which leads to higher expression levels of many X-linked genes in females, contributes to sex-related differences in many tissue types and consequently in health and disease [Carrel and Willard, 2005]. Of note, many nuclear encoded mitochondrial proteins are X-linked, and have been reported to escape from XCI [Mishra & Nyomba, 2019], which may contribute to sex-related differences in the mitochondria in the context of renal physiology and pathophysiology.

CHAPTER II

Study Rationale, Hypothesis and Objectives

2.1 Study Rationale

Biological sex is increasingly recognized as an important variable in many aspects of renal biology and pathobiology, as preclinical and clinical studies have reported sex-related differences in renal structure, function, and in renal diseases [Bairey Merz et al. 2019; Clotet-Freixas et al. 2024; Layton and Sullivan, 2019]. However, despite clear evidence that biological sex influences the course, prevalence, and incidence of many renal diseases [Lauretta et al. 2018], little is known about the mediators involved in these processes and the molecular mechanisms underlying these differences. A better understanding of sex-related differences in renal biology may open new opportunities for better management and treatment of renal diseases, a growing health problem worldwide.

Prohibitin 1 (PHB1) is an evolutionarily conserved, ubiquitously expressed, and a pleiotropic membrane-associated protein, which localizes to the mitochondrial and the plasma membranes [Ikonen et al. 1995; Kim et al. 2013]. However, the molecular basis of the pleiotropic attributes of PHB1 remain unclear. Tissue-specific transgenic mouse models of PHB1 and a mutant-PHB1^{Y114F} mouse model have revealed its role in sex-related differences in adipose and immune functions [Ande et al. 2014; Ande et al. 2016; Nguyen et al. 2016; Xu et al 2018]. However, the mechanisms involved remain largely unknown. Moreover, it is not known whether PHB1 has a role in sex-related differences in other cell and tissue types. To explore this at the systemic level, our laboratory focused on two key evolutionarily conserved post-translational modification sites in PHB1 (the Cys^{69} and Tyr^{114} residues) that have been implicated in its intracellular trafficking and membrane-signaling functions [Ande and Mishra, 2010; Kim et al. 2013; Lee et al. 2010; Chowdhury et al. 2016]. The palmitoylation of the Cys^{69} residue facilitates its plasma membrane localization and subsequent phosphorylation at the Tyr^{114} residue in relation to membrane signaling function [Ande and Mishra, 2010; Ande et al. 2009; Kim et al. 2013]. For this reason, it would be reasonable to speculate that the loss of Cys^{69} palmitoylation or Tyr^{114} phosphorylation likely have common or overlapping downstream effects in a particular cell type. However, the Cys^{69} residue in PHB1 is present only in vertebrates, whereas the Tyr^{114} residue is

conserved in both vertebrates and invertebrates, as well as in yeast [**Figure 2.1**] [Mishra et al. 2006; Qu et al. 2020]. This would indicate that the Tyr^{114} residue in PHB1 may undergo phosphorylation in a Cys^{69} independent manner and have other functions (apart from membrane signaling).

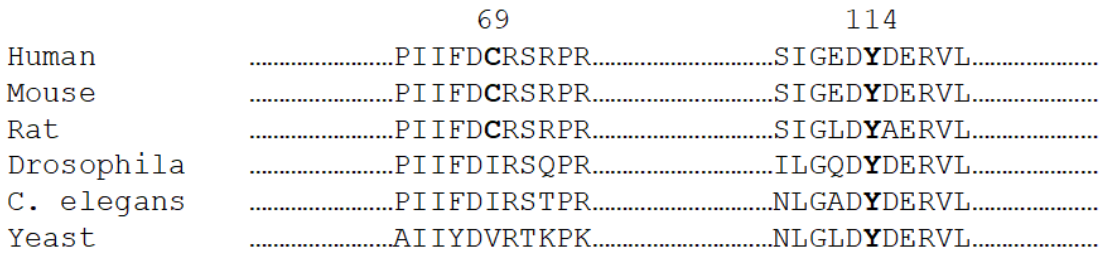


Figure 2.1. A part of PHB1 protein sequence alignment from different species (including vertebrates, invertebrates and yeast) spanning the conserved Cys^{69} and Tyr^{114} sites (in bold) containing motifs. Number at the top indicate the location of the conserved residues in human and mouse, which are identical.

This scenario may lead to differences in their downstream phenotypic effects when targeting them separately at the systemic level in animal models. This is because the loss of palmitoylation at the Cys^{69} residue would interfere with only the Cys^{69}-linked membrane signaling function of the Tyr^{114} site, whereas a direct loss of Tyr^{114} phosphorylation would affect both its membrane signaling and other known and unknown intracellular functions. For example, the phosphorylation of PHB1 at the Tyr^{114} site has been implicated in its intracellular trafficking between the mitochondria and the nucleus (independent of plasma membrane-related, cell-signaling functions) [Lee et al. 2010; Chowdhury et al. 2016]. Of note, the thiol group (-SH, also known as the sulfhydryl group) in the cysteine residue is versatile, and subjected to many posttranslational modifications (for example, S-acylation, S-nitrosylation, S-sulfenylation, and S-sulfhydration). Emerging evidence suggests that this is also the case for the Cys^{69} residue in PHB1 [Ande and Mishra, 2010; Qu et al. 2020; Yang et al. 2014; Wu et al. 2019]. It is possible that the thiol group of Cys^{69} may be used differently in different cellular compartments and in different cell types, and contribute to the pleiotropic function of PHB1. This is because different posttranslational modifications at a common residue in a protein occur in a mutually exclusive manner [Ande et al. 2013].

To explore the importance of the evolutionarily conserved Cys^{69} and Tyr^{114} residues in PHB1 and to discern their relationship in the pleiotropic attributes of PHB1, including in sex-

related differences, our laboratory developed a whole-body *Phb1-Ki*^{C69A} and *Phb1-Ki*^{Y114F} knock-in mouse model (together referred to as the *Phb1-Ki* mice unless specified separately). Consistent with previous findings from the PHB1 and mutant-PHB1^{Y114F} transgenic mouse models, both the *Phb1-Ki* mouse models displayed sex-related differences in metabolic and immune phenotypes [Bernier, 2024; Shah, 2023; Bhatia et al. 2024; Dehkordi et al. 2024], signifying the importance of Cys^{69}- and Tyr^{114}-linked functions in sexually dimorphic feature of PHB1. Interestingly, during their phenotypic characterization, a sex-related difference in their kidney sizes were found in the *Phb1-Ki* mice compared with the wild-type mice, revealing that PHB1 plays a role in sex-related differences in renal biology [Bhatia et al. 2024]. The *Phb1-Ki* mouse models created an opportunity to advance our understanding of sex-related differences in renal biology, especially regarding the role of PHB1 in these processes, which is virtually unknown in current literature. This gap in knowledge provided the impetus for this thesis project.

2.2 Hypothesis

The Cys^{69} and Tyr^{114} residues in PHB1 play a role in mediating its effects on sex-related differences in renal biology.

2.3 Specific Objectives

1. To characterize sex-related differences in renal tissues from the female and male *Phb1-Ki* and wild-type mice.
2. To explore the potential mechanisms involved in sex-related differences in renal tissues from the female and male *Phb1-Ki* and wild-type mice.

CHAPTER III

Materials & Methods

3.1 Materials

Various antibodies, buffers and reagents used in this thesis project are listed in the following Tables.

Table 3.1. List of antibodies used for Western immunoblotting and immunohistochemistry. In general, listed primary antibodies are raised in rabbit unless specified otherwise. Dilution of primary and secondar antibodies used are as per the recommendation by the manufacturers.

Antibody	Source	Catalog No	Dilution used
Anti-PHB1	Cell Signaling Technology	2426S	1/1000
Anti-PHB2	Cell Signaling Technology	14085	1/1000
Anti-Prohibitin (for IHC)	Abcam Inc.	ab28172	1/500
Anti-Rabbit IgG (secondary)	Cell Signaling Technology	7074S	1/2000
Anti-Mouse (IgG) (secondary)	Cell Signaling Technology	7076	1/2000
Anti-AMPK	Cell Signaling Technology	5831T	1/1000
Anti-phospho-AMPK	Cell Signaling Technology	50081T	1/1000
Anti-DRP1	Cell Signaling Technology	8570S	1/1000
Anti-OPA1	Cell Signaling Technology	67589S	1/1000
Anti-LC3A/B	Cell Signaling Technology	D3U4C	1/1000

Anti-polyubiquitin	Cell Signaling Technology	3936T	1/1000
Anti-COX-IV	Cell Signaling Technology	PA529992	1/1000
Anti-beta actin	Cell Signaling Technology	4967	1/1000
Anti-IGF1-R	Cell Signaling Technology	3027	1/1000
Anti Phospho-mTOR	Cell Signaling Technology	5536	1/1000
Anti-Akt	Cell Signaling Technology	4685S	1/1000
Anti phospho-Akt	Cell Signaling Technology	2965	1/1000
Anti-podocin	Sigma-Millipore	P0372	1/1000

Table 3.2. List of buffer compositions [Bollag et al., 1996].

Buffer	Composition
RIPA buffer	50 mM Tris base, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, HCl to pH 8.0.
5X Sample buffer	60 mM Tris base, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromophenol blue, HCl to pH 6.8.
SDS-PAGE Running buffer	25 mM Tris base, 192 mM glycine, 0.1% SDS, HCl to pH 8.3.
Transfer buffer	15.6 mM Tris base, 120 mM glycine.
TBST wash buffer	10 mM Tris base, 150 mM NaCl, 0.1% Tween-20, HCl to pH 7.5

Table 3.3. List of reagents

Reagent	Source
Acrylamide	Fisher Scientific
Ammonium persulfate	Fisher Scientific
Bis-acrylamide	Fisher Scientific
Blotting-grade blocker (non-fat dry milk)	Bio-Rad
Bovine serum albumin	Sigma-Aldrich
Bromophenol blue	Bio-Rad
Chloroform	Sigma-Aldrich
Clarity™ Western ECL Substrate	Bio-Rad
cOmplete™, mini, EDTA-free protease inhibitor cocktail	Roche
Dithiothreitol	Invitrogen
Formalin	Fisher Scientific
Glycine	Fisher Scientific
Isopropanol	Fisher Scientific
Methanol	Fisher Scientific
Mitochondria/cytosol protein extraction kit	Abcam Inc.
PBS	Sigma
Pierce™ 660nm protein assay reagent	Thermo Scientific
PhosSTOP (phosphatase inhibitors)	Roche
PVDF membrane	Bio-Rad
ReadyPrep Protein Extraction Kit (Signal)	Bio-Rad
SDS	Bio-Rad

Tris base	Fisher Scientific
Triton X-100	Fisher Scientific
Tween-20	Sigma

3.2 Experimental Design: Experimental design for the proposed specific objectives is depicted in Figure 3.1.

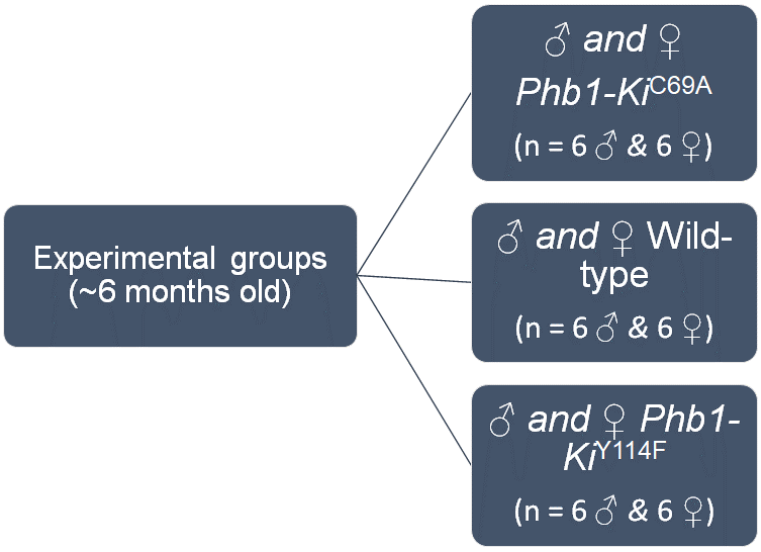


Figure 3.1. Experimental design for the specific objectives 1 and 2 (as described in 2.3).

3.3 Methods

3.3.1 The Development of *Phb1-Ki* Mouse Models & Their Genotyping

The *Phb1-Ki* mouse models were developed in-house at the Genetic Modeling Center at the University of Manitoba. The gRNA targeting the PHB1 gene within the mouse, along with the donor vector carrying a knock-in model cassette and Cas9 mRNA were injected together into fertilized eggs to generate the desired genetic modification in the resultant offspring. The resultant animals were identified by a polymerase chain reaction (PCR) and confirmed by DNA sequencing [Bernier, 2024; Shah, 2023].

Genotyping of the *Phb1-Ki* mice was performed using ear-punch samples received from the mouse breeding facility at the Central Animal Care Services (University of Manitoba). Samples were lysed overnight in 300 µl in lysis buffer at 40-60 °C and then subjected to centrifugation at 13,000 rpm for 13 minutes. The collected supernatant was added to 300 µl of isopropanol for precipitation of DNA. The samples were then centrifuged again at 13,000 rpm for 12 minutes, the supernatant obtained was discarded and the desired DNA was isolated. The obtained DNA was air dried for around 30 minutes and was re-suspended in 100 µl of double distilled water. The obtained DNA was subjected to PCR (**Table 3.4**).

Table 3.4. PCR cycling conditions for genotyping of the *Phb1-Ki* mice.

Knock-in mice	**Primers**	**PCR condition**	
Phb1-Ki^{C69A}	F 5’ AGCTGTCATCTTTGACCGATT-3’ R 5’ ACCTCTGTATCCTAGATGTCTCC-3’	94 ºC 3m 94 ºC 30s 60 ºC 30s X 40 cycles 72 ºC 30s Amplicon: 233 bp	Initial Denaturation Denaturation Annealing Extension
Phb1-Ki^{Y114F}	F 5’-ATTCAGGCTGCGCAACTGTTG-3’ R 5’-TAGAAATCCAGCGGCGTATCTCA-3’	94 °C 3m 94 °C 30s 60 °C 30s X 33 cycles 65 °C 50s Amplicon 254 bp	Initial Denaturation Denaturation Annealing Extension

F – forward primer; R – reverse primer; bp – base pair

Experimental animals were housed in a controlled environment with a 12-h light, 12-h dark cycle at 22 ºC with a standard chow diet (Lab Diet, St. Louise, MO) and water *ad libitum*. All animal experiments were conducted as per the Animal Use Protocol #24-023 approved by University of Manitoba’s Animal Care and Use Committee. All animal experiments followed the Canadian Council on Animal Care (CCAC) guidelines for animal research.

3.3.2 Blood and Tissue Collection

Homozygous male and the female *Phb1-Ki* mice and wild-type (WT, procured from in-house breeding at the Central Animal Care Services, University of Manitoba) mice of about 6 months of age were anesthetized using 4% isoflurane, followed by euthanasia in a CO_2-filled chamber. Their body weight was recorded and both kidneys from each animal were harvested, weighed, and processed for subsequent analysis. One kidney was flash frozen in liquid nitrogen for subsequent analysis of gene and protein expression levels, and the other kidney was processed for histological analysis and electron microscopy as described in the following sections. Blood was collected from the heart and was allowed to clot at room temperature for 30 minutes, and then centrifuged at 1500 g for 12 minutes at 4°C. The collected supernatant serum was stored at -80°C for subsequent analysis.

3.3.3 Histomorphometry

Kidney tissues from the male and female *Phb1-Ki* mice and wild-type mice were fixed in 4% formalin solution for around 12 hours at room temperature, followed by dehydration through an ascending series of ethanol and embedment in a paraffin block [Ande et al., 2016, Bassi and Mishra, 2022]. Hematoxylin and eosin were used to stain slices of 5 µm thickness. Subsequently, the slides were examined with Evos (XL Core AMEX 1000, Invitrogen) [Ande et al., 2014, 2016, Bassi and Mishra, 2022]. The mount of kidney sections and areas of the medulla and cortex were captured by using different magnifications (4x, 10x and 40x). In addition to this, from each group, 6 animals were used with 10-12 sections selected per animal, and differences in glomeruli and renal tubules' shape, size, and compositions were compared between biological sexes and all three genotypes. For renal histomorphometry, 40 serial sections per kidney were cut in the mid-region, and every eighth section was chosen for hematoxylin-eosin staining, i.e. a total of five sections per kidney [Nguyen et al. 2015; Xu et al. 2018]. Renal tubular and glomerulus areas were manually selected using ImageJ's Cell Magic Wand tool before automatic analysis, which assessed tubular and glomerular cell numbers and areas.

3.3.4 Immunohistochemistry

The paraffin-embedded tissue sections were used for immunohistochemistry, where sections were first deparaffinized and rehydrated in xylene and a descending concentration series of ethanol for 10 minutes each [Ande et al. 2016; Nguyen et al. 2016; Bassi and Mishra, 2022]. For antigen unmasking, slides were kept in a citrate buffer, followed by boiling for around 10 minutes. To avoid background staining, all the sections were incubated and blocked with BSA for 1 hour at room temperature. An optimum amount of the primary antibody was added using a blocking serum (TBST containing 2% BSA+3% Normal goat serum) and incubated overnight at 4℃ [Bassi and Mishra, 2022, Ande et al. 2016]. Excess primary antibody was washed using PBS thrice for 5 minutes each, followed by incubation in a secondary antibody for 1 hour at room temperature, followed by washing with PBS thrice for 5 minutes each. To confirm protein specificity of the antibody used, primary antibody step was omitted as a negative control. The sections were counterstained using hematoxylin, dehydrated through an ascending series of alcohol and xylene, and sections were mounted with a coverslip using a mounting solution [Bassi and Mishra, 2022]. The stained sections were visualized under Evos (XL Core AMEX 1000, Invitrogen).

3.3.5 Periodic Acid Schiff's (PAS) Staining

Renal section mounted slides were deparaffinized in xylene and hydrated in a decreasing concentration of ethanol, as in H & E staining, for histological analysis. The sections were then oxidized in periodic acid solution (1%) for 5-10 minutes and rinsed with at least two changes of distilled water [Matsumoto et al. 2021]. The sections were treated with Schiff's reagent for approximately 30 minutes and then rinsed in running tap water for approximately 10 minutes [Matsumoto et al. 2021]. The sections were counterstained with hematoxylin for approximately 5 minutes, followed by dehydration in increasing concentration of alcohols, and sections were allowed to clear in two xylene baths. The sections were mounted in dibutyl phthalate in xylene (DPX) and histomicrographs were captured using Evos (XL Core AMEX 1000, Invitrogen).

3.3.6 Electron Microscopy

The transmission electron microscopy analysis of renal tissues from different experimental groups was performed using a Philips CM10 at 80 kV at the Histomorphology & Ultrastructural Imaging Platform in the Faculty of Health Sciences at the University of Manitoba [Bassi and Mishra, 2022; Bassi et al. 2022]. Briefly, the renal tissue samples were excised into approximately 1 mm^3 and fixed with 3% glutaraldehyde in 0.1 M Sorensen's buffer for 3 h. After fixation, the renal tissues were re-suspended in 5% sucrose in 0.1 M Sorensen's buffer and then embedded in EPON™ resin. Subsequently, analysis was performed on ultra-thin sections (100 nm) stained with uranyl acetate and counterstained with lead citrate [Bassi and Mishra, 2022; Bassi et al. 2022].

3.3.7 Western Immunoblotting

Renal tissue samples were lysed with a RIPA lysis buffer. For this, 10 mg of renal tissue and 500 µl of the RIPA lysis buffer were added and homogenized completely using a tissue homogenizer. Protein concentrations were determined using a Bradford assay, and 20 µg of protein was loaded for each experimental group, separated on a SDS-PAGE gel of about 10-12%. Subsequently, separated samples were transferred to a polyvinylidene difluoride (PVDF) membrane, followed by blocking with 5% non-fat milk or BSA prepared in TBST. A specific primary antibody was added and incubated overnight at 4 °C, followed by washing with TBST for 10 minutes thrice, and the blot was further incubated with specific secondary antibody for 1 hour at room temperature. Finally, the membranes were given final washing with TBST for 10 minutes thrice at room temperature, and the blots were captured using an enhanced chemiluminescent substrate (Bio-Rad, Canada) and Bio-Rad Imaging system [Xu et al. 2022; Bassi et al. 2022]. Equal loading of protein was confirmed by reprobing membranes with housekeeping protein beta-actin or cell compartment-specific marker protein as applicable.

3.3.8 Subcellular Fractionation

Renal tissues from each experimental group were subjected to isolation of mitochondrial and cytosolic fractions using the Millipore Mitochondrial and Cytosolic Protein Extraction kit following the manufacturer's instructions [Xu et al. 2022; Bernier, 2024]. The fractions were run on 12% SDS-PAGE gels, followed by Western blotting for both PHB1 and PHB2. The fractions

were confirmed with compartment-specific protein markers ─ mitochondrial fractions with COX-IV and cytosolic fractions with beta-actin [Xu et al. 2022; Bernier, 2024].

3.3.9 Quantification and Statistical Analysis

Quantification of protein band densities from the Western blots and cell numbers were performed using Image*J* software (https://imagej.nih.gov/ij/) and QuoPath software. Counting cells from PAS staining was also performed by manual counting. GraphPad Prism 10.4.1 software was used for statistical analysis in all experiments (where applicable). Variables were analyzed by two-way ANOVA with sex and genotype as factors. For *post hoc* analysis, Tukey's correction for multiple comparisons was used to determine the significance of differences between sexes and between genotypes. $P < 0.05$ was considered significant.

CHAPTER IV

Results

4.1 The *Phb1-Ki* Mice Display Decreased Body Weight but Increased Kidney Weight

The male and the female *Phb1-Ki* mice consistently displayed lower body weights and reduced visceral adipose tissue mass (not shown) in comparison with the age- and sex-matched wild-type mice (**Figure 4.1**). Moreover, the difference in the body weight between sexes within the *Phb1-Ki* genotype was reduced when compared with the wild-type mice (**Figure 4.1**). Interestingly, an increase in kidney size was observed in both the male and the female *Phb1-Ki* mice compared with respective wild-type mice. Because age-matched male and female mice significantly differ in their body weight, which is alterd in *Phb1-Ki* mice, therefore, normalized kidney weight (i.e., the ratio of their kidney to body weight) was used for data analysis (**Figure 4.1**). A significant increase in kidney weight was found in the *Phb1-Ki*^{C69A} mice when compared with the wild-type mice (**Figure 4.1**). A further increase in kidney weight was observed in the *Phb1-Ki*^{Y114F} mice (**Figure 4.1**), which was significantly higher than the *Phb1-Ki*^{C69A} mice (**Figure 4.1**). This initial finding revealed the importance of Cys^{69}- and Tyr^{114}-linked functions of PHB1 in renal biology and provided a hint for a potential link between the two PTM sites, and possibly an additional effect of the Tyr^{114}-linked function on tissue and body weight between sexes in mice.

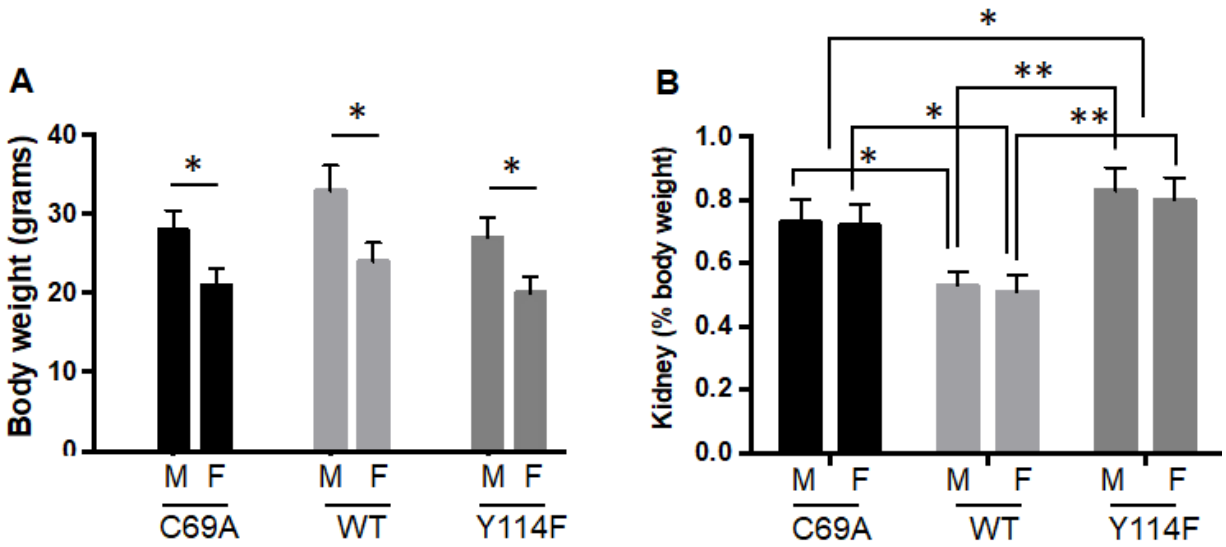
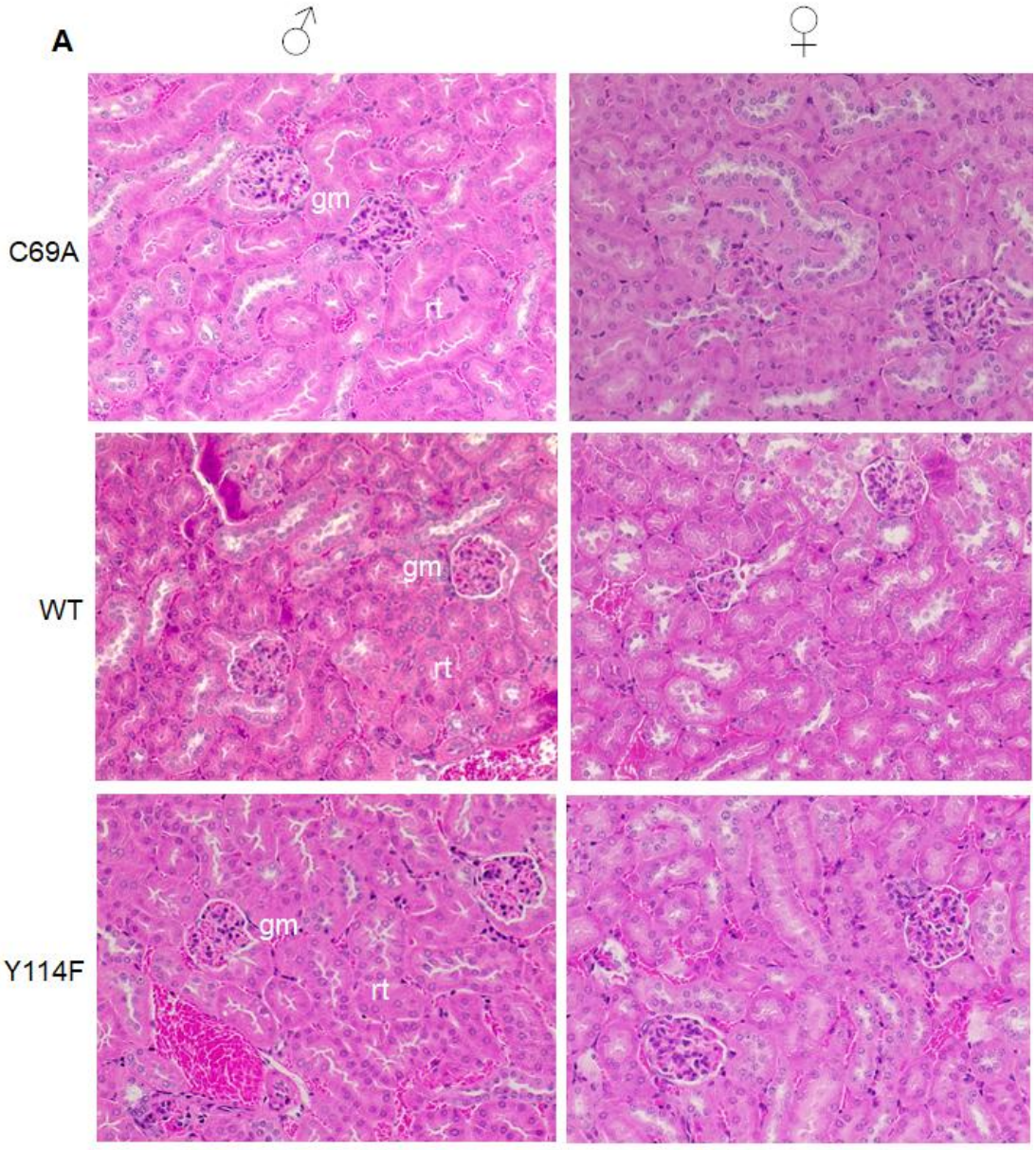


Figure 4.1. The *Phb1-Ki* mice display decreased body weight but increased kidney weight compared with wild-type mice. Histograms showing body and kidney weights of age-matched male and female *Phb1-Ki* mice and wild-type mice. Average whole-body mass (A), and ratio of kidney to body mass (B) are shown by sex and genotype. Data are presented as mean ± SD. (n = 18-20 mice/experimental group). Two-way ANOVA, body weight: $P < 0.05$ for genotype, sex and non-significant for interaction. Kidney weight: $P < 0.05$ for genotype, sex, and non-significant for interaction. % kidney: $P < 0.05$ for genotype and non-significant for sex and interaction. For multiple comparisons, Tukey's post-test was applied. Only significant differences are labelled/shown. M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

4.2 Histological analysis of kidneys from *Phb1-Ki* mice and wild-type mice

To determine whether increase in kidney weights in *Phb1-Ki* mice are due to changes in its cellular composition and characteristics, histological analysis was performed using H & E staining of 5 µm thin kidney sections. In general, a difference in the tubular epithelial cells and glomerular size were noticeable between *Phb1-Ki* mice and wild-type mice, which were relatively larger in *Phb1-Ki* mice in comparison with wild-type mice (**Figure 4.2**). In addition, quantification of hematoxylin stained nuclei revealed an increase in tubular epithelial cells in *Phb1-Ki* mice when compared sex-matched wild-type mice (**Figure 4.2**) indicating their potential contribution in observed differences in their kidney weights. A difference in hematoxylin-stained nuclei number in the tubular epithelial cells was also observed between sexes in wild type and *Phb1-Ki*^{C69A} mice without a significant difference in *Phb1-Ki*^{Y114F} mice (**Figure 4.2**). However, Ki-67 positive cells which is a marker for cell proliferation and apoptotic nuclei were not dtected in all experimental groups (not shown). The quantification of glomerular cells is described in the section 4.5.


A
♂
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C69A
WT
Y114F
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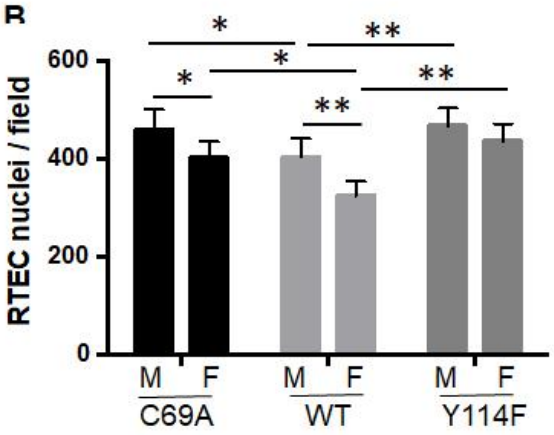
B
RTEC nuclei / field
600
400
200
0
M F
C69A
M F
WT
M F
Y114F
*
**

Figure 4.2. Histological analysis of kidneys from the *Phb1-Ki* mice and wild-type mice. (A) Photomicrographs showing H & E stained kidney sections from age-matched male and female *Phb1-Ki* mice and wild-type mice. (B) Histograms showing quantification of renal tubular nuclei / field (Magnification 40 X). Data are presented as mean ± SD. (n = 6). Two-way ANOVA, RTEC: $P < 0.05$ for genotype, sex and P non-significant for interaction. For multiple comparisons, Tukey's post-test was applied. Only significant differences are labelled/shown. M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type, gl – glomerulus, rt – renal tubule. *$P < 0.05$; **$P < 0.01$.

4.3 Mutant-PHB1 Protein Levels in Renal Tissues from the *Phb1-Ki* Mice

4.3.1: Western Immunoblotting

Mutating key post-translational modification sites in a protein may affect the protein's stability, and consequently, its levels, as well as its intracellular distribution and localization. To determine if this occurs in the mutant PHB1^{C69A} and PHB1^{Y114F}, an immunoblot analysis of the renal lysates was performed using a validated anti-PHB1 antibody (Cell Signaling Technology). No differences in PHB1 levels were observed between sexes and genotypes (**Figure 4.3**), suggesting that mutating the Cys^{69} with an Ala and Tyr^{114} with Phe residue separately does not influence PHB1 protein levels in renal tissues. However, an increasing trend was observed in *Phb1-Ki*^{C69A} mice in comparison with wild-type mice whereas an opposite trend was noticeable in *Phb1-Ki*^{Y114F} mice (**Figure 4.3**). In addition, PHB2 levels were examined, as both proteins form heterodimers and have been reported to influence each other's levels [Ko et al. 2010; Supale et al. 2013]. Again, no difference in PHB2 levels was observed between sexes within genotype and between genotypes (**Figure 4.3**) suggesting that the Cys^{69} and Tyr^{114} PHB1 mutants do not affect PHB2 levels in renal tissues in the *Phb1-Ki* mice.

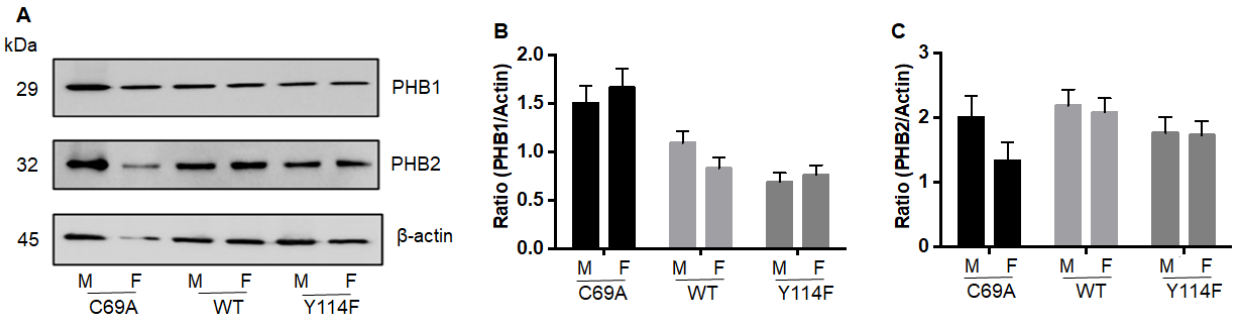

Figure 4.3. PHB1 protein levels in renal tissues from the *Phb1-Ki* mice and wild-type mice. (A) Immunoblots showing PHB1 and PHB2 protein levels in renal tissue samples from age-matched *Phb1-Ki* mice wild-type mice. Beta-actin immunoblot is shown as a loading control. (B & C) Histograms showing quantification of protein bands by densitometry. Data are presented as mean ± SD. (n = 3-4). kDa – kilo Dalton, M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

In addition, PHB1 and PHB2 levels in the mitochondrial fraction of renal tissues were also examined, because both proteins are primarily localized to the mitochondria, where they form heterodimeric mega-complexes [Ikonen et al. 1995; Merkwirth et al. 2012] and high mitochondrial contents in renal tissue [Hoogstraten et al. 2024]. Unexpectedly, a selective and substantial difference in PHB1 levels was observed between sexes and genotypes (**Figure 4.4**).

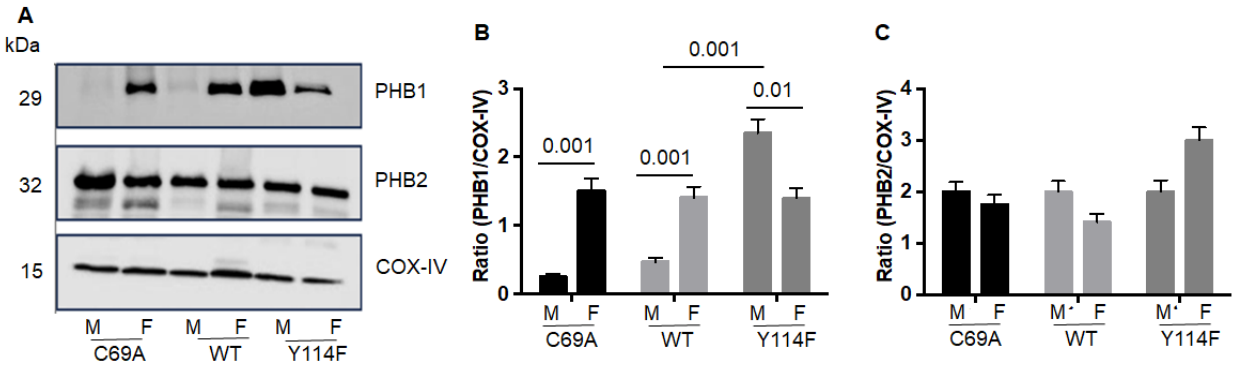

Figure 4.4. (A) Immunoblots showing PHB1 and PHB2 protein levels in the mitochondrial fraction of renal tissues from the *Phb1-Ki* mice and wild-type mice. COX-IV immunoblot is shown as a loading control. (B &C) Histograms showing quantification of protein band densities. Data are presented as mean ± SD. (n = 3). Two-way ANOVA, PHB1: $P < 0.001$ for genotype and sex.

P <0.01for interaction. PHB2: *P* non-significant. For multiple comparisons, Tukey's post-test was applied. Only significant differences are labelled/shown. kDa – kilo Dalton, M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

In the wild-type mice, PHB1 levels were significantly higher in the female mice in comparison with the male mice (**Figure 4.4**). A similar pattern was observed between the female and the male *Phb1-Ki*^{C69A} mice (**Figure 4.4**). In both cases, a weak PHB1 band was observed in the male mice in comparison with their female counterparts (**Figure 4.4**), but not within the mitochondrial marker COX-IV as a loading control (**Figure 4.4**). Interestingly, an opposite trend was observed in the *Phb1-Ki*^{Y114F} mice, wherein PHB1 levels were significantly decreased in the female mice compared with their male counterparts (**Figure 4.4**). No difference in PHB2 levels were found in the female mice across three genotypes (**Figure 4.4**). Together, this finding suggests the importance of the Cys^{69} and Tyr^{114} residues in PHB1 in the regulation of its level in the mitochondria, which largely appears to be independent of PHB2. Moreover, this finding suggests that the loss of the Tyr^{114} site in PHB1 likely alters its mitochondrial localization or antibody-recognizable epitope change in the male mice but not in the female mice, whereas the loss of the Cys^{69} site in PHB1 appears to enhance the level of PHB2 only in the male mice but not in the female mice (**Figure 4.4**).

4.3.2: Immunohistochemical analysis of PHB1 in Renal Histological Sections

To attain a better understanding of PHB1 protein levels in different renal cell types (tubular epithelial and glomerular cells, as per current knowledge in literature on PHB1 in renal biology) [Ising and Brinkoetter, 2017; Ye et al. 2015], an immunohistochemistry was performed using a validated anti-PHB1 antibody (Abcam Canada).

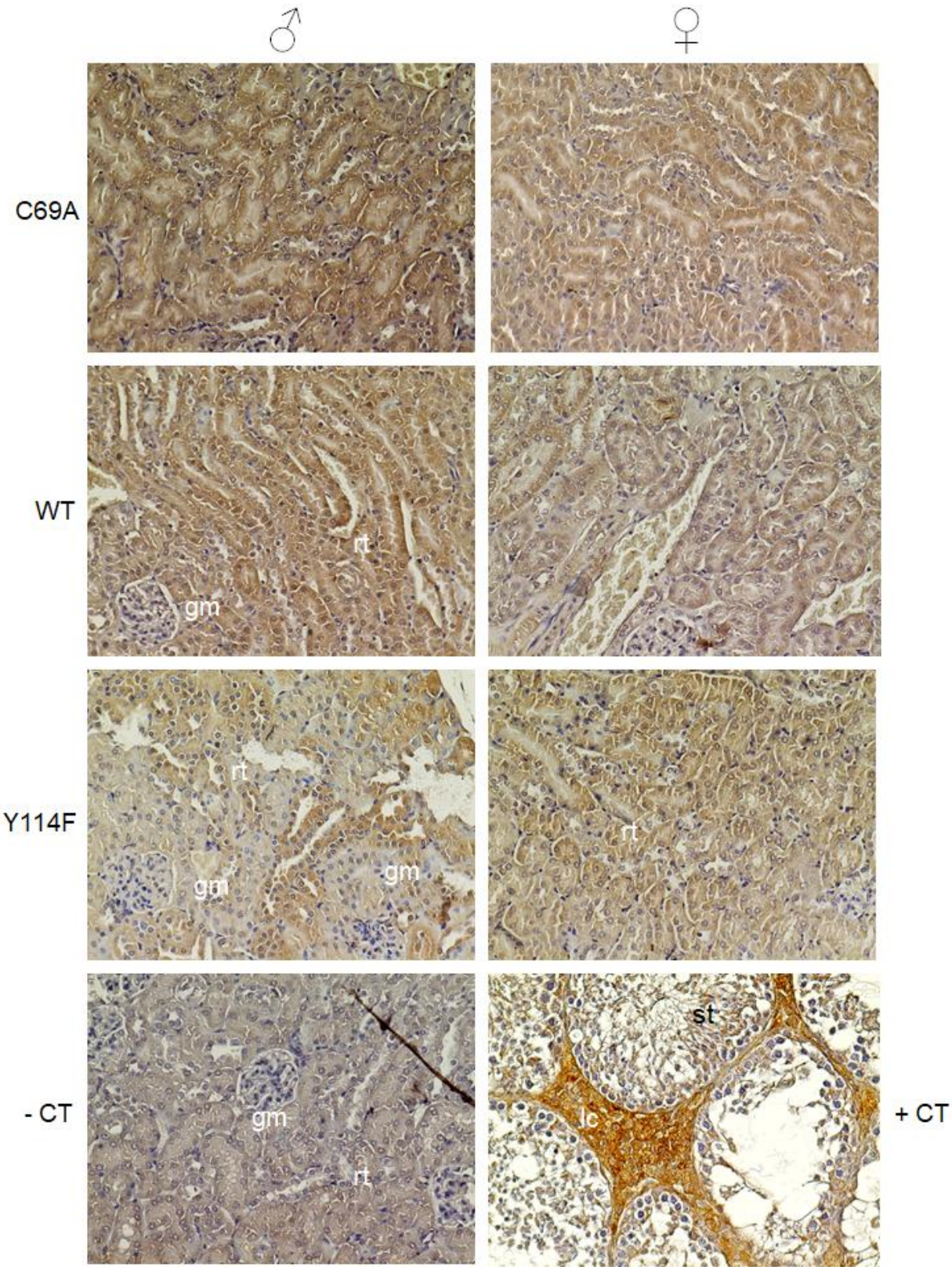
♂
♀
C69A
WT
Y114F
- CT
+ CT
rt
gm
st
lc

Figure 4.5. Immunohistochemical analysis of PHB1 in kidney sections from the *Phb1-Ki* mice and wild-type mice. Negative (omission of primary antibody) and positive (testicular Leydig cells) controls are shown in the lower panel. (n = 3). gl – glomerulus, rt – renal tubule, lc – Leydig cells, st – seminiferous tubule, M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type, - CT – negative control, +CT – positive control.

Table 4.1. A summary of relative PHB1 staining intensity by immunohistochemistry between different experimental groups.

Genotype	Male	Female
Phb1-KiC69A	+++	++
Wild-type	++	+
Phb1-KiY114F	+	+

The intensity of PHB1 staining in the renal tubular cells was distinctly higher than the glomerular cells (**Figure 4.5**). Moreover, a similar PHB1 staining pattern was observed between the female and male wild-type mice and *Phb1-Ki* mice (**Figure 4.5**); however, the staining was found to be more intense in the *Phb1-Ki*^{Y114F} mice (**Figure 4.5, Table 4.1**). This data indicates that the protein levels of the PHB1^{C69A} mutant is not altered in the major renal cell types in *Phb1-Ki* mice when compared with the wild-type mice, which was found to be higher in the *Phb1-Ki*^{Y114F} mice, including in glomerular cells (**Figure 4.5**). In general, the renal tubular cells stained more intensely, matching their high mitochondrial contents compared to the glomerular cells, which were lightly stained (**Figure 4.5**). This data suggests that PHB1 protein levels in tubular epithelial cells and glomerular mesangial cells and podocytes matches with their mitochondrial contents, respectively. However, a difference in PHB1 levels, as observed in the mitochondrial fractions between the male and the female wild-type and *Phb1-Ki*^{C69A} mice, was not observed by immunohistochemistry. However, a relatively intense staining in the *Phb1-Ki*^{Y114F} renal samples (**Figure 4.5, Table 4.1**) correlated with its increased levels observed in the mitochondrial fraction by immunoblotting (**Figure 4.4**).

4.4 Periodic Acid Schiff's (PAS) Staining of Renal Tissues

Next, a PAS staining of renal histological sections was performed, which is used to evaluate basement membrane integrity of the glomerular capillary loops and tubular epithelium [Matsumoto et al. 2021]. In general, an intense PAS staining of the glomeruli was discernable from other parts in the renal samples across genotypes in both sexes (**Figure 4.6**). The capillary loops of the wild-type glomerulus are well-defined and thin. The endothelial cells are seen clearly within the capillary loops. In the male wild-type mice, the glomeruli were compact, and the majority of their component cells (mesangial and podocytes) were darkly stained with hematoxylin counterstaining, whereas in the female mice, a mixture of lightly and darkly stained nuclei were observed (**Figure 4.6**). The glomeruli in the male *Phb1-Ki*^{Y14F} mice displayed significantly increased cell numbers and intercellular space, as well as a mixture of lightly and darkly stained nuclei, as well as a thick basement membrane (**Figure 4.6**), whereas in the female mice, the glomeruli predominantly darkly stained cells, and only a few lightly stained cells were observed (**Figure 4.6**). A difference in Bowman's space was also observed between genotypes, which were relatively wider and more apparent in the male *Phb1-Ki* mice than the female *Phb1-Ki* mice and wild-type mice (**Figure 4.6**). In addition, the glomeruli in the *Phb1-Ki*^{Y114F} mice were found to be larger than the wild-type mice in both sexes (**Figure 4.6**), whereas in the *Phb1-Ki*^{C69A} mice, the glomeruli were intermediate in size between the wild-type and *Phb1-Ki*^{Y114F} mice. Notably, signs of immune cell infiltration were observed in the *Phb1-Ki*^{Y114F} mice (**Figure 4.6**), which was not observed in the wild-type mice.

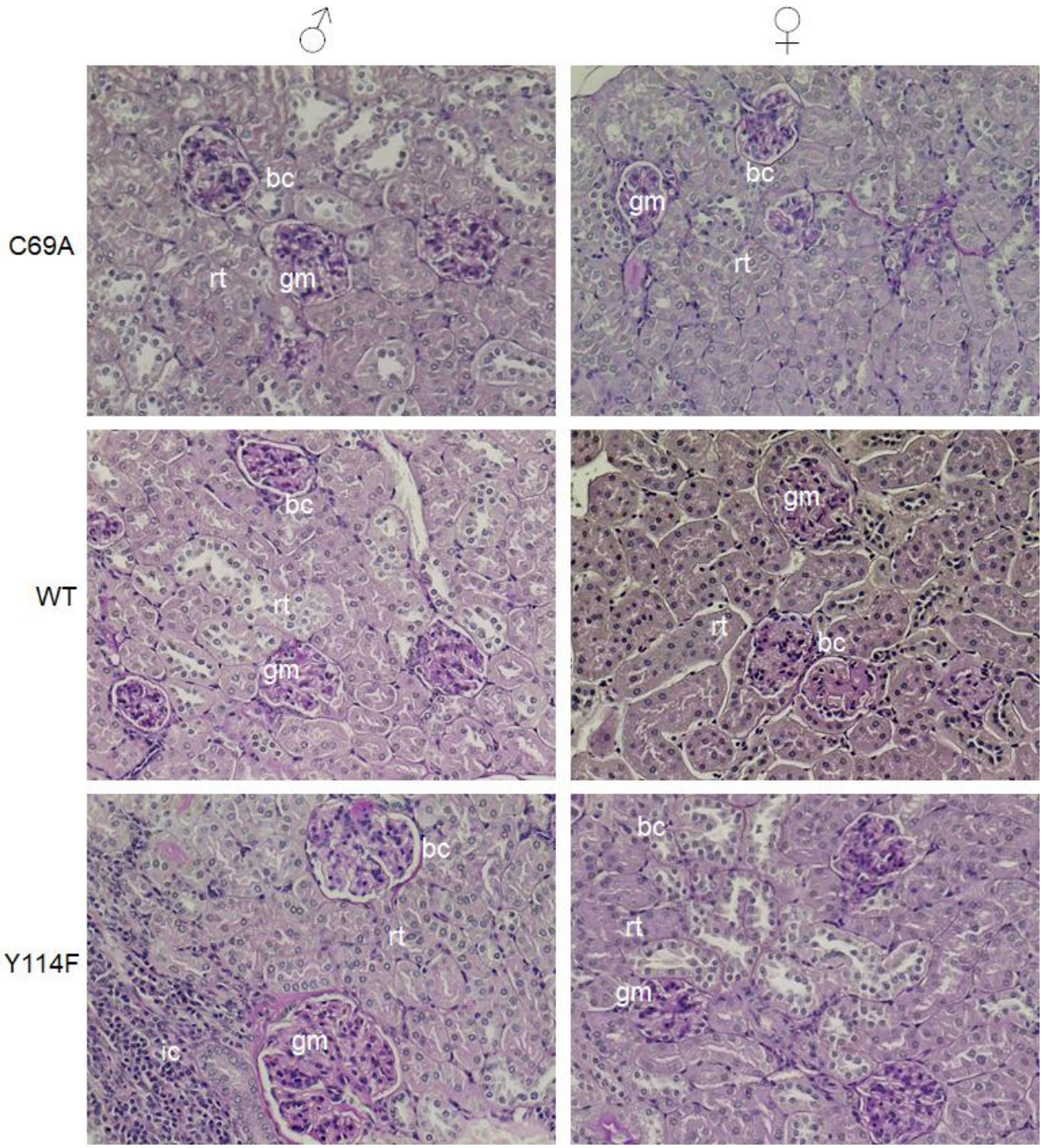

Figure 4.6. Representative photomicrographs showing periodic acid Schiff's (PAS) staining of renal sections from the *Phb1-Ki* mice and wild-type mice. (n = 3). gl – glomerulus, bc, Bowman's capsule, ic – immune cells, rt – renal tubule, M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

4.5 Quantification of glomerular cells and podocytes confirmation by IHC

Next, the quantification of podocytes was performed, which showed sex differences in their numbers between genotypes, which was higher in the male and female *Phb1-Ki* mice when compared with the respective wild-type mice (**Figure 4.7**). To further confirm the identity of glomerular cell types undergoing sex-specific changes in *Phb1-Ki*, an immunohistochemistry was performed using an anti-podocin antibody (a podocyte-specific marker), which confirmed that the cells with darkly stained nuclei were indeed podocytes (**Figure 4.7**).

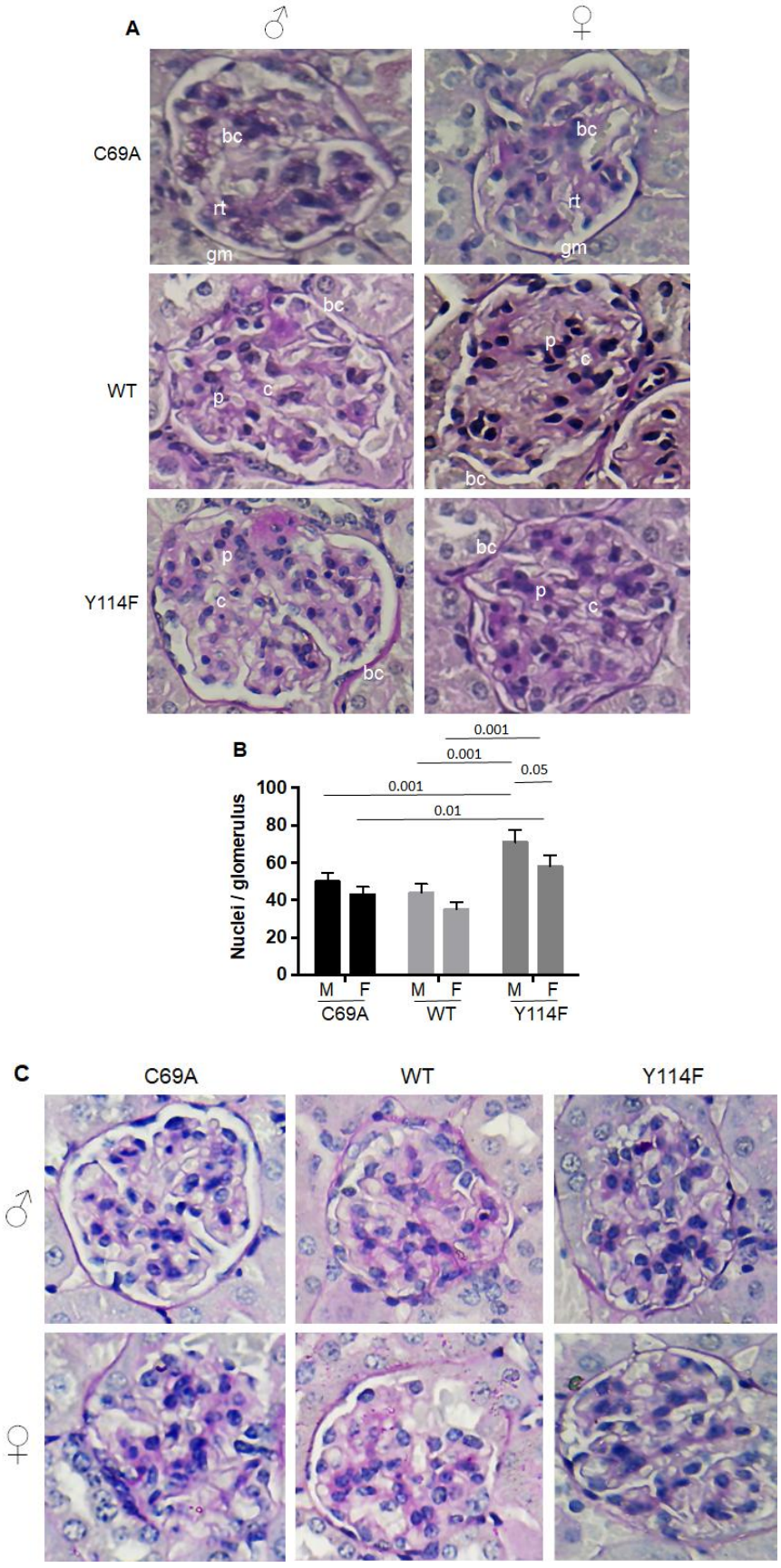
A
♂
♀
C69A
WT
Y114F
bc
rt
gm
p
c
B
Nuclei / glomerulus
100
80
60
40
20
0
0.001
0.001
0.05
0.001
0.01
M F
C69A
M F
WT
M F
Y114F
C
C69A
WT
Y114F
♂
♀

Figure 4.7. (A) Representative photomicrographs of PAS stained glomeruli. (B) Histograms showing quantification of glomerular cells. Data are presented as mean ± SD. Two-way ANOVA, glomerular cells: $P < 0.05$ for genotype and sex. P non-significant for interaction. For multiple comparisons, Tukey's post-test was applied. Only significant differences are labelled/shown. (C) Photomicrographs showing podocytes as determined by anti-podocin specific antibody. (n = 6). M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

4.6 Markers of Mitochondrial Dynamics are Altered Renal Tissues from the *Phb1-Ki* Mice

Renal tubular cells are enriched in their mitochondrial contents, and maintenance of mitochondrial homeostasis requires a balance between mitochondrial biogenesis, fission, fusion, and mitophagy [Bhargava and Schnellmann, 2017; Mishra and Singh, 2023]. PHB1 has been implicated in these processes [Ande et al. 2016]. Moreover, mitochondrial homeostasis requires a balance between mitochondrial dynamics and mitochondrial energetics that ensures the maintenance of properly functioning mitochondria [Bhargava and Schnellmann, 2017; Hoogstraten et al. 2024] – an important consideration in relation to the role of PHB1 in mitochondrial biology and mitochondria in renal biology. Therefore, renal samples from the *Phb1-Ki* mice were examined for the markers of mitochondrial fusion (OPA1) and fission (DRP1) using western immunoblotting. No differences in OPA1 and DRP1 levels were found between male and female wild-type mice (**Figure 4.8**).

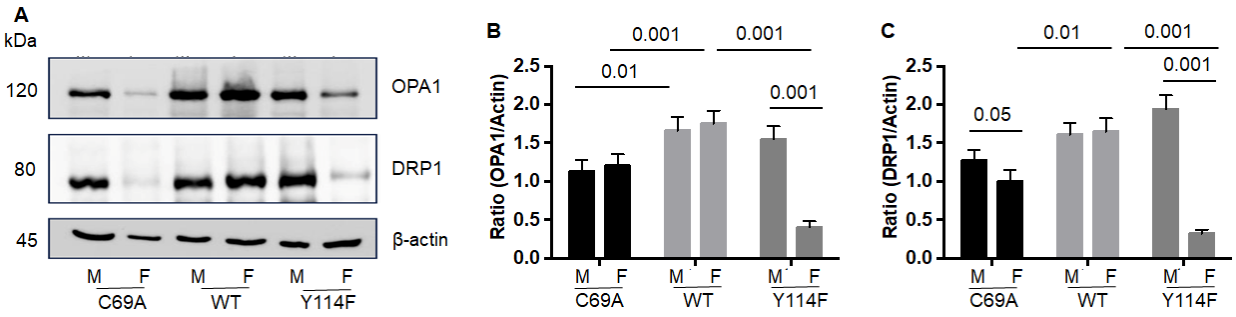

Figure 4.8. Analysis of the Markers of Mitochondrial Dynamics in Renal Tissues from the *Phb1-Ki* Mice. (A) Immunoblots showing protein levels of markers of mitochondrial dynamics in renal samples from *Phb1-Ki* and wild-type mice. Beta-acting immunoblot is shown as a loading control. (B & C) Histograms showing quantification of protein bands by densitometry. Data are

presented as mean ± SD. (n = 3). Two-way ANOVA, OPA1 & DRP1: $P < 0.05$ for genotype, sex and $P < 0.01$for interaction. For multiple comparisons, Tukey's post-test was applied. Only significant differences are labelled/shown. kDa – kilo Dalton, M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

In the *Phb1-Ki* mice, OPA1 and DRP1 levels were significantly higher in males than females mice. (**Figure 4.8**). Moreover, the expression levels of both proteins significantly decreased in the female *Phb1-Ki* mice when compared with the respective male *Phb1-Ki* mice (**Figure 4.8**). A similar trend was found in DRP1 levels between the female and the male wild-type mice (**Figure 4.8**); whereas in the *Phb1-Ki* mice, DRP1 levels substantially decreased in the female mice but not in the male mice (**Figure 4.8**). In addition, DRP1 levels were found to be increased in the male *Phb1-Ki*^{Y114F} mice compared with the male wild-type and *Phb1-Ki*^{C69A} mice (**Figure 4.8**). Together, this data suggests that the markers of mitochondrial dynamics are differently altered in the female and the male *Phb1-Ki* mice, suggesting the importance of the Cys^{69} and Tyr^{114}-linked function in their regulation in the male and the female mice.

4.7 IGF1-R-mTOR Signaling is Altered in Renal Tissues from the *Phb1-Ki* Mice

The mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) signaling pathways are known to play roles in various aspects of renal biology, and their dysregulation has been reported in renal pathophysiology [Bhargava and Schnellmann, 2017], including the presence of mitochondrial abnormalities in kidney diseases [Hoogstraten et al. 2024]. Of note, PHB1 has been reported to modulate the activation level of Akt, mTOR and AMPK in various cell and tissue types [Mishra et al. 2010; Ande et al. 2016], including renal tissues. For example, the loss of prohibitin-1/2 (PHB1/2) in podocytes results in a disturbed mitochondrial structure and an increase in insulin/IGF-1 signaling, leading to mTOR activation [Ising and Brinkkoetter, 2017]. The role of PHB in mTOR signaling has also been reported in renal tubular epithelial cells. Moreover, the palmitoylation of PHB1 at the Cys^{69} site facilitates its plasma membrane localization and subsequent phosphorylation at the Tyr^{114} residue, which in turn negatively modulates insulin and the insulin-like growth factor and immune cell signaling [Ande

and Mishra, 2010; Ande et al. 2009]. Moreover, IGF1-R levels have been reported to be dysregulated in renal tissue from the podocyte-specific knockout mouse model of PHB2 [Ising et al. 2015]. Therefore, the expression level of IGF1-R was examined first (an upstream signaling molecule in the Akt and mTOR pathway). In the wild-type mice, IGF1-R band intensity was faint in the male mice, whereas a relatively better band density was observed in the female mice (**Figure 4.9**). Interestingly, IGFI-R levels substantially increased in the male *Phb1-Ki* mice when compared with the male wild-type mice (**Figure 4.9**).

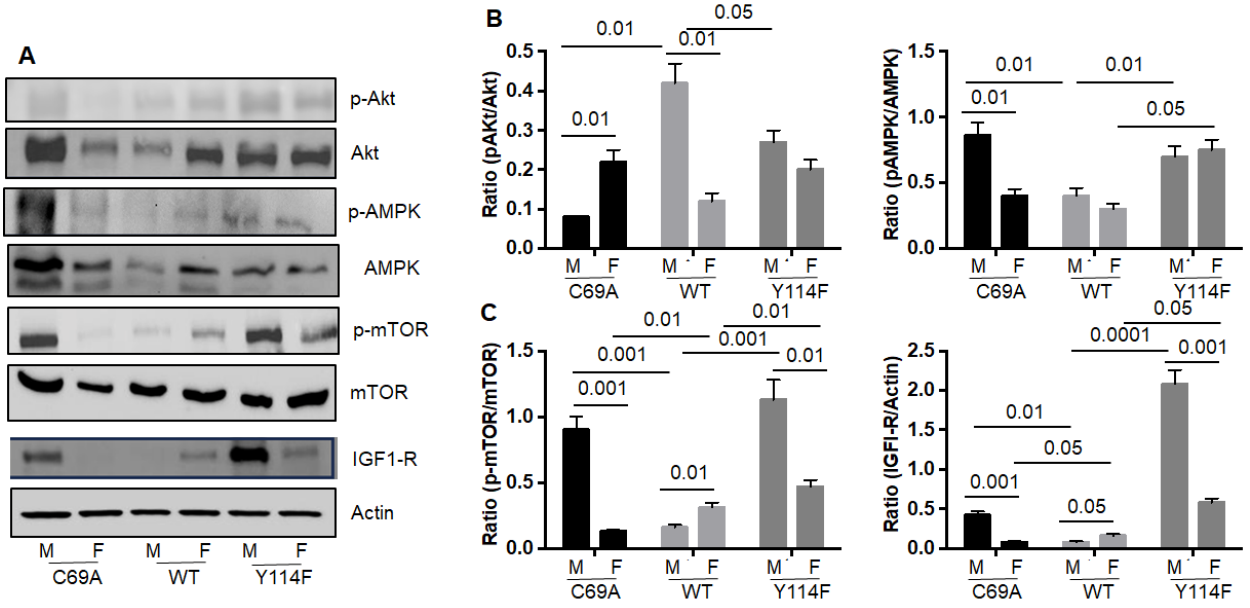

Figure 4.9. IGF1-R-mTOR signaling is altered in renal tissues from the *Phb1-Ki* Mice. (A) Immunoblots showing phosphorylation levels of different cell signaling intermediates in renal lysates from male and female *Phb1-Ki* mice and wild-type mice. (B & C) Histograms showing quantification of protein bands by densitometry. Data are presented as mean ± SD. (n = 3). Two-way ANOVA, pAkt: $P < 0.05$ for genotype, $P < 0.01$ for sex, and $P < 0.01$ for interaction. AMPK: $P < 0.05 – 0.01$ for genotype and sex, and not significant for interaction. p-mTOR $P < 0.001$ for genotype and sex, $P < 0.01$ for interaction. IGFI-R: $P < 0.05$ for genotype, $P < 0.01$ for sex, and $P < 0.01$ for interaction. For multiple comparisons, Tukey's post-test was applied. Only significant differences are labelled/shown. M – male, F – female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

In addition, a difference in IGF1-R level between the male *Phb1-Ki*^{C69A} mice and *Phb1-Ki*^{Y114F} mice was apparent, which was substantially higher in the male *Phb1-Ki*^{Y114F} mice (**Figure 4.9**). In the female *Phb1-Ki* mice, IGFI-R band intensity was faint in the *Phb1-Ki*^{C69A} mice, whereas a relatively better band intensity was observed in the *Phb1-Ki*^{Y114F} mice (**Figure 4.9**). Together, this evidence suggests a sexually dimorphic effect of PHB1 mutants on IGF1-R levels in renal tissue. This finding prompted to examine its downstream signaling intermediates. A similar p-mTOR band pattern was observed between sexes and genotypes matching IGFI-R levels (**Figure 4.9**) indicating a potential link between the two. Next, the activation levels of Akt and AMPK in renal tissues were examined using their phosphorylation site-specific antibodies. In general, the p-Akt band intensity was weak but statistically significant between genotype and sexes (wild-type and *Phb1-Ki*^{C69A} mice) but not in *Phb1-Ki*^{Y114F} mice (**Figure 4.9**), However, a moderate increase in p-Akt level in the male *Phb1-Ki*^{Y14F} mice was noticeable when compared with other genotypes (**Figure 4.9**), which is consistent with previous reports on the role of Tyr^{114} phosphorylation in the regulation of p-Akt negatively [Ande et al. 2009]. The p-AMPK level showed a similar pattern in the female wild-type mice and female *Phb1-Ki*^{C69A} mice, whereas it was moderately upregulated in the male and female *Phb1-Ki*^{Y114F} mice and in the male *Phb1-Ki*^{C69A} mice (**Figure 4.9**). Thus, the relative importance of p-Akt and p-AMPK appears to be less than the p-mTOR in the *Phb1-Ki* mice. Together, this data suggests that PHB1^{C69A} and PHB1^{Y114F} mutants have a similar sex-specific effect on IGF1-R-mTOR signaling; however, the effect was more pronounced in the latter than the former models.

4.8 TEM Analysis Revealed Impaired Mitochondrial Features in the RTEC of *Phb1-Ki* Mice

Mitochondria plays a critical role in supporting renal physiology, and impaired mitochondrial structure and function are often an underlying factor in common renal diseases [Bhargava and Schnellmann, 2017], including diabetic nephropathy and chronic kidney disease, whereas PHB1 and PHB2 have an important role in various aspects of mitochondrial biology [Merkwirth et al. 2012; Ko et al. 2013; Supale et al. 2013]. Therefore, mitochondrial phenotypes in renal tubular epithelial *Phb1-Ki* mice was explored because of their vital role in this cell type, and the well-known role of PHB1 in mitochondrial biology at large. Moreover, the glomerular phenotype in *Phb1-Ki* mice was also examined because of the known role of the PHB domain

protein podocin and PHB1 in podocyte biology [Ising and Brinkkoetter, 2017; Völker et al. 2013]. The TEM analysis revealed a substantial change in the mitochondrial features of tubular epithelial cells in the *Phb1-Ki* mice when compared with wild-type mice. This includes a decrease in mitochondrial number, shape alterations, cristae damages and reduced cristae compactness in both *Phb1-Ki* mice in comparison with the wild-type mice (**Figure 4.10**). These changes in mitochondrial features were more pronounced (between sexes) in the male *Phb1-Ki* mice than their female counterparts (**Figure 4.10**) and between genotypes in the *Phb1-Ki*^{Y114F} mice than the *Phb1-Ki*^{C69A} mice (**Figure 4.10**). In addition, lipid droplets were observed in tubular cells from the male *Phb1-Ki*^{C69A} mice, whereas autophagic and mitophagic vacuoles were more common in the female *Phb1-Ki*^{C69A} mice (**Figure 4.10**). In the tubular cells of the male *Phb1-Ki*^{Y114F} mice, an accumulation of damaged mitochondria were commonly observed, which was not observed in any other experimental groups (**Figure 4.10**). The mitochondrial characteristics of the female *Phb1-Ki*^{Y114F} mice were very similar to the female *Phb1-Ki*^{C69A} mice (**Figures 4.10 & 4.11**).

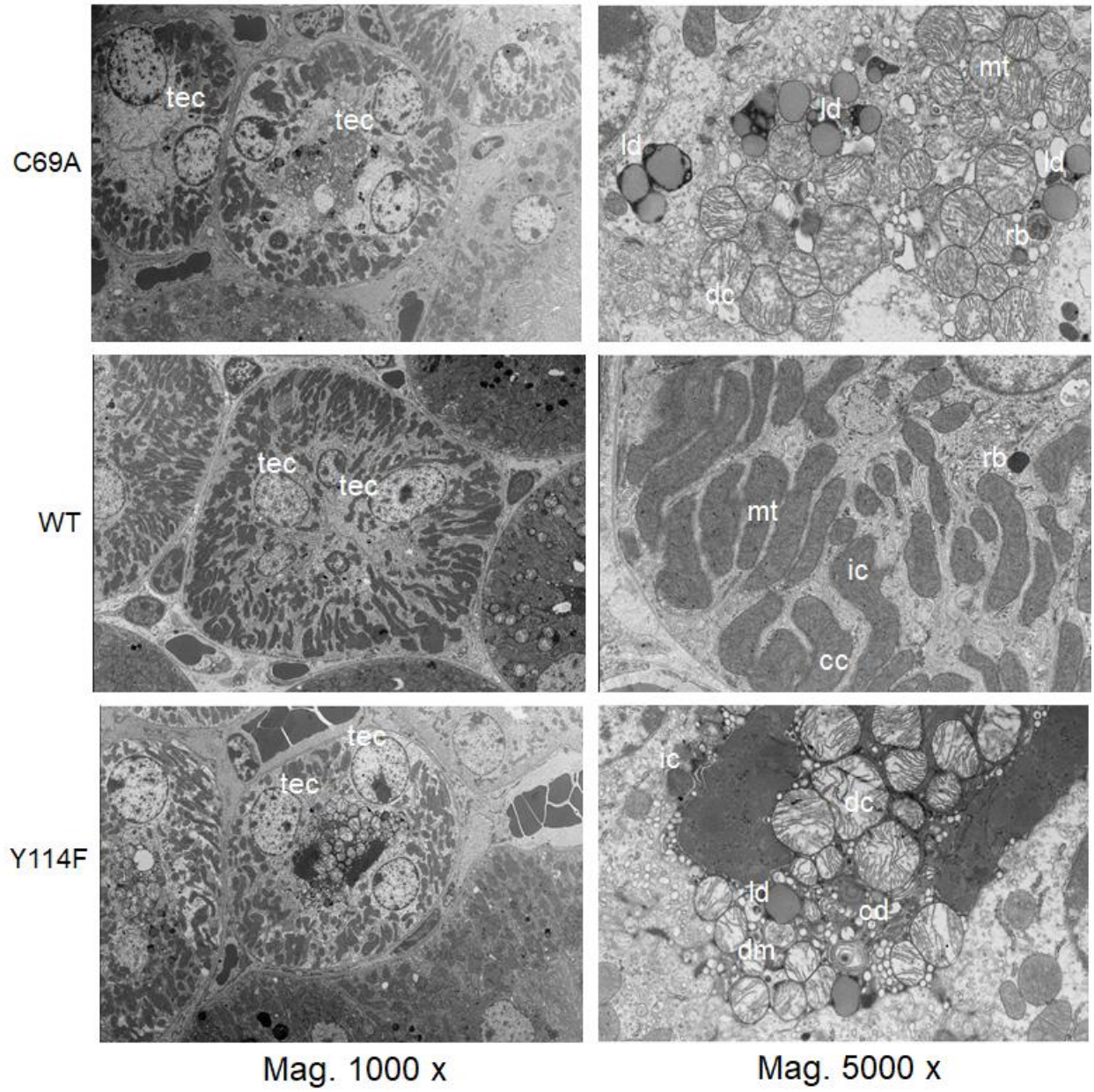

Figure 4.10. TEM analysis of the RTEC from the male *Phb1-Ki* mice and wild-type mice. (n = 5). C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type, tec – tubular epithelial cells, ld – lipid droplets, mt – mitochondria, rb – residual bodies, cc – compact cristae, dc – damaged cristae.

Together, this data suggests that PHB1 plays a role in sex-related differences in renal tubular cell mitochondria. This finding also indicates an interrelated and independent role of Cys^{69}- and Tyr^{114}-

linked functions in renal tubular cell mitochondria, because of the similarities and differences between the two *Phb1-Ki* genotypes, which differently manifested in the female and the male *Phb-Ki* mice.

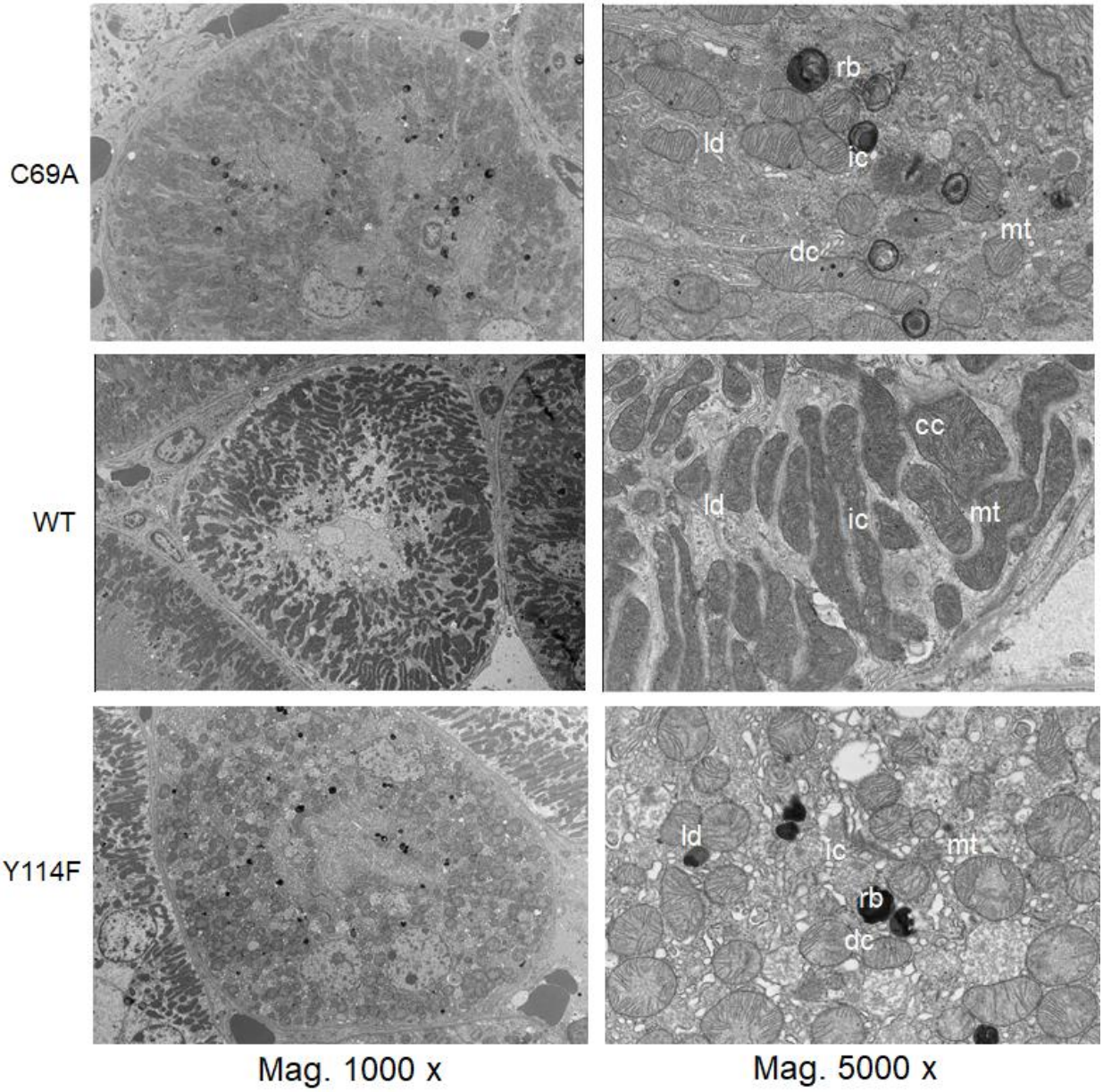

Figure 4.11. TEM analysis of the RTEC from the female *Phb1-Ki* mice and wild-type mice. (n = 5). C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type, tec – tubular epithelial cells, ld – lipid droplets, mt – mitochondria, residual bodies, cc – compact cristae, damaged cristae.

4.9 TEM Analysis Revealed Impaired Glomerular Structure in the *Phb1-Ki* Mice

Studies have shown that PHB1 and PHB2 play a role in glomerular biology, where they confer an extra-mitochondrial function in podocytes as they localize to the slit diaphragm and thereby stabilize the unique intercellular contact between podocytes required to maintain an effective filtration barrier [Ising et al. 2016]. Moreover, podocyte-specific PHB2 knockout mice develop proteinuria and glomerulosclerosis, and eventually exhibit a total loss of renal function [Ising and Brinkkoetter, 2017]. Therefore, within the scope of this thesis, glomeruli from the *Phb1-Ki* mice were also examined using transmission electron microscopy. In general, a negative impact on the glomerular filtration bed between the endothelial cell and podocytes, as well as the intercellular contact between podocytes, were observed in the *Phb1-Ki* mice when compared with the wild-type mice, which were more pronounced in the male *Phb1-Ki* mice than the female *Phb1-Ki* mice (**Figures 4.12 & 4.13**).

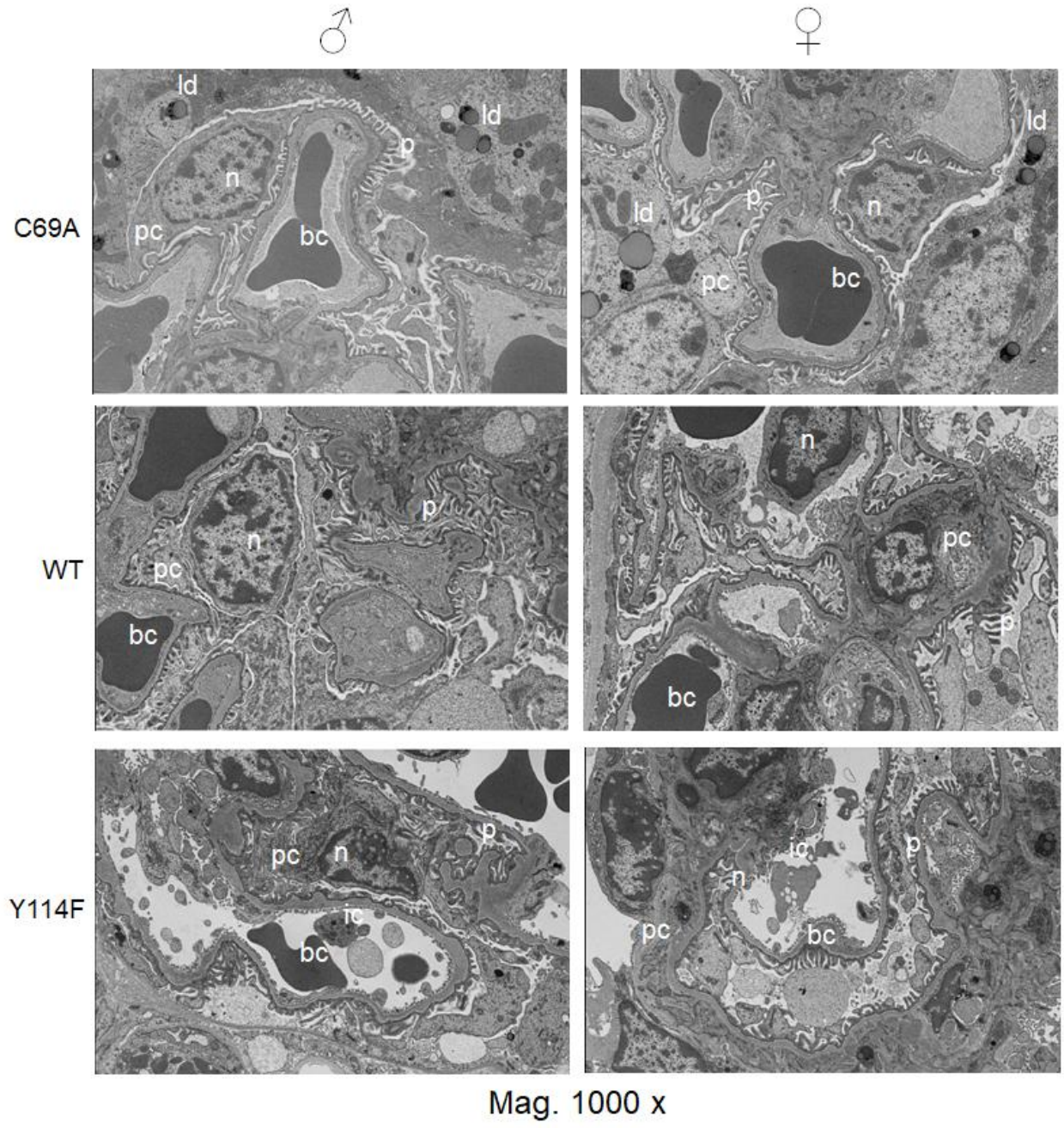

Figure 4.12. TEM analysis of glomerulus from the male and female *Phb1-Ki* mice and wild-type mice. (n = 5). C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type, bc – blood capillary, ld – lipid droplets, n– nucleus, pc – podocyte, p - podia.

Like tubular epithelial cells, lipid droplets were also observed in mesangial cells and podocytes in the *Phb1-Ki*^{C69A} mice, whereas signs of immune cell infiltration were more apparent in the *Phb1-Ki*^{Y114F} mice (**Figures 4.12 & 4.13**). Moreover, in *Phb1-Ki*^{C69A} mice but not in *Phb1-Ki*^{Y114F}mice blood capillaries were found to be dilated, and endothelial processes involved in inter-digitation with podocytes were less developed in the *Phb1-Ki* mice when compared with the wild-type mice (**Figures 4.12 & 4.13**). In summary, the manifestation of cellular dysregulation in endothelial cells, mesangial cells, and podocytes were more pronounced in the male mice than the female mice in both *Phb1-Ki* mice, suggesting the potential role of PHB1's Cys^{69}- and Tyr^{114}-linked functions in sex-related differences in glomerular biology.

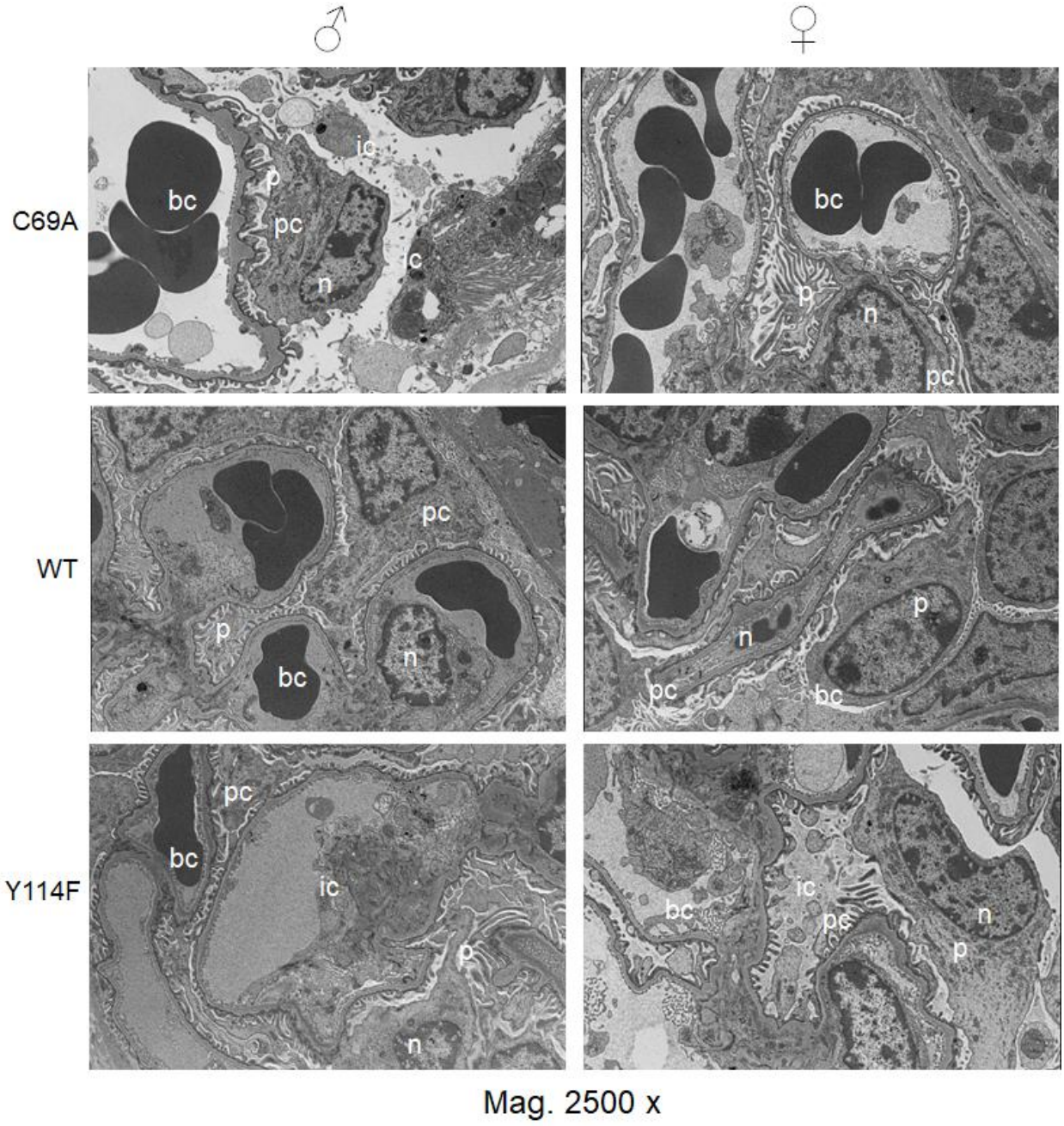

Figure 4.13. TEM analysis of glomerulus from the male and female *Phb1-Ki* mice and wild-type mice. (n = 5). C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type, bc – blood capillary, ld – lipid droplets, n– nucleus, pc – podocyte, p - podia.

4.10 *Phb1-Ki* Mice Display Sex Differences in Ubiquitin Proteasome Activity

Mutations in genes encoding enzymes of the ubiquitin proteasome system result in renal disease, and mutations in kidney-specific genes result in ubiquitin–proteasome dysfunction that leads to renal disease [Meyer-Schwesinger, 2019; Jenkins et al. 2020], suggesting that the ubiquitin proteasome system plays an important role in kidney biology and pathobiology. Moreover, the ubiquitin proteasome system is an important component of autophagy and mitophagy, and growing evidence suggests that PHB1 and PHB2 play a critical role in mitophagy [Meyer-Schwesinger, 2019; Xu et al. 2019]. These evidences, along with an increase in autophagic and mitophagic vacuoles and the accumulation of damaged mitochondria in tubular epithelial cells from the *Phb1-Ki* mice, as observed in TEM analysis, provided an impetus to examine the ubiquitin proteasome activity levels in the renal samples from the *Phb1-Ki* mice. An analysis of renal tissue lysates by western immunoblotting using an anti-polyubiquitin antibody revealed an apparent sex difference in poly-ubiquitinated proteins, which was substantially higher in the female wild-type mice than the male mice (**Figure 4.14**).

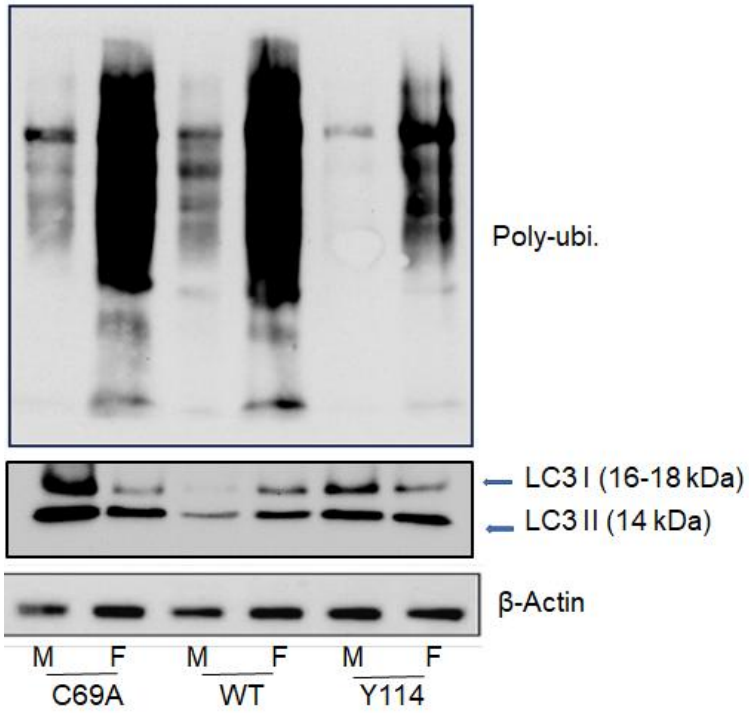

Figure 4.14. *Phb1-Ki* mice display sex differences in ubiquitin proteasome activity and mitophagy marker LC3 I/II. Beta-actin blot is shown as a loading control. M – male, F -female, C69A – *Phb1-Ki*^{C69A}, Y114F – *Phb1-Ki*^{Y114F}, WT – wild-type.

Interestingly, the levels of poly-ubiquitinated proteins were substantially reduced in the female and the male *Phb1-Ki*^{Y114F} mice when compared with the wild-type mice, respectively (**Figure 4.14**). Such a decrease in poly-ubiquitinated proteins were not observed in the female and the male *Phb1-Ki*^{C69A} mice when compared with the wild-type mice (**Figure 4.14**). Moreover, a difference was observed in poly-ubiquitinated protein levels between the *Phb1-Ki*^{C69A} and *Phb-Ki*^{Y114} mice, which was higher in the former and weaker in the latter (**Figure 4.14**). Together, this data suggests that the activity of the ubiquitin proteasome system is differently altered in renal samples from the female and the male *Phb1-Ki*^{C69A} and *Phb1-Ki*^{Y114F} mice, which inversely correlated by the accumulation of damaged mitochondria in the *Phb1-Ki*^{Y114F} mice, as observed in the analysis of renal tissues by electron microscopy (**Figure 4.10**), directly correlated with the increased IGF1-R-mTOR levels present in the *Phb-Ki*^{Y114F} mice (**Figure 4.9**).

4.11 Analysis of Autophagy & Mitophagy Markers in Renal Tissues from the *Phb1-Ki* Mice

Sex-related differences in autophagic and mitophagic vacuoles, as observed by electron microscopy and in poly-ubiquitinated protein levels by immunoblotting in the renal samples, prompted me to examine the activation status of autophagy and mitophagy markers in them. Analysis of renal lysates from different genotypes by immunoblotting showed a clear sex differences in the cleavage of LC3 I band in the wild-type mice, which was significantly higher in the female mice than in the male mice (**Figure 4.14**). Interestingly, an opposite trend was observed in the *Phb1-Ki* mice (**Figure 4.14**). An increased accumulation of damaged mitochondria in the *Phb1-Ki*^{Y114F} mice despite higher LC3 I cleavage activity would mean blockage in the mitophagy pathway downstream of LC3 I/II. Collectively, this finding suggests that PHB1 plays a role in sex-related differences in the regulation of ubiquitin proteasome activity, and in the autophagy and mitophagy in renal tissues of the mice, and revealed the importance of the PHB1 Cys^{69} and Tyr^{114}-linked function in these processes.

CHAPTER V

Discussion

This research aimed to investigate the previously unknown role of PHB1 in sex-related differences in renal biology that was observed during the phenotypic characterization of the *Phb1-Ki* mouse models in our laboratory. In this thesis, the research findings showed that the *Phb1-Ki* mice display an increase in their renal mass (% of body weight). In brief, the *Phb1*^{Y114F} mice showed a significantly larger renal mass than the *Phb1*^{C69A} mice and wild-type mice. Further analysis of their renal tissues showed structural differences in the renal tubules and glomeruli. Particularly, the dysregulation of the mitochondrial structure and accumulation of damaged mitochondria in renal tubular epithelial cells, which were more pronounced in the male *Phb1-Ki*^{Y114F} mice. In addition, the infiltration of immune cells was observed in the renal tissue from the male *Phb1*-Ki^{Y114F} mice. Moreover, lipid droplets were frequently encountered in renal tubular epithelial cells from the *Phb1-Ki*^{C69A} mice, but not in the *Phb1-Ki*^{Y114F} and the wild-type mice. These cellular changes in renal tissues from the *Phb1-Ki* mice may have contributed to their relatively increased mass compared to the respective wild-type control mice. Notably, sex-related differences in the proteasome system were apparent between the wild-type male and female mice, which was differently affected in renal tissues from the *Phb1-Ki* mice and inversely correlated with the accumulation of damaged mitochondria in renal tubular epithelial cells. In addition, sex-related differences in IGF1-R-mTOR singling and in the levels of mitochondrial dynamics and mitophagy markers were found in the *Phb1-Ki* mice, which were differently altered in the *Phb1-Ki*^{C69A} mice and *Phb1-Ki*^{Y114F} mice. Collectively, these research findings suggest that PHB1 plays a role in renal biology, including in the renal tubular and the glomerular compartments, which are consistent findings with emerging evidence in literature [Schurek et al. 2014; Zhou et al. 2012; Zhou et al. 2013; Ising and Brinkkoetter, 2017]. Particularly, unraveling the importance of Cys^{69} and Tyr^{114}-linked function of PHB1 in sex-related differences in renal biology.

The last two decades have seen a growing recognition and emphasis for advancing our knowledge of biological sex-related differences in health and disease. Historically, our poor understanding in this field appears to be largely due to the lopsided consideration of the male sex in experimental and study design, including in the fields of renal biology and pathobiology. One reason for this lack of knowledge is that most basic science studies are conducted using male

kidneys. Indeed, in three leading journals that publish research on kidney physiology and pathophysiology, male to female ratios in basic studies are 5:1 (American Journal of Physiology - Renal Physiology), 11:1 (Kidney International) and 16:1 (Journal of the American Society of Nephrology) [Sandberg et al. 2017]. Emerging evidence suggests that sex-related differences are prevalent in many aspects of renal physiology and pathophysiology [Bairey Merz, 2019]. However, our knowledge of various factors that mediate sex-related differences remains poor. Consequently, our understanding of the underlying mechanisms involved in these processes remains limited. The research findings presented herein provide new insight in sex-related differences in renal biology, especially the role of a novel player, PHB1, in it, which needs further investigations. Strong evidence exists in current literature suggesting that PHB1 has both mitochondrial and extra-mitochondrial membrane-signaling functions [Ande et al. 2014, Ande et al. 2016, Kim et al. 2013, Qu et al. 2020]. However, a major challenge in discerning the cell compartment-specific function of PHB1 is our limited understanding of PHB1 biology to selectively target its cell compartment-specific functions and limitations associated with the knockout approach in this context. Moreover, tissue-specific knockout mouse models of PHB1 at the systemic level have not proven helpful in this context because of a severe mitochondrial phenotype, rapid deterioration in body physiology postnatally, and premature death [Ko et al. 2010; Merkwirth et al. 2012; Supale et al. 2013].

Based on current literature regarding the link between the Cys^{69} and Tyr^{114} sites in the membrane-signaling function of PHB1 [Ande et al. 2017], the effects of mutant $PHB1^{C69A}$ and $PHB1^{Y114F}$ should be similar. However, substantial similarities (e.g., mTOR and AMPK signaling) and differences (e.g., accumulation of lipid droplets and damaged mitochondria) between the *Phb1-Ki*^{C69A} and *Phb1-Ki*^{Y114F} mice in their renal phenotypes indicate that both sites likely have cell type-specific interconnected and independent functions. This is logical in the context of the evolutionary acquisition of both sites in vertebrates and invertebrates [**Figure 2.1**] as described in the study rationale (Chapter II).

The discrepancies between PHB1 and PHB2 levels in the mitochondrial fraction from renal tissues, as observed by immunoblotting between the male and female wild-type mice and the *Phb1-Ki*^{C69A} mice, is difficult to explain in the context of current information in literature about hetero-dimerization and the interdependence of both proteins in the mitochondrial biology [Merkwirth et

al. 2012; Supale et al. 2013; Mishra, 2019]. However, such differences between sexes in wild-type mice and the *Phb1-Ki*^{C69A} mice was not observed via immunohistochemistry. This may be due to differences in antigen epitopes used to raise the PHB1 antibodies used in this study, which may be altered in the PHB1^{C69A} mutant form. However, a lack of such differences in total cell lysates by immunoblotting using the same antibody (as in the analysis of the mitochondrial fraction), but in the mitochondrial fraction of the wild-type mice by immunoblotting, would indicate a mitochondria-specific change in PHB1 and PHB1^{C69A}. This notion may also apply to an inverse sex-related difference in PHB1^{Y114F} mutant levels in the male and the female *Phb1-Ki*^{Y114F} mice, as observed in the mitochondrial fraction. Further analysis of mitochondrial PHB1 using tandem mass spectrometry is required to better define the importance of both posttranslational modification sites in sex-related differences in mitochondrial biology in the kidneys.

Moreover, similar sex-specific changes in IGF1-R and p-mTOR levels in the *Phb1-Ki*^{C69A} and *Phb1-Ki*^{Y114F} mice are consistent with the link between these two post-translational modification sites in the role of PHB1 in its membrane-signaling function. However, a significant difference in the amplitude of the effect of PHB1^{C69A} and PHB1^{Y114F} mutants on IGFI-R levels would imply an additional effect of the latter, independent of the Cys^{69}-linked function. This notion also applies to biological sex-related differences in mitochondrial characteristics, as observed between the *Phb1-Ki*^{C69A} and *Phb1-Ki*^{Y114F} mice by electron microscopy, as well as in ubiquitin proteasome activity by immunoblotting. As the ubiquitin-proteasome cascade affects nearly all cellular processes and displays sex-related differences in many tissue types, their differential regulation in the male and the female *Phb1-Ki* mice has opened an exciting prospect in this field, which warrants further investigations. Of note, PHB1 and PHB2 are known to interact with ubiquitin ligase in relation to mitophagy [Wei et al. 2017]; however, whether the post-translational modification of PHB1 / PHB2 play a role in this interaction is currently unknown. In addition, PHB2 has been shown to be a dysregulated protein in hyperglycemia-induced disruption in mitochondrial homeostasis, which involve its ubiquitination and proteasomal degradation [Liu et al. 2014]. Thus, it is possible that dysregulation of PHB1 and PHB2 may influence proteasome activity and ubiquitination of target proteins. In conclusion, the similarities and differences in renal phenotypes observed between the *Phb1-Ki*^{C69A} and *Phb1-Ki*^{Y114F} mice is consistent with the initial hypothesis that (i) the Cys^{69}- and Tyr^{114}-linked function of PHB1 plays a role in sex-related differences and (ii) the Tyr^{114} phosphorylation site has Cys^{69}-dependent and –independent functions.

However, it is important to note that although palmitoylation at the Cys^{69} residue leads to phosphorylation at Tyr^{114} site, other PTMs occur at the Cys^{69} residue, which may affect PTMs at other sites. Therefore, phenotypes of *Phb1-Ki*^{C69A} mice may include those that are not mediated by Tyr^{114} phosphorylation and phenotypes of Phb1-Ki^{Y114F} may include both Cys^{69}-depedent and -independent effects. For example, a greater effect in Phb1-Ki^{Y114F} compared with Phb1-Ki^{C69A} (e.g., kidney weight and IGF1-R levels in male mice) may be due to Cys^{69}-independent effect of Tyr^{114} phosphorylation contributes to the greater effect in *Phb1-Ki*^{Y114F} mice. In this case, both Cys^{69}-dependent and -independent phosphorylation of Tyr^{114} may have the same direction effect. However, the mutant Tyr^{114}-specific effect as observed in relation to ubiquitin proteasome activity is Cys^{69}-independent effect of Tyr^{114}-phospho-mutant.

5.1 Significance

Chronic renal diseases are a major health problem worldwide. Structural and functional abnormalities in tubular cell mitochondria are important components of prevailing renal diseases, including chronic kidney disease, diabetic nephropathy and end-stage chronic kidney disease. These research findings provide new insights in our understanding of sex-related differences in renal biology, as revealed by the loss-of-function mutations in PHB1, affecting mitochondrial and proteasome homeostasis and damaging renal tissues, and causing lipid droplets accumulation amd triggering immune cell infiltration. As mitochondrial dysregulation is a pivotal event in many renal diseases, the ability to improve mitochondrial attributes pertaining to mitochondrial dynamics offer a novel precision tool to maintain kidney structure and function during metabolic dysregulation, and induce tolerance to metabolic diseases.

5.2 Study Limitations

There are many limitations in this study. A few examples are:

1) A lack of functional studies in *Phb1-Ki* mice to determine the effects of structural abnormalities on their kidney function.

2) An investigation in the gonadectomized mice with and without steroid hormone replacement therapy to define the relationship between PHB1 and sex steroid hormones in sex-related differences in renal physiology and pathophysiology has not yet been undertaken.
3) The potential role of PHB2 in causing the observed sex-related differences in renal tissues from the *Phb1-Ki* mice may not be ruled out because of the conjoint role of PHB1 and PHB2 in mitochondrial biology.

Further investigations are necessary to address these limitations.

5.3 Future Research Directions

1) Cellular and structural changes in the renal tubules and glomeruli in *Phb1-Ki* mice are indicative of the cell type- and cell compartment-specific role of the Cys^{69}- and Tyr^{114}-linked function of PHB1 in renal biology. It would be interesting to determine their response to experimentally induced obesity, type 2 diabetes, and type 1 diabetes-associated metabolic dysregulation in the development and progression of chronic and diabetic kidney diseases.
2) As structural and functional abnormalities in the mitochondria are common features of acute kidney disease, chronic kidney disease and diabetic nephropathy, it would be interesting to know how *Phb1-Ki* mice would respond to experimentally-induced acute and chronic kidney diseases.
3) To better define the relationship between PHB1 and sex steroid hormones in sex-related differences in renal biology would require an investigation of the gonadectomized male and female *Phb1-Ki* mice with and without hormone replacement therapy.
4) Apart from renal biology, the development of the *Phb1*^{C69A} and *Phb1*^{Y114F} knock-in mouse models, along with previously developed transgenic mouse models, have created an opportunity to discern the cell compartment- and cell type-specific functions of PHB1 and advance our understanding of this ubiquitously expressed, evolutionarily conserved, and pleiotropic protein.

CHAPTER VI

Conclusions

The research work presented in this thesis has revealed the role of PHB1 and its Cys^{69}- and Tyr^{114}-linked functions in sex-related differences in renal biology. These differences in the male and female *Phb1-Ki* mice correlate, in part, with mitochondrial characteristics in renal tubular epithelial cells and glomerular structure, as well as changes in IGF1R-mTOR and ubiquitin proteasome activity in renal tissues from the *Phb1-Ki* mice, signifying a role of PHB1in their regulation.

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