MITOCHONDRIAL DYSFUNCTION AND THE ROLE OF SIRT5 IN RENAL DISEASE

PhD Thesis

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Statement of originality

I, Timo Nicolas Haschler, declare that the work presented in this thesis is my own. I also confirm that information derived from other sources has been indicated as such in the thesis.
Abstract

Kidney disease is a global health concern with high mortality for which effective therapies are lacking. Accumulating evidence has identified excessive mitochondrial fragmentation causing mitochondrial dysfunction as a central pathologic feature of kidney disease. Recently, the NAD\(^+\)-dependent lysine desuccinylase/demalonylase sirtuin 5 (SIRT5) has emerged as a key regulator of mitochondrial form and function but its role in the kidney is unknown.

Immunostaining of normal human kidneys showed increased expression of SIRT5 in mitochondria-rich tubules. Subsequent immunostaining of murine kidneys from an ischaemic renal injury (IRI) (AKI model) and folic acid nephritis (FAN) (CKD model) model revealed that IRI increased SIRT5 expression, while FAN decreased expression.

An \textit{in vitro} oxygen/nutrient-deprivation (OND) model was developed in human proximal tubular epithelial cells (hPTECs) to mimic \textit{in vivo} renal ischaemia. OND increased SIRT5 expression in hPTECs. SIRT5 was depleted (by RNAi) and hPTECs were exposed to OND followed by assessment of mitochondrial form and function using confocal/ transmission electron microscopy, Seahorse, FACS and WB. SIRT5 depletion impaired cellular energy metabolism, disrupted mitochondrial fission/fusion dynamics, induced mitochondrial fragmentation and enhanced mitophagy. This effect was exacerbated by OND. SIRT5 depletion also increased mitochondrial swelling and decreased mitochondrial respiration after OND.

\textit{Sirt5}\(^{-/-}\) and wild-type mice underwent IRI surgery (40min) (AKI model), or received a single injection of with folic acid (240\(\mu\)g/g BW) or vehicle control (CKD model). IRI and FAN kidneys were harvested after 24h and 14d, respectively, and analysed by qPCR, IHC and WB. \textit{Sirt5}\(^{-/-}\) mice were protected from IRI and showed mildly aggravated injury in the FAN model. Analysis of renal \textit{Sirt5}\(^{-/-}\) mitochondria revealed a reduction in complex II activity, a central driver of reperfusion injury in IRI, hinting that this may be one potential mechanism that alleviates IRI. Mitochondrial function has been shown to be impaired in \textit{Sirt5}\(^{-/-}\) kidneys suggesting that this may have exacerbated FAN.

Taken together, the data showed that SIRT5 is an ischaemia-inducible enzyme in murine kidneys and hPTECs. This together with the observation that SIRT5 depletion caused mitochondrial dysfunction \textit{in vitro} in hPTECs as well as \textit{in vivo} in murine kidney suggested that the increase in SIRT5 levels is aimed at enhancing mitochondrial function. Loss of SIRT5 alleviated IRI and exacerbated FAN in mice \textit{in vivo} indicating that impaired mitochondrial function may on one hand reduce acute ischaemic injury, and on the other, aggravate chronic nephrotoxic injury.
Impact statement

Acute and chronic kidney diseases (AKI and CKD) are a global health concern with high morbidity and mortality. In the UK, AKI accounts for up to 18% of emergency hospital admissions and the prevalence of CKD (stage 3-5) is estimated to be up to 7% of the general population >16 years of age. The annual cost of AKI and CKD to the NHS is ~£2.47 billion, which is around 2% of the annual budget. In comparison, the combined cost of treating myocardial infarction and strokes is £174-178 million per annum. To date, there are no curative treatments for kidney disease which highlights the unmet need to identify new therapeutic strategies for AKI and CKD. Accumulating evidence has identified mitochondrial dysfunction and the consequent metabolic disturbance as a central pathophysiologic feature of both AKI and CKD. The NAD⁺-dependent enzyme SIRT5 has emerged as important regulator of mitochondrial energy metabolism, however, its function in the kidney was unknown. This thesis sought to investigate the role of SIRT5 in healthy kidneys as well as in AKI and CKD.

Academically, this project contributed to the understanding of SIRT5 biology in both mouse and human kidneys. Using a translational approach, it was shown that SIRT5 is an ischaemia-inducible enzyme in murine kidneys and human proximal tubular epithelial cells (hPTECs), the kidney cell type primarily affected by injury. SIRT5 depletion in hPTECs caused mitochondrial dysfunction as indicated by structural and functional changes of the organelle suggesting an important role for SIRT5 as a metabolic regulator in these cells. The in vitro data formed the basis of a successful grant application to St Peters Trust which allowed the analysis of SIRT5 function in vivo using Sirt5⁻/⁻ mice. Taking the in vitro study forward to an in vivo model was a crucial step. In contrast to our predictions from the in vitro data, the in vivo study showed that SIRT5 ablation did not exacerbate but rather alleviated ischaemic AKI suggesting that slight impairment of mitochondrial function may not necessarily be detrimental in acute renal ischaemia. Interestingly, Sirt5⁻/⁻ kidneys exposed to a chronic nephrotoxic injury showed mildly exacerbated injury which suggested that the impact of SIRT5 ablation on kidney disease may highly depend on the type and duration of the insult (acute ischaemic versus chronic nephrotoxic).

The idea that mitochondria may drive acute ischaemic injury is not entirely new. However, by identifying SIRT5 as target enzyme the present research adds an important piece to the puzzle with respect to finding new therapeutic targets in the treatment of AKI and CKD. In the case of ischaemic AKI, this may also be exploited commercially, in particular, as small molecule inhibitors of SIRT5 have been developed.
The data described in this thesis were presented at national as well as international conferences and were well received by the scientific community (academic and commercial). In addition, a manuscript has been submitted for publication which is currently under review and which will allow wider dissemination of the findings.

With respect to future work, although a first in man proof of principle clinical trial to test whether SIRT5 modulation may pre-empt AKI development after e.g. cardiac surgery or benefit patients with CKD is still some way off, these data will help to secure follow-on funding. This is key to further dissect the consequences of targeting SIRT5 pharmacologically and thus, assess its potential as therapeutic target in kidney disease. The identification of a new treatment for acute and/or chronic kidney disease would have a profound impact on the quality of life and longevity of a very substantial number of patients.
Acknowledgements

First and foremost, I would like to thank AstraZeneca and the Royal Free Charity for funding and supporting this work.

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A very special thanks to Dr Harry Horsley, who helped me not only with confocal imaging and image analyses but was always there for me. Thanks Harry, for your in-depth knowledge, constant support and friendship.

My thanks also to Dr Paul Simons, who taught me a lot about CRISPR/Cas9 and with whom I attempted to generate SIRT5 knockout hPTECs. I want to thank you Paul, for your in-depth genetics knowledge, support and the use of your laboratory.

I also want to thank Duncan Moore, Jessica Weedon, Mark Neal and Chloe Bidault in the Comparative Biology Unit, Royal Free Campus, for breeding and maintaining the colony of Sirt5-/- mice. In particular I would like to thank Prof. Alan Salama for teaching me surgical techniques and Mark Neal for helping me with the folic acid model.

Thanks too to our collaborators Glenn Anderson and Dr Monika Balys, at the Institute for Child Health, for the sample processing and electron microscopy.

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Manuscripts under review

Published reviews

Peer-reviewed abstracts


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Amount: £11,000
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Amino acid</td>
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<tr>
<td>ACMS</td>
<td>α-amino-β-carboxymuconate-ε-semialdehyde</td>
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<td>ACMSD</td>
<td>ACMS decarboxylase</td>
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<td>ACS2</td>
<td>Acetyl-coA synthetase 2</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>AIF</td>
<td>Apoptosis-inducing factor</td>
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<td>AKI</td>
<td>Acute kidney injury</td>
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<td>ALDOB</td>
<td>Aldolase B</td>
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<td>AMP</td>
<td>Adenosine monophosphate</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<td>AngII</td>
<td>Angiotensin II</td>
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<td>ANT</td>
<td>Adenine nucleotide translocator</td>
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<td>Activating protein 1</td>
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<td>Aquaporin 1 and 2</td>
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<td>ATG4</td>
<td>Autophagy-related protein 4</td>
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<td>Adenosine triphosphate</td>
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<td>Brown adipose tissue</td>
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<td>β-OHB</td>
<td>β-hydroxybutyrate</td>
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<td>β-ME</td>
<td>β-mercaptoethanol</td>
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<td>BNIP3</td>
<td>B-cell lymphoma 2 19 kilodalton interacting protein 3</td>
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<td>BNIP3L/NIX</td>
<td>BNIP3-ligand</td>
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<td>BRD4</td>
<td>Bromodomain 4</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BW</td>
<td>Body weight</td>
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<td>cADPR</td>
<td>Cyclic ADP ribose</td>
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<td>Calcineurin</td>
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<td>Calbindin-D-28k</td>
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<td>Collecting duct</td>
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<td>CD3</td>
<td>Cluster of differentiation 3</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>ChIP-seq</td>
<td>Chromatin immunoprecipitation-sequencing</td>
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<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>CL</td>
<td>Contralateral</td>
</tr>
<tr>
<td>CLEAR</td>
<td>Coordinated lysosomal expression and regulation</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>CLN</td>
<td>Cardiolipin</td>
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<td>Complete medium</td>
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<td>CoCl₂</td>
<td>Cobalt chloride</td>
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<td>CoQ2/10</td>
<td>Coenzyme Q2/10</td>
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<td>CPS1</td>
<td>Carbamoyl phosphate synthetase 1</td>
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<td>CPT1α</td>
<td>Carnitine palmitoyltransferase 1α</td>
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<td>CR</td>
<td>Caloric restriction</td>
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<td>CSE</td>
<td>Cigarette smoke extract</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>Cyt C</td>
<td>Cytochrome c</td>
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<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
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<td>DAPI</td>
<td>4′,6-Diamidino-2-phenylindole</td>
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<td>DBA</td>
<td>Dolichos biflorus agglutinin</td>
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<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DCIP</td>
<td>2,6-dichloroindophenol</td>
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<tr>
<td>DN</td>
<td>Dominant negative</td>
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<tr>
<td>DUB</td>
<td>Deubiquitinating enzyme</td>
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<tr>
<td>ΔΨₘ</td>
<td>Mitochondrial membrane potential</td>
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<tr>
<td>Δp</td>
<td>Proton-motive force</td>
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<tr>
<td>ΔpH</td>
<td>pH gradient</td>
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<td>DOX</td>
<td>Doxycycline</td>
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<td>DRP1</td>
<td>Dynamin-related protein 1</td>
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<tr>
<td>DT</td>
<td>Distal tubule</td>
</tr>
<tr>
<td>DTECs</td>
<td>Distal tubular epithelial cells</td>
</tr>
<tr>
<td>E-box</td>
<td>Enhancer box</td>
</tr>
<tr>
<td>EC</td>
<td>Epithelial cell</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EHHADH</td>
<td>Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase</td>
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<td>ESRD</td>
<td>End stage renal disease</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERRα</td>
<td>Estrogen-related receptor-α</td>
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<td>ERRE</td>
<td>ERR response element</td>
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<td>ETC</td>
<td>Electron transport chain</td>
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<td>ETF-QO</td>
<td>Electron transfer flavoprotein-ubiquinone oxidoreductase</td>
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<td>Abbreviation</td>
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<tr>
<td>FA</td>
<td>Folic acid</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FAD</td>
<td>Flavine adenine nucleotide</td>
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<td>FAN</td>
<td>Folic acid nephropathy</td>
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<td>FAO</td>
<td>Fatty acid oxidation</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>FCCP</td>
<td>Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone</td>
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<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
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<tr>
<td>FIS1</td>
<td>Fission 1</td>
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<td>Fn-EDA</td>
<td>Fibronectin-extra domain A</td>
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<td>FoxO3a</td>
<td>Forkhead box O3a</td>
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<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<td>FUNDC1</td>
<td>FUN14 domain containing 1</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GECI</td>
<td>Genetically-encoded calcium indicator</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>GLS</td>
<td>Glutaminase</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
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<tr>
<td>GPDH</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
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<tr>
<td>gRNA</td>
<td>Guide RNA</td>
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<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
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<td>HADHA</td>
<td>Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase</td>
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<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HEK293T</td>
<td>Human embryonic kidney 293, SV40-transformed</td>
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<td>HFD</td>
<td>High fat diet</td>
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<td>HIF1/2</td>
<td>Hypoxia-inducible factor 1 and 2</td>
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<td>HK-2</td>
<td>Human kidney-2</td>
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<td>HKC-8</td>
<td>Human kidney clone-8</td>
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<td>H+L</td>
<td>Heavy and light chain</td>
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<td>HMGCS2</td>
<td>3-Hydroxy-3-methylglutaryl-CoA synthase 2</td>
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<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
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<td>HRE</td>
<td>Hormone response element</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>IDH1/2</td>
<td>Isocitrate dehydrogenase 1 and 2</td>
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<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<td>IF</td>
<td>Immunofluorescence</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IMM</td>
<td>Mitochondrial inner membrane</td>
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<tr>
<td>IMS</td>
<td>Intermembrane space</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>IRI</td>
<td>Ischaemic renal injury</td>
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<td>K</td>
<td>Lysine</td>
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<td>KAc</td>
<td>K-acetylation</td>
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<td>K-acetyl transferases</td>
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<td>KDACs</td>
<td>K-deacetylases</td>
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<td>KDIGO</td>
<td>Kidney Disease Improving Global Outcomes</td>
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<td>KGlut</td>
<td>K-glutarylation</td>
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<td>KIM-1</td>
<td>Kidney injury molecule-1</td>
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<td>K-succinylation</td>
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<td>LBD</td>
<td>Ligand-binding domain</td>
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<td>LC3</td>
<td>Microtubule-associated proteins 1A/1B light chain 3 B</td>
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<td>LCAD</td>
<td>Long-chain acyl-CoA dehydrogenase</td>
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<td>LDHA</td>
<td>Lactate dehydrogenase</td>
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<td>LIR</td>
<td>LC3 interacting region</td>
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<td>LOH</td>
<td>Loop of Henle</td>
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<tr>
<td>mc</td>
<td>Monoclonal</td>
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<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
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<tr>
<td>MERCs</td>
<td>Mitochondria endoplasmic reticulum contacts</td>
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<td>MFF</td>
<td>Mitochondrial fission factor</td>
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<td>MiD49/51</td>
<td>Mitochondrial dynamics protein 49/51</td>
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<td>MiTF</td>
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<td>mitoQC</td>
<td>Mitochondrial quality control</td>
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<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>mTORC1/2</td>
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<td>Mitochondrial permeability transition</td>
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<td>mV</td>
<td>Millivolt</td>
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<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>NAAD</td>
<td>Nicotinic acid adenine dinucleotide</td>
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<tr>
<td>NAM</td>
<td>Nicotinamide</td>
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<td>NAMN</td>
<td>Nicotinic acid mononucleotide</td>
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<td>Nicotinic acid phosphoribosyltransferase</td>
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<td>NADS</td>
<td>Nicotinamide adenine dinucleotide synthase</td>
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<td>NADH:ubiquinone oxidoreductase subunit A9</td>
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<td>NGS</td>
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<td>Normal human kidney</td>
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<td>NHR</td>
<td>Nuclear hormone receptor</td>
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<td>Nicotinamide adenine mononucleotide</td>
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<td>NMN adenylyltransferase</td>
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<td>NR</td>
<td>Nicotinamide riboside</td>
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<td>NRK1/2</td>
<td>NR kinase 1/2</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>OD</td>
<td>Oxygen deprivation</td>
</tr>
<tr>
<td>OGD</td>
<td>Oxygen and glucose deprivation</td>
</tr>
<tr>
<td>OMA1</td>
<td>m-AAA protease 1 homolog</td>
</tr>
<tr>
<td>OMM</td>
<td>Mitochondrial outer membrane</td>
</tr>
<tr>
<td>OND</td>
<td>Oxygen and nutrient deprivation</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy 1</td>
</tr>
<tr>
<td>I/s-OPA1</td>
<td>Long/Short-OPA1</td>
</tr>
<tr>
<td>OPTN</td>
<td>Optineurin</td>
</tr>
<tr>
<td>OTC</td>
<td>Ornithine transcarbamoylase</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
</tbody>
</table>
O$_2^-$  Superoxide
PARP  Poly-ADP-ribose polymerases
PAS  Periodic acid Schiff
pc  Polyclonal
PE  Phosphatidylethanolamine
PGC1  Peroxisome proliferator-activated receptor-γ co-activator 1
PGE$_2$  Prostaglandin E$_2$
PHDs  Prolyl-hydroxylases
PINK1  Phosphatase and tensin homolog-induced putative kinase 1
polyUB  Poly-ubiquitination
PPAR  Peroxisome proliferator-activated receptor
PPRE  PPAR response element
PRC  PGC1-related co-activator
PT  Proximal tubule
PTECs  Proximal tubular epithelial cells
PTM  Post-translational modification
pUB  Phospho-ubiquitination
Q  Ubiquinone
QH$_2$  Ubiquinol
qPCR  Quantitative polymerase chain reaction
QPRT  Quinolinate phosphoribosyltransferase
RNAi  RNA interference
ROS  Reactive oxygen species
RT  Room temperature
S  Serine
s.c.  Subcutaneous
SCr  Serum creatinine
SDHA  Succinate dehydrogenase, subunit A
SGLT2  Sodium glucose transporter 2
shRNA  Short hairpin RNA
siRNA  Small interfering RNA
SIRT1-7  Sirtuin 1-7
SNP  Single nucleotide polymorphism
SOD1/2  Superoxide dismutase 1 and 2
SQSTM1/p62  Sequestosome 1
STZ  Streptozotocin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TAL</td>
<td>Thick ascending limb</td>
</tr>
<tr>
<td>TAX1BP</td>
<td>Tax-binding protein 1</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS supplemented with Tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TDL</td>
<td>Thin descending limb</td>
</tr>
<tr>
<td>TDO</td>
<td>Tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>TFB</td>
<td>Mitochondrial transcription factor B</td>
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<tr>
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<td>TFEC</td>
<td>Transcription factor EC</td>
</tr>
<tr>
<td>TFE3</td>
<td>Transcription factor E3</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>THP</td>
<td>Tamm Horsfall protein</td>
</tr>
<tr>
<td>TIM23</td>
<td>Translocase of inner mitochondrial membrane 23</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TOM20</td>
<td>Translocase of outer mitochondrial membrane 20</td>
</tr>
<tr>
<td>TPP</td>
<td>Tetraphenylphosphonium</td>
</tr>
<tr>
<td>tRNA&lt;sup&gt;ile&lt;/sup&gt;</td>
<td>Isoleucine transfer RNA</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td>TTFA</td>
<td>2-thienyltrifluoroacetone</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type two diabetes mellitus</td>
</tr>
<tr>
<td>UB</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UBD</td>
<td>Ubiquitin binding domain</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureter obstruction</td>
</tr>
<tr>
<td>VLCAD</td>
<td>Very long-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>VR</td>
<td>Vasa recta</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>YME1L</td>
<td>yme1-like protein</td>
</tr>
<tr>
<td>ZKSCAN3</td>
<td>Zinc finger with KRAB and SCAN domains 3</td>
</tr>
</tbody>
</table>
# List of figures

## Chapter 1

| Figure 1.1: The structure of the nephron | 3 |
| Figure 1.2: Mitochondrial quality control | 16 |
| Figure 1.3: Mitophagy | 21 |
| Figure 1.4: The electron transport chain (ETC) | 26 |
| Figure 1.5: Regulation of the NAD⁺/NADH ratio | 29 |
| Figure 1.6: *De novo* NAD⁺ biosynthesis pathway | 31 |
| Figure 1.7: NAD⁺ biosynthesis via salvage pathways | 32 |
| Figure 1.8: Classification of the SIRT family | 36 |

## Chapter 3

| Figure 3.1: Immunostaining of human renal cortex and medulla for PGC1α, SIRT5 and SIRT3 | 58 |
| Figure 3.2: Co-localisation of PGC1α and segment-specific markers in the human kidney | 60 |
| Figure 3.3: Co-localisation of SIRT5 and segment-specific markers in the human kidney | 62 |
| Figure 3.4: Co-localisation analysis of SIRT3 in the human kidney | 63 |
| Figure 3.5: Nuclear localisation of SIRT5 in the distal tubule of the human kidney | 64 |
| Figure 3.6: Co-localisation of TOM20 with AQP1 and CBD in the human renal cortex | 65 |
| Figure 3.7: Co-localisation of SIRT5 with TOM20 in the human kidney | 66 |
| Figure 3.8: Ageing-associated reduction in mitochondrial mass in the human renal cortex | 67 |
| Figure 3.9: Quantitative analyses of PGC1α and SIRT5 expression in normal human kidneys | 68 |
| Figure 3.10: SIRT5 expression increases after ischaemia in PTECs *in vivo* | 69 |
| Figure 3.11: SIRT5 expression decreased in chronic kidney disease | 70 |
| Figure 3.12: SIRT3/5 and PGC1α expression along the human nephron | 72 |

## Chapter 4

| Figure 4.1: I-OPA1 proteolysis by OMA1 causes mitochondrial fragmentation | 81 |
| Figure 4.2: qPCR gene expression analyses of the OND model | 94 |
| Figure 4.3: OND induces autophagy | 96 |
| Figure 4.4: HBSS, hypoxia and OND induce mitophagy | 97 |
| Figure 4.5: OND stimulates I-OPA1 degradation | 98 |
| Figure 4.6: OND stimulates YME1L degradation | 99 |
| Figure 4.7: OND increases SIRT5 mRNA and protein levels *in vitro* | 100 |
| Figure 4.8: SIRT5 gene transcription is not regulated by the HIF1 pathway | 101 |
| Figure 4.9: mTORC1 inhibition increases SIRT5 mRNA expression | 102 |
| Figure 4.10: Putative MiTF/TFEB and ZKSCAN3 binding sites in the human SIRT1 and SIRT5 gene promoters | 103 |
| Figure 4.11: SIRT5 gene expression is not regulated by the transcription factors TFEB or TFE3 | 104 |
| Figure 4.12: SIRT5 knockdown impairs cellular energy metabolism | 106 |
| Figure 4.13: SIRT5 RNAi induces mitochondrial fragmentation | 108 |
| Figure 4.14: SIRT5 knockdown disrupts the mitochondrial fission/fusion machinery | 110 |
| Figure 4.15: SIRT5 knockdown alters I-OPA1 processing by OMA1 and YME1L | 111 |
| Figure 4.16: SIRT5 knockdown aggravates mitochondrial swelling in OND | 113 |
| Figure 4.17: SIRT5 knockdown aggravates OND-induced mitochondrial dysfunction | 114 |
Figure 4.18: Mitochondrial and lysosomal co-labelling to assess mitophagy............... 115
Figure 4.19: SIRT5 depletion enhances OND-induced mitophagy. .............................. 116
Figure 4.20: SIRT5 knockdown aggravates OND-induced reduction in mitochondrial mass ................................................................................................................ 117
Figure 4.21: SIRT5 knockdown may enhance mitochondria ER contact sites (MERCs). ... 119
Figure 4.22: Super-resolution confocal image analysis of MERCs. .............................. 120
Figure 4.23: Co-localisation of Ca$^{2+}$ dyes Rhod2-AM and Fluo4-AM with Mitotracker dye in hPTECs .................................................................................................................. 121
Figure 4.24: Rhod2-AM and Fluo4-AM primarily accumulate in extra-mitochondrial vesicles. ......................................................................................................................... 122
Figure 4.25: Proposed model of how SIRT5 depletion affects mitochondrial function and exacerbates OND-induced mitochondrial dysfunction in hPTECs. .......... 136

Chapter 5

Figure 5.1: Loss of SIRT5 results in increased renal lysine succinylation and malonylation................................................................. 148
Figure 5.2: Effect of SIRT5 ablation on mitochondrial structure in PTs ................. 148
Figure 5.3: Effect of SIRT5 ablation on mitochondrial structure in DTs. ............... 149
Figure 5.4: Effect of SIRT5 ablation on podocyte foot processes. ......................... 149
Figure 5.5: Effect of Sirt5 deletion on renal function ................................................. 150
Figure 5.6: Histological analyses of WT and Sirt5$^{-/-}$ kidneys exposed to renal ischaemia. ......................................................................................................................... 151
Figure 5.7: Sirt5 deletion alleviates IRI and the decline in renal function ............... 152
Figure 5.8: Effect of IRI on mitochondrial mass-/ biogenesis-associated markers..... 153
Figure 5.9: Sirt mRNA expression in WT and Sirt5$^{-/-}$ kidneys......................... 154
Figure 5.10: SIRT5 ablation impairs complex II activity. ........................................ 155
Figure 5.11: Effect of FA on Sirt mRNA expression in WT and Sirt5$^{-/-}$ kidneys. .... 157
Figure 5.12: Histological characterisation of WT and Sirt5$^{-/-}$ FA kidneys. ........... 158
Figure 5.13: Effect of FA injection on renal expression of Ngal and Kim1 in WT and Sirt5$^{-/-}$ kidneys ................................................................. 159
Figure 5.14: Effect of FA-injection on immune cell infiltration in WT and Sirt5$^{-/-}$ kidneys ................................................................. 161
Figure 5.15: Effect of FA injection on mRNA expression of pro-inflammatory markers in WT and Sirt5$^{-/-}$ kidneys. ................................................................. 162
Figure 5.16: Vimentin expression in WT and Sirt5$^{-/-}$ kidneys in response to FA .... 163
Figure 5.17: Collagen deposition in WT and Sirt5$^{-/-}$ kidneys in response to FA ...... 164
Figure 5.18: Expression of pro-fibrotic markers in FA-treated WT and Sirt5$^{-/-}$ kidneys ......................................................................................................................... 165
Figure 5.19: Expression of mitochondrial pro-fusion proteins in WT and Sirt5$^{-/-}$ kidneys in response to FA ................................................................. 167
Figure 5.20: Expression of the I-OPA1 degrading enzyme YME1L in WT and Sirt5$^{-/-}$ kidneys in response to FA ................................................................. 168
Figure 5.21: Expression of autophagy and mitochondrial marker proteins in WT and Sirt5$^{-/-}$ kidneys in response to FA ................................................................. 169
Figure 5.22: Renal function in FA-treated WT and Sirt5$^{-/-}$ kidneys. ................... 171
Figure 5.23: Proposed model of the role of SIRT5 in AKI and CKD ...................... 185

Appendix

Appendix Figure 1: SIRT5 overexpression increased viability of cisplatin-treated hPTECs ......................................................................................................................... 208
Appendix Figure 2: BRD4 inhibition by JQ1 affects SIRT1, 3 and 5 mRNA levels ....... 209
List of tables

Chapter 3
Table 3.1: Antibodies and fluorescence stains ............................................................ 55

Chapter 4
Table 4.1 PCR primers for quantitative gene expression analyses .............................. 88
Table 4.2: PCR primers for mtDNA quantification ......................................................... 90
Table 4.3: Antibodies for immunofluorescence analyses ............................................. 91
Table 4.4: Antibodies for Western blot analyses ......................................................... 92

Chapter 5
Table 5.1: Antibodies for immunohistochemical analyses ......................................... 142
Table 5.2: PCR primers for quantitative gene expression analyses .......................... 144
Table 5.3: PCR primers for mtDNA quantification ..................................................... 145
Table 5.4: Antibodies for Western blot analyses ....................................................... 146

Appendix
Appendix Table 1: MiTF/TFEB and ZKSCAN3 binding sites .................................... 207
Table of contents

Statement of originality ................................................................. i
Abstract ......................................................................................... ii
Impact statement ........................................................................... iii
Acknowledgements ......................................................................... v
List of abbreviations ...................................................................... viii
List of figures ................................................................................ xiii
List of tables .................................................................................. xviii

Chapter 1 Introduction ..................................................................... 1
  1.1 The kidney in health and disease ........................................... 2
      1.1.1 The structure of the kidney ........................................... 2
      1.1.2 Acute kidney disease ............................................... 4
      1.1.3 Chronic kidney disease ........................................... 4
      1.1.4 Mitochondrial dysfunction in kidney disease .............. 5
      1.1.5 Murine models of acute and chronic kidney disease .... 10
  1.2 Mitochondria ........................................................................ 11
      1.2.1 Mitochondrial biogenesis ........................................... 11
      1.2.2 Mitochondrial dynamics .......................................... 14
      1.2.3 Mitophagy ............................................................. 19
      1.2.4 Mitochondrial interplay with other cellular organelles ... 23
  1.3 Cellular bioenergetics ............................................................ 24
      1.3.1 Glycolysis .............................................................. 24
      1.3.2 Oxidative phosphorylation ....................................... 24
      1.3.3 NAD+ metabolism ................................................ 29
  1.4 Post-translational modifications .......................................... 34
  1.5 The Sirtuins ......................................................................... 36
      1.5.1 Mitochondrial sirtuins ............................................. 37
      1.5.2 SIRT SNPs and the link to human disease ................. 42
  1.6 Aims and objectives ............................................................. 44

Chapter 2 General Materials and Methods .................................. 45
  2.1 Chemicals ........................................................................... 46
2.2 Mouse breeding, maintenance and renal injury models ................. 46
  2.2.1 Unilateral ischaemia and reperfusion injury ................................. 46
  2.2.2 Bilateral ischaemia and reperfusion injury .................................. 47
  2.2.3 Folic acid nephropathy .............................................................. 47
2.3 Molecular biological analyses ......................................................... 48
  2.3.1 Kidney samples and immunostaining .......................................... 48
  2.3.2 Gene expression analyses by RT-qPCR ...................................... 50
  2.3.3 Western blot analyses ............................................................. 51
  2.3.4 Transmission electron microscopy ............................................ 52
  2.3.5 Statistical analyses ......................................................... 52

Chapter 3 SIRT5 is expressed in murine as well as human kidneys and
its expression is affected by acute and chronic injury .................. 53
  3.1 Introduction ..................................................................................... 54
  3.2 Specific aims .................................................................................. 55
  3.3 Methods .......................................................................................... 55
    3.3.1 Mouse breeding, maintenance and renal injury models .......... 55
    3.3.2 Immunohistochemistry and immunofluorescence ................. 55
  3.4 Results ............................................................................................ 58
    3.4.1 PGC1α, SIRT5 and SIRT3 localise to mitochondria-rich nephron
        segments in the human kidney .................................................... 58
    3.4.2 Mitochondrial abundance decreases with age in the human kidney .... 65
    3.4.3 Ageing does not affect SIRT5 expression in the human kidneys .... 67
    3.4.4 SIRT5 levels are regulated by acute and chronic renal injury .... 68
  3.5 Discussion ....................................................................................... 71
  3.6 Conclusion ....................................................................................... 77

Chapter 4 SIRT5 depletion exacerbates OND-induced mitochondrial
dysfunction in hPTECs ................................................................. 78
  4.1 Introduction ..................................................................................... 79
  4.2 Specific aims .................................................................................. 82
  4.3 Methods .......................................................................................... 82
    4.3.1 Cells and cell culture ............................................................ 82
    4.3.2 In vitro simulation of ischaemia ............................................. 83
    4.3.3 SIRT5 knockdown using siRNA ........................................... 83
    4.3.4 TFEB and TFE3 knockdown using siRNA ......................... 84
    4.3.5 Assessment of the mitochondrial membrane potential ........ 84
    4.3.6 Assessment of mitochondrial morphology ........................... 85
    4.3.7 ATP quantification .............................................................. 86
    4.3.8 Assessment of mitochondrial structure by electron microscopy .... 86
    4.3.9 Assessment of mitochondrial function by respirometry .......... 87
6.4 Future work .......................................................................................... 199
  6.4.1 Ex vivo investigations in human renal tissue .................................. 200
  6.4.2 In vitro investigations ................................................................. 200
  6.4.3 In vivo investigations ................................................................. 203

Appendix ............................................................................................... 206

References.............................................................................................. 210
Chapter 1

Introduction
Acute and chronic kidney disease is a major global health burden and so far, there are no curative treatments. Mitochondrial dysfunction has emerged as central pathophysiological feature of acute and chronic kidney disease.\(^3\),\(^4\) In recent years, the sirtuins (SIRTs) have been identified as vital stress sensors that promote metabolic adaptation and thereby, are assumed to maintain mitochondrial homeostasis during injury.\(^5\),\(^6\)

Due to the complexity of the topic and to gain a better understanding of the underlying mechanisms the introductory Chapter 1 is split into five Sections: The first Section (1.1) is dedicated to the kidney in health and disease and addresses why mitochondrial dysfunction is a major driver of renal disease. The second Section (1.2) focuses on the pathways which regulate mitochondrial form and function as well as turnover, and reveals how these three aspects are linked. Thus, this Section provides a theoretical background for the processes driving mitochondrial dysfunction discussed in 1.1. The third Section (1.3) focuses on cellular energy metabolism (glycolysis and oxidative phosphorylation) with a special emphasis on nicotinamide adenine dinucleotide (NAD\(^+\)), a vital co-factor which facilitates energy metabolism. The subsequent Section (1.4) addresses the role of post-translational modifications (PTMs) and explains how PTMs impact on cellular bioenergetics. The final Section (1.5) focuses on the SIRTs, NAD\(^+\)-dependent lysine (K) deacylases (KDACs), which have recently emerged as major regulators of cellular bioenergetics and highlights why boosting SIRT activity may be a new therapeutic option to treat acute and chronic kidney diseases.

1.1 The kidney in health and disease

1.1.1 The structure of the kidney

The kidneys play an essential role in the regulation of body electrolyte and fluid balance and facilitate blood pressure homeostasis.\(^7\) The normal adult kidney filters about 180 litres of blood per day and only 1% of filtered solutes are excreted in the urine, while 99% are reabsorbed and return to the circulation.\(^7\) This process is facilitated by renal nephrons, the filtration units of the kidneys. Human kidneys contain on average 1 million nephrons, although the individual numbers vary between 200,000 and >2.5 million.\(^8\) Structurally, each nephron consists of a renal corpuscle, a blood filtration unit, which contains the glomerulus (tuft of capillaries) and a glomerular capsule (so called Bowman’s capsule); and, a tubular unit comprising the proximal tubule (PT), loop of Henle (LoH), distal tubule (DT), which consists of the distal convoluted tubule and connecting tubule; and, the collecting duct (CD) (Figure 1.1).\(^9\) The glomerulus is exclusively responsible for filtration of the blood (~25% of cardiac output) producing
primary urine, while the tubules are required for reabsorption of the glomerularly-filtered electrolytes as well as water.(7) The PTs consist of three segments (S1-S3) depending on their localisation within the kidneys and are referred to as early proximal convoluted (S1), late proximal convoluted and early straight (S2) and straight (S3) tubules.(9) The LoH, which follows the PT S3, comprises the thin descending limb (TDL), thin ascending limb and thick ascending limb (TAL).(9)

Figure 1.1: The structure of the nephron. The nephron is a functional unit of the kidney and consists of a renal corpuscle and a tubular unit. The corpuscle consists of a glomerulus (1) surrounded by the Bowman’s capsule (2) and is responsible for blood filtration, thereby, producing primary urine. The tubular unit consists of proximal tubule, segments S1-3 (3-5), the loop of Henle, consisting of the thin descending limb (6), thin ascending limb (7) and thick ascending limb (8), thereafter, the distal convoluted tubule (9), the connecting tubule (10) and the collecting duct (11).

Over 65% of solutes (amino acids, electrolytes and low molecular weight proteins), 80% of filtered bicarbonates and all glucose in the primary urine are reabsorbed via the PTs.(7) Notably, the majority of water reabsorption (~80%) takes place in the PTs and
the TDL.(10, 11) In the TAL, which is water-impermeable, ions including sodium, potassium and chloride are reabsorbed.(12, 13) Adjoining to the TAL is the DT and later on, the CD, which are responsible for the reabsorption of the remaining sodium, chloride and water, respectively.(13) Several CDs finally merge to a papillary duct, which eventually drain the urine into the ureter.(13)

1.1.2 Acute kidney disease

Acute kidney injury (AKI) is a major global health concern and is associated with high morbidity and mortality. In the UK AKI accounts for up to 18% of emergency hospital admissions and is a key risk factor for the development of kidney failure.(14) In addition, AKI is predicted to be the underlying cause of about 100,000 hospital deaths every year.(15) The annual estimated cost of AKI to the NHS is £1.02 billion, which is around 1% of the annual budget.(16) The most common cause of AKI is renal hypoperfusion (90% of the cases) which may be due to volume depletion (e.g. as a consequence of cardiac surgery), hypotension or may be drug-induced.(17) Additional causes are nephrotoxic agents (e.g. cisplatin, radiocontrast media and aminoglycosides) and sepsis.(17) AKI causes a rapid decline in renal function within hours or days, and is clinically characterised by reduced urine output (oliguria or anuria; <0.5ml/kg/h for more than 6h) and an increase in serum creatinine levels (SCr) (rise of >26µmol/l or greater within 48h).(14) Risk factors for AKI are advanced age, diabetes, a history of AKI and most importantly, pre-existing chronic kidney disease (CKD), which, at least in part, may explain the increased incidence of AKI in the elderly.(18, 19) Early detection and intervention are vital to limit disease progression and determine patient outcome. Given that a substantial amount of AKI arises from surgery-induced renal hypoperfusion it should be stressed that particular at-risk individuals would benefit from a pre-emptive treatment to prevent subsequent AKI. At present, there are no approved preventative or curative treatments for AKI. Current treatments are primarily supportive (e.g. dopamine and selective dopamine-receptor agonists such as Fenoldopam, to increase renal blood flow) which highlights the pressing need for new therapeutic strategies.

1.1.3 Chronic kidney disease

CKD, which pre-disposes to AKI, is equally a worldwide health problem and can lead to end-stage renal disease (ESRD).(20) Furthermore, CKD is a major driver of cardiovascular disease (CVD) (20), the global leading cause of death (21). Interestingly, in patients with CKD the risk of developing CVD is greater than the risk of progression to
ESRD and the need for renal replacement (20, 22), which highlights the importance of managing the disease and treating associated illnesses. The annual CKD-related cost to the NHS is thought to be ~£1.45 billion, with over 50% spent on renal replacement therapy.(23) In comparison, the combined cost of treating myocardial infarction and strokes is £174-178 million per annum. In England, the prevalence of CKD (stage 3-5) is estimated to be up to 7% of the general population >16 years of age.(24) Unlike AKI, CKD is a slowly progressive disease which causes a gradual loss of kidney function over time and can remain undetected for years. Risk factors for CKD include reduced nephron number at birth, advanced age (>65 years), AKI, nephrotoxic agents (e.g. cisplatin, radiographic media and amino- glycosides) and underlying diseases (i.e. type 2 diabetes mellitus (T2DM) and hypertension).(25) The Kidney Disease Improving Global Outcomes (KDIGO) initiative categorised CKD into 5 stages based on the glomerular filtration rate (GFR) and the degree of albuminuria, which are both measures of renal function.(25) Currently there are no curative treatments for CKD and interventions are aimed at slowing disease progression by e.g. managing hypertension (angiotensin-converting enzyme (ACE) inhibitors such as Enalapril or angiotensin 2 receptor blockers (ARBs) such as Telmisartan). (25) Of note, recent studies have shown that patients with diabetic nephropathy benefit from treatment with the sodium-glucose transporter 2 (SGLT2) inhibitor Canagliflozin, which reduced CVD mortality.(26) Targeting SGLT2 may, therefore, be a promising therapeutic approach to alleviate diabetes-induced CKD. This is of particular importance as T2DM is a major risk factor for CKD (up to 40% of T2DM patients will develop CKD) (26) and diabetic nephropathy is the predominant form of CKD (approximately 30-50%), followed by hypertension-induced CKD (up to 27%) (27). Nonetheless, although the current advances in treating diabetic nephropathy appear promising, it has to be emphasised that given the overall prevalence of CKD worldwide there is still an unmet need for further therapeutic strategies for CKD treatment.

1.1.4 Mitochondrial dysfunction in kidney disease

Mitochondria, which are regarded the powerhouse of the cell, are highly dynamic structures, crucial for adenosine triphosphate (ATP) production by oxidative phosphorylation (OXPHOS), calcium homeostasis, and regulation of apoptosis as well as the regulation of reactive oxygen species (ROS).(28) After the heart, the kidneys are the organs with the highest oxygen consumption rate and mitochondrial abundance.(3, 29) It is estimated that the kidneys use around 10% of the total oxygen although they constitute only ~1% of the total body weight.(30) The reason for this is the extent of ATP-
dependent reabsorption/excretion processes carried out in the renal nephron driven by the basolateral Na⁺/K⁺-ATPase (3), which require high ATP production (primarily (~95%) generated by oxidative metabolism) (30). The majority of active reabsorption takes place in the proximal tubular (PT) epithelial cells (ECs) (PTECs) and distal tubular (DT) ECs (DTECs), which explains the high mitochondrial density found in both segments. (3) PTs contribute around 50% to the total renal mass. (31) Using the mitochondrial cristae membrane surface and calculating the number of membrane respiratory chain units as well as oxygen consumption it was estimated that PTECs produce ~14µmol ATP/min, DTECs ~6µmol ATP/min and the remaining nephron <1µmol ATP/min. (32) Intriguingly, PTECs and DTECs differ markedly in their substrate specificity for generating ATP. (33) While both nephron segments equally depend on FAO, PTECs have a very low glycolytic capacity relative to DTECs (34, 35), however, show an enhanced ability to produce glucose (by gluconeogenesis) (32).

Bearing in mind that PTECs and DTECs are highly metabolically active and depend on mitochondrial function, it is not surprising that disruption with this system (referred to as mitochondrial dysfunction) has emerged as central pathophysiologic feature of both AKI and CKD. (3, 4) Tubular disorders of the renal nephron caused by mitochondrial dysfunction can arise from: (i) genetic mutations leading to OXPHOS dysfunction; or, (ii) cellular insults (e.g. hypoperfusion causing ischaemia), which impair mitochondrial function.

(i) Genetic mutations. Mutations in nuclear and/or mitochondrial genes encoding components of the electron transport chain (ETC) or mutations in genes key to mitochondrial biogenesis can lead to renal disorders. (3) Typically, inherited mitochondrial diseases which arise from OXPHOS defects manifest in tissues dependent on oxidative metabolism such as brain and muscle. (36) Therefore, it is quite rare, that genetic forms of mitochondrial dysfunction purely manifest in a renal phenotype rather than being a part of a systemic disease. (3) However, two mitochondrial tubular disorders have been described which arise from genetic mutations: The first is induced by mutations in the gene coding for the mitochondrial isoleucine transfer RNA (tRNAle). (37) It is thought to predominantly affect distal tubules as patients display pronounced hypomagnesaemia. (37) The second genetically-induced renal disorder is autosomal dominant Fanconi syndrome. (38) This disorder results from a mutation in the gene coding for the peroxisomal bifunctional enzyme (EHHADH), which is key to peroxisomal fatty acid oxidation (FAO), and primarily affects PTECs. (38) Disease-causing mutations in the EHHADH gene mistarget the enzyme to the mitochondria where EHHADH forms a dominant-negative complex with the mitochondrial trifunctional enzyme (HADHA), which interferes with FAO and ATP generation. (38) Consequently, Na⁺/K⁺-ATPase
activity is diminished, which is key to maintaining the chemical gradient needed for reabsorption of solutes from the primary urine.(38)

(ii) Cellular insults. The major cause of mitochondria-associated tubulopathies are cellular insults including hyperglycaemia, sepsis, and ischaemia as well as nephrotoxic compounds (3, 4), manifesting in metabolic and structural changes in the organelle (39-41). Mitochondrial form and function are tightly-linked and changes in mitochondrial architecture have a dramatic impact on bioenergetics and vice versa.(42, 43) Due to the complexity of the process, the following paragraphs will only focus on the cause, consequences and putative treatments of the various aspects of mitochondrial dysfunction: mitochondrial architecture, mitochondrial degradation/biogenesis and mitochondrial bioenergetics; as well as cell death mechanisms. The detailed mechanisms underlying mitochondrial dysfunction will be discussed in Section 1.2.

Mitochondrial architecture. Mitochondria are highly dynamic organelles which undergo constant fission and fusion.(44) In particular, excessive mitochondrial fragmentation (caused by an imbalance in fusion and fission) emerged as early sign of tubular injury and a major feature of renal damage.(39-41) Two tightly-linked elements, the mitochondrial membrane potential (ΔΨ<sub>M</sub>) and the ATP pool, are central determinants of mitochondrial architecture and their reduction, as occurs during i.e. ischaemia, is a fundamental driver of mitochondrial fragmentation.(45, 46) Interestingly, it emerged that in HeLa cells exposed to oxygen and glucose deprivation or chemical anoxia (using cyanide) (47) as well as cardiomyocytes exposed to anoxia (48), the F1/F0-ATP synthase (complex V) maintains the ΔΨ<sub>M</sub> by consuming (glycolytic) ATP and by doing so, counteracts excessive fission. This, however, requires an ability to switch metabolically from aerobic to anaerobic metabolism to maintain and not further deplete, the cellular ATP pool (48), thereby highlighting the significance of extra-mitochondrial ATP generation to mitochondrial form and function. In the kidney, PTECs lack the ability to sufficiently switch to anaerobic glycolysis, which renders this nephron segment highly susceptible to necrotic cell death during ischaemia.(45, 49, 50) Conversely, DTECs, which are more metabolically flexible, can shift to anaerobic glycolysis and withstand injury by evading an ATP shunt.(45) Nonetheless, chronic hypoxia will drive DTEC death with apoptosis rather than necrosis being the predominant form.(50) Beyond bioenergetic consequences, excessive fragmentation leads to cytochrome C (Cyt C) release and provokes tubular epithelial cell apoptosis.(39) Increased Cyt C release is a central mechanism in ischaemic renal injury (IRI)-induced damage to human renal allografts.(51) A major driver of mitochondrial fragmentation (by facilitating fission) is the GTPase dynamin-related protein 1 (DRP1).(44) Inhibition of DRP1 through gene knockout or pharmacological intervention, impairs mitochondrial fragmentation, Cyt C
release and thus, apoptosis.(46) Using inducible PT-specific Drp1-/- mice, Perry et al. showed that inhibition of fission before and after IRI reduces renal damage by improving mitochondrial function post-injury, thereby, underscoring the therapeutic potential of manipulating mitochondrial fission.(52) These investigators also reported that deletion of Drp1 prevented IRI-induced downregulation of peroxisome proliferator-activated receptor γ (PPARγ)-coactivator 1α (PGC1α), sirtuin 3 (SIRT3) and nicotinamide phosphoribosyltransferase (NAMPT), all of which are involved in mitochondrial metabolism.

Mitophagy and mitochondrial biogenesis. Increased mitochondrial fragmentation and bioenergetic disturbance render mitochondria susceptible to degradation by mitophagy.(42) Although mitophagy is a vital process which facilitates mitochondrial quality control (mitoQC) (53), it is also a driver of mitochondrial mass reduction (54). In the kidney, mitophagy protects cells during injury (e.g. ischaemia and nephrotoxins) and thereby, improves renal function.(55-57) In line with this, activating mitophagy has been proposed to alleviate renal disease.(54) However, specifically activating mitophagy has proved difficult. A more general approach currently being investigated is the induction of autophagy using the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) inhibitor rapamycin, which has been shown to reduce injury in models of kidney disease.(58) However, this is only relevant to CKD as mTOR activity is important for tubular regeneration in AKI.(58)

In mice it was recently shown that PTs are the nephron segment with the highest rates of mitophagy (indicating high mitochondrial turnover).(59) This is an important observation as it may provide an additional explanation as to why PTs are exceptionally sensitive to injury: To maintain mitochondrial mass and consequently bioenergetic output, cells have to generate new mitochondria (mitochondrial biogenesis).(4, 60) This process is largely driven by the transcriptional coactivator, PGC1α.(61) Multiple studies have shown that acute and chronic renal injury cause a decline in PGC1α levels in PTECs and thereby, drive disease progression.(62, 63) Conversely, increasing PGC1α levels genetically (PT-specific, inducible) has been found to reduce renal injury and thereby, help to maintain tubular function post-insult in murine models of AKI (64, 65) and CKD.(62, 63) These findings, therefore, hint that increasing mitochondrial biogenesis (via PGC1α) might be a viable therapeutic approach. Although thus far no specific small molecular activator for PGC1α has been identified, it has been shown that formoterol, a β2-adrenergic receptor agonist, increases PGC1α levels in murine kidneys (66) and improves renal function after ischaemic AKI (67). In addition, it has been suggested that activating transcription factors (TFs) downstream of PGC1α such as PPARα (e.g. with Fenofibrate), may be an alternative way to circumvent this issue and
alleviate kidney disease in both mice (63) and men (68). Although this may be one therapeutic approach it should be noted that as yet, no curative treatment for kidney disease targeting mitophagy or mitochondrial biogenesis has been identified.

**Mitochondrial bioenergetics.** As previously mentioned, mitochondrial form and function are tightly interlinked and preventing mitochondrial fragmentation in kidney disease has been shown to preserve renal function by enhancing mitochondrial function.(52) While this is an indirect way of improving mitochondrial function, it has been suggested that targeting mitochondria directly by using e.g. mitochondria-targeted antioxidants such as MitoQ or SS-31 which reduce mitochondrial ROS, may be an option. MitoQ is mitochondrial coenzyme Q10 (CoQ10) conjugated to a lipophilic cation (Tetraphenylphosphonium (TPP)) which results in accumulation in the mitochondrial matrix based on the $\Delta \Psi_M$ (69), while SS-31 is thought to interact with cardiolipin in the inner mitochondrial membrane (IMM).(70) Both compounds have been shown to improve renal function in kidney disease.(71, 72) However, a recent study by Gottwald et al. demonstrated that at high concentrations MitoQ induces pronounced mitochondrial swelling and depolarisation in PTECs (in kidney cortex slices) causing significant mitochondrial toxicity, which urges caution with respect to using this compound in patients.(73)

In addition to using antioxidants, another putative approach has been proposed to boost mitochondrial function by activating sirtuins (SIRTs).(6) SIRTs are NAD$^+$-dependent KDACs which have been shown to promote mitochondrial metabolism.(6) A SIRT1 activator, SRT1720, has been shown to alleviate experimental kidney disease in mice (74, 75) hinting that activating SIRTs may be a new therapeutic option. Targeting individual SIRTs specifically has proved challenging.(76) However, specific activation of a single SIRT family member may not be required given that multiple SIRTs including SIRT1, 3 and 6, have been shown to be renoprotective.(77) Therefore, a more general strategy currently under investigation is to boost NAD$^+$ levels. NAD$^+$ is an important co-factor required for a multitude of enzymatic reactions (glycolysis or OXPHOS) and increasing NAD$^+$ levels has been shown to alleviate AKI in mice.(78, 79) The Parikh group recently showed that increasing NAD$^+$ levels (via the precursor nicotinamide (NAM)) decreases susceptibility to ischaemic AKI in mice as well as in patients undergoing cardiac surgery (80) suggesting that increasing NAD$^+$ levels may be beneficial in kidney disease. Although this approach has shown some promise, very limited human data have been generated to date, which is why further investigations are required.

**Cell death mechanisms.** As mentioned in the section on mitochondrial architecture, excessive mitochondrial fragmentation can lead to tubular cell apoptosis via
Cyt C release. Apoptosis is only one of several forms of cell death, which can be differentiated based on distinct morphologic alterations. Apoptosis is classified as type I cell death and apoptotic cells display specific features including cell shrinkage, apoptotic body formation, DNA fragmentation and chromatin condensation. Type II cell death is referred to as autophagic cell death and is characterised by large-scale vacuolisation of cellular organelles and cytosolic components. Type III cell death, known as necrosis, can be identified by swelling of cellular organelles, loss of membrane integrity and absence of phagocytic lysosomal involvement. Apoptosis and autophagic cell death are regulated forms of cell death while necrosis is an uncontrolled form of cell death. In the kidney, renal tubular necrosis is the primary form of cell death induced by acute ischaemia as well as nephrotoxic insults and a hallmark of tissue injury. Of note, there are also regulated forms of cell death with a necrotic phenotype adding another layer of complexity. One form of regulated necrosis is mitochondrial permeability transition (MPT)-driven necrosis. The MPT pore is a megachannel consisting of proteins on the mitochondrial outer/inner membranes. MPT pore opening can be triggered by several events including cytosolic Ca\(^{2+}\) overload, decreased ΔΨ\( _{M} \) and severe oxidative stress. Opening of the MPT pore results in increased Ca\(^{2+}\) influx into the mitochondrial matrix, which disrupts the chemiosmotic gradient and thus, impairs ETC function; and, leads to efflux of Cyt C as well as other pro-apoptotic factors, thereby, activating apoptotic pathways. Notably, prolonged MPT pore opening significantly raises Ca\(^{2+}\) levels in the mitochondrial matrix, which osmotically drives the influx of water into the organelle. This causes mitochondrial swelling as well as membrane rupture, and eventually manifests as necrotic cell death.

Taken together, these observations emphasise the therapeutic potential of manipulating mitochondrial architecture, metabolism, mitophagy and biogenesis for the treatment of renal disorders. However, no curative treatment for AKI or CKD targeting mitochondrial form/function has been found so far which highlights the need for further studies to assess whether this is a viable therapeutic strategy.

1.1.5 Murine models of acute and chronic kidney disease

Ischemic renal injury (IRI) as a model of AKI. A commonly used and robust model to induce AKI in mice is ischemic renal injury (IRI). IRI is a surgical model in which the renal artery is clamped for a period of 20 - 45 min to cause ischaemic injury and subsequently released, which causes reperfusion injury. This model can be carried out as unilateral ischaemia (without contralateral nephrectomy) or as bilateral ischaemia (with contralateral nephrectomy). Although AKI-to-CKD transition has been
proposed (87), IRI is primarily used as an AKI model as it appears that murine kidneys can regenerate quite quickly and recover renal function 7d post injury (86).

**Folic acid nephropathy (FAN) as model of CKD.** FAN is a widely-used nephrotoxic model of CKD.(88) In this model, a single high dose of folic acid (>240µg/g body weight) dissolved in 0.3M bicarbonate buffer is injected in mice intraperitoneally causing renal damage.(88) Proposed mechanisms leading to injury are folic acid crystal formation in the tubular lumen, which result in tubular obstruction and cause tubular cell necrosis.(89) Notably, Stallons et al. proposed mitochondrial dysfunction as a central mechanism driving CKD progression in this model.(90)

1.2 Mitochondria

1.2.1 Mitochondrial biogenesis

Mitochondria are highly dynamic organelles which are essential for cellular energy metabolism, calcium homeostasis and involved in the regulation of cell death.(28) Mitochondria possess their own circular genome, which is exclusively inherited maternally.(91) In vertebrates, the mitochondrial DNA (mtDNA) is ~16.5kb and contains 13 protein-coding genes which all encode essential subunits of the ETC complexes.(92) Both, the H- and L-mtDNA strands contain promoter sequences and enhancers, which are recognised by the mitochondrial transcription machinery: the mitochondrial RNA polymerase, the mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B (TFB).(92, 93) Of note, TFAM is not only a TF but is also responsible for mtDNA replication, further highlighting the importance of this factor in mitochondrial biogenesis.(94)

*De novo* generation of mitochondria is not possible, which is why mitochondrial fission is an essential prerequisite for mitochondrial biogenesis.(95, 96) In addition, mitochondrial biogenesis relies on an intricate transcriptional network that facilitates the synthesis of mitochondrial components needed for the assembly of new mitochondria from pre-existing ones.(95, 96) When mitochondrial biogenesis is initiated, mitochondria undergo replicative fission giving rise to two healthy daughter mitochondria.(96) These subsequently integrate newly synthesised mitochondrial proteins as well as lipids and mature to new, fully functional mitochondria.(96) As mitochondria possess only a limited genetic repertoire, mitochondrial biogenesis requires a major contribution from nuclear genes.(97) The transcriptional regulation of the nuclear genes will be discussed in the next Section.
1.2.1.1 Transcriptional regulation of mitochondrial biogenesis

The nuclear transcriptional network driving mitochondrial biogenesis involves nuclear TFs and nuclear hormone receptors (NHRs) both of which are activated by the transcriptional co-regulator family of peroxisome proliferator-activated receptor-γ co-activators (PGCs). Therefore, PGC1 family proteins are regarded as central regulators of mitochondrial biogenesis. The PGC1 family comprises three members: PGC1α, PGC1β and PGC1-related co-activator (PRC).(98) Overexpression experiments in vivo (99) and in vitro (100), have shown that PGC1α can stimulate virtually all aspects of mitochondrial biogenesis. Similar roles have been demonstrated for PGC1β (101, 102) and PRC (103-106), highlighting their central function in promoting mitochondrial biogenesis. Of note, tissue expression analyses revealed that protein levels of PGC1α and β as well as their targets, peak in highly metabolically-active tissues including brown adipose tissue (BAT), heart and kidneys (107-111), while PRC is ubiquitously expressed (61). All three PGC1 family proteins contain a leucine-rich motif (LXXLL) through which they interact with TFs and NHRs involved in mitochondrial biogenesis to promote transcription of downstream target genes.(61) Examples of interaction partners are the (i) TFs, nuclear respiratory factor 1/2 (NRF1/2); and (ii) NHRs, PPARα and estrogen-related receptor (ERR)α.(61)

(i) Nuclear TFs. NRF1 and NRF2 are well-characterised TFs, which bind to specific target sequences within promoters of genes involved in mitochondrial biogenesis and function.(93) NRF1/2 targets are TFAM (112), subunits of the ETC complexes I-IV (93) and Cyt C (113) as well as translocase of outer membrane 20 (TOM20) (114). In particular, the NRF1/TFAM-axis is a good example of how nuclear TFs link the expression of nuclear-encoded mitochondrial genes to an increase in mtDNA and thus, drive biogenesis.(93)

(ii) NHRs. NHRs are fundamental mediators of mitochondrial biogenesis that act as molecular switches to control transcription (115, 116). They are structurally characterised by a ligand-binding domain (LBD) as well as a DNA-binding domain (DBD) which facilitate binding to specific hormone response elements (HREs).(115) In the case of PPARs and ERRs, these elements are referred to as PPAR response elements (PPREs) and ERR response elements (ERREs), respectively.(115) ERRα and PPARα are central transcriptional regulators of enzymes involved in mitochondrial fatty acid metabolism including carnitine palmitoyltransferase 1α (CPT1α) and hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (HADHA).(109, 116, 117) Of interest, although it is generally accepted that the PPARs/ERRs promote mitochondrial metabolism it has not yet been resolved whether their impact on
mitochondrial biogenesis and function arises from primary or secondary effects. Beyond the positive contribution to mitochondrial FAO, ERRα directly controls mitochondrial architecture through transcriptional regulation of mitofusin1/2 (MFN1/2), which facilitate mitochondrial fusion.\(^{(118, 119)}\) NHRs can be activated by distinct ligands. While no endogenous ligand for ERRs \(^{(109)}\) have been found, PPARs are activated by fatty acids \(^{(120, 121)}\) providing a mechanism through which changes in cellular lipid metabolism directly affect the transcriptional regulation of mitochondrial FAO and potentially, mitochondriogenesis.

1.2.1.2 Regulation of PGC1α to promote mitochondriogenesis

While the mechanisms though which PGC1α regulates downstream transcription are fairly well established, it is not entirely resolved how PGC1α itself is controlled. The current literature indicates a tight regulation on a (i) transcriptional as well as (ii) post-translational level:\(^{(122)}\)

(i) Transcriptional regulation of PGCα. Metabolic challenges such as nutrient deprivation or exercise, have been shown to increase the expression of PGC1α.\(^{(123, 124)}\) As a result PGC1α increases mitochondrial OXPHOS capacity, which in turn facilitates metabolic adaptation by switching from glycolysis to FAO.\(^{(125)}\) In recent years, the microphthalmia-associated TF (MiTF)/ transcription factor EB (TFEB) (MiTF/TFEB) family of TFs has emerged as central regulator of PGC1 gene transcription.\(^{(126-128)}\) This TF family comprises 4 members, MiTF, TFEB, TFE3 and TFEC \(^{(129, 130)}\), of which TFEB is regarded the master regulator of autophagy and also lysosomal biogenesis.\(^{(130, 131)}\) Autophagy is a vital catabolic process used to deliver damaged cellular organelles and components including mitochondria, misfolded proteins or pathogens, to the lysosome for degradation.\(^{(132)}\) In addition, autophagy is a key survival mechanism during starvation that facilitates the mobilisation of nutrients by degrading intracellular proteins, lipids and carbohydrates.\(^{(132)}\) MiTF/TFE proteins homo- or heterodimerise and bind the DNA at enhancer (E)-boxes (CANNTG) close to transcriptional start sites (TSS).\(^{(133-135)}\) TFEB primarily binds to a specific type of E-box-containing sequence, namely coordinated lysosomal expression and regulation (CLEAR) sites (gtCACGTGac).\(^{(133-135)}\) MiTF/TFE family proteins are tightly regulated through mTOR-complex 1 (mTORC1) and calcineurin (CaN)-mediated phosphorylation/ dephosphorylation that control their distinct intracellular localisation.\(^{(133)}\) mTORC1-mediated TFEB/3 and MiTF phosphorylation enables 14-3-3 protein binding and results in cytosolic retention.\(^{(136)}\) This process can be reversed by the calcium (Ca\(^{2+}\))-dependent phosphatase CaN, which promotes nuclear translocation of TFEB/3.\(^{(133)}\)
Specific stimuli which inhibit mTORC1 or activate CaN such as rapamycin or starvation, enable MiTF/TFEB cytosol-to-nucleus shuttling to induce target gene transcription.(136) Interestingly, PGC1α was found to contain a number of CLEAR sites suggesting it is a central TFEB target gene.(127, 137) In line with this, genetic ablation of TFEB impaired induction of PGC1α expression during nutrient deprivation (127, 137), while overexpression of TFEB increased PGC1α/β expression (138). Thus, the TFEB/PGC1α-axis exemplifies how changes in nutrient availability directly impact on mitochondrial form and function. Notably, Mansueto et al. recently showed that in addition to the ability to signal through PGC1α/β, TFEB drives mitochondrial biogenesis per se in a PGC1α/β-independent manner by targeting genes including TFAM, NRF2 and NADH:Ubiquinone oxidoreductase subunit A9 (NDUFA9; subunit of ETC complex I).(138)

(ii) Post-translational regulation of PGC1α. PGC1α can also be regulated through post-translational modifications (PTMs). For example, during starvation AMP/ATP and NAD+/NADH ratios rise, which directly activate the kinase, AMP-activated protein kinase (AMPK), and the deacetylase, SIRT1.(123) AMPK-mediated phosphorylation (139) and SIRT1-driven deacetylation (140) increase the activity of PGC1α and drive expression of nuclear-encoded mitochondrial genes. Intriguingly, in a recent publication, Shin et al. reported that AMPK also increases the activity of TFEB and in addition, identified SIRT1 as TFEB target gene.(141)

Together these observations reveal an intricate, interconnected regulatory model of mitochondrial biogenesis during starvation driven by TFEB, PGC1α, AMPK and SIRT1.

1.2.2 Mitochondrial dynamics

Mitochondria are highly dynamic organelles that constantly undergo structural changes (fusion and fission). As discussed in Section 1.2.1, mitochondrial dynamism is a central mechanism that facilitates mitochondrial biogenesis. Changes in mitochondrial architecture have additional functions including (i) cellular adaptation to bioenergetic requirements; and, (ii) mitochondrial turnover.

(i) Bioenergetic adaptation. Metabolic changes such as nutrient availability alter mitochondrial architecture, which enables bioenergetic adaptation to the environment.(44) When exposed to excess nutrients, mitochondria start to fragment and reduce their capacity and efficiency of ATP production.(142) In contrast, in a state of starvation, mitochondria fuse and become elongated, which promotes ATP generation.(44) Given the impact of fragmentation on mitochondrial bioenergetics, it is not surprising that increased fragmentation, as seen in obesity as well as cardiometabolic
disorders including diabetes and cancer, is regarded a hallmark of cellular dysfunction.(44)

(ii) Mitochondrial life cycle. Apart from bioenergetic adaptation, fusion and fission dynamics serve another fundamental role, namely, the regulation of the mitochondrial life cycle. As mentioned in Section 1.1.1, healthy mitochondria can undergo replicative fission forming two daughter mitochondria that mature to fully functional new organelles, a process key to mitochondrial biogenesis.(96) In addition, mitochondria can undergo asymmetric fission. This process allows the removal of dysfunctional components and ultimately, facilitates mitoQC (Figure 1.2).(42) While it is not entirely clear how replicative fission is induced, it has emerged that the underlying mechanism of asymmetric fission relates to the mitochondrial membrane potential, ΔΨ_M. Asymmetric fission gives rise to two daughter mitochondria, which differ in their ΔΨ_M – one with a normal ΔΨ_M and one with a reduced ΔΨ_M.(42) If the depolarised daughter mitochondrion fails to recover its ΔΨ_M and instead, displays a further decrease in the ΔΨ_M, it will not be able to fuse again.(42) Hence, it will undergo further fragmentation followed by autophagic degradation (mitophagy) while the healthy daughter mitochondrion will fuse with other organelles and re-join the network.(42) Interestingly, in an acute stress setting such as nutrient depletion, mitochondria undergo stress-induced mitochondrial hyperfusion whereby they are protected from autophagic proteolytic degradation.(43) However, chronic interference with this process by inhibition of fission, results in the accumulation of oxidised mitochondrial proteins and ultimately reduces cellular respiration highlighting the importance of a functioning fission system to mitoQC.(42)
Mitochondria are dynamic organelles that undergo fusion and fission, a process which is highly determined by the mitochondrial membrane potential ($\Delta \Psi_M$). **Fusion:** Mitochondria with healthy $\Delta \Psi_M$ can undergo fusion. This process is divided in mitochondrial outer membrane (OMM) and mitochondrial inner membrane (IMM) fusion. OMM fusion is mediated by the OMM-associated GTPases mitofusin 1 (MFN1) and MFN2. IMM fusion is determined by optic atrophy 1 (OPA1) which exists as long (l)-OPA1 and short (s)-OPA1 and a balance of the two is central to mitochondrial fusion. The peptidases m-AAA protease 1 homolog (OMA1) and yme1-like protein (YME1L) can both cleave l-OPA1 to s-OPA1, however, only OMA1 can transform all l-OPA1 to s-OPA1 thereby promoting IMM fission. In healthy mitochondria, ATP levels are high which leads to activation of YME1L and subsequent degradation of OMA1 to promote IMM fusion. When ATP levels drop, YME1L activity is decreased and OMA1 degrades YME1L, which ultimately promotes s-OPA1 accumulation and OMM fission. **Fission:** Mitochondrial fission is a mechanism central to mitochondrial biogenesis (replicative fission) as well as the elimination of damaged components (asymmetric fission). Fission is driven by the dynamin-related protein 1 (DRP1) (red circles), which binds to its receptors (fission 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial dynamics protein 49/51 (MiD49/51) on the mitochondrial outer membrane (OMM) and thus, triggers fission. Replicative fission of a healthy mitochondrion gives rise to two daughter mitochondria that grow into two healthy mitochondria with normal $\Delta \Psi_M$. Asymmetric fission gives rise to two daughter mitochondria – one with a normal $\Delta \Psi_M$ and one with a reduced $\Delta \Psi_M$. If the dysfunctional mitochondrion fails to recover its $\Delta \Psi_M$ it will not be able to fuse again and therefore, undergo autophagic degradation (mitophagy) or induce apoptosis due to cytochrome c (Cyt C) release. Figure adapted from Fischer et al., 2012.(143)
Mitochondrial fusion and fission dynamics are orchestrated by highly conserved dynamin-related proteins (DRPs), a protein family with characteristic guanosine triphosphatase (GTPase) activity.\(^{(144)}\) Of note, DRPs are large GTPases, meaning that their enzymatic activity is induced by oligomerisation (dimerisation or tetramerisation) and therefore, dependent on reaching a critical mass.\(^{(145, 146)}\) The next Section is dedicated to the discussion of the mitochondrial fusion and fission machinery to provide a better understanding of the regulation of mitochondrial architecture:

### 1.2.2.1 Mitochondrial fusion

Mitochondrial fusion is driven by MFN1/2 as well as optic atrophy 1 (OPA1). These proteins are anchored in either the mitochondrial outer membrane (OMM) or mitochondrial inner membrane (IMM), respectively, and consequently, are responsible for either OMM or IMM fusion.\(^{(44)}\) Genetic ablation of MFN1/2 \(^{(147)}\) or OPA1 \(^{(148)}\) results in embryonic lethality due to unopposed fission, underscoring the importance of a functioning mitochondrial fusion system.

**OMM fusion.** MFNs are integrated into the OMM with their C-terminal GTPase and N-terminal coiled-coil structure exposed to the cytoplasm.\(^{(149, 150)}\) They act as membrane anchors, which tether adjacent OMMs together. Upon, MFN/MFN-binding, GTP hydrolysis leads to conformational changes and hence, initiates fusion.\(^{(151)}\) Of note, MFN2 has also been detected in the endoplasmic reticulum (ER) membrane and detailed analysis revealed that MFN-mediated ER-mitochondrial contacts are important for mitochondrial Ca\(^{2+}\) signalling.\(^{(152, 153)}\)

**IMM fusion and fission.** As yet, little is known about the exact mechanisms behind IMM fusion. Nevertheless, one protein, OPA1, has been shown to impact IMM dynamism.\(^{(44)}\) In contrast to MFN1/2, which purely promote fusion, OPA1 proteins have the ability to regulate both fusion and fission.\(^{(44)}\) Tissue- and cell-specific alternative splicing of \(OPA1\) gene products generate various long OPA1 (l-OPA1) isoforms, which are all characterised by a N-terminal membrane anchor.\(^{(44)}\) This anchor can be cleaved off by specific IMM peptidases giving rise to soluble, short OPA1 (s-OPA1) proteins.\(^{(154)}\) While l-OPA1 proteins are regarded as purely pro-fusion molecules, s-OPA1 proteins seem predominantly to promote IMM fission.\(^{(154)}\) Of note, l/s-OPA1 oligomers have emerged as a central component of the IMM and contribute to cristae formation.\(^{(155, 156)}\) Consequently, interference with the balanced l/s-OPA1 system has detrimental effects on IMM ultrastructure and thus, compromises mitochondrial respiration.\(^{(155, 156)}\)
To date, two IMM peptidases have been identified, which can cleave l-OPA1: (1) overlapping with the m-AAA protease 1 homolog (OMA1) and (2) yme1-like protein (YME1L). Both enzymes remove the N-terminal transmembrane domain, however, they differ in their specific cleavage sites (S1 and S2): OMA1 cleaves at the S1 site whereas YME1L cleaves at the S2 site of L-OPA1. Of interest, the S1 cleavage site can be detected in all translated L-OPA1 isoforms, while the S2 cleavage site is only present in about 50% of the isoforms (splice variants). Only OMA1 but not YME1L, is capable of converting all l-OPA1 isoforms to s-OPA1, and strongly promotes fission. To add another layer of complexity, it has emerged that YME1L and OMA1 are able to degrade and thus regulate, each other in an ATP-dependent manner. It has been shown that YME1L but not OMA1 requires ATP to function properly. This gives rise to a mechanistic model in which the cellular energy status directly affects l-/s-OPA balance, and hence, mitochondrial form and function. Under physiological conditions, OMA1 and YME1L activity are balanced leading to equal amounts of l-OPA1 and s-OPA1. However, cellular stress such as ischaemic injury, deplete cellular ATP levels thereby, reducing YME1L activity. This is followed by OMA1-mediated YME1L degradation. The subsequent drop in YME1L disrupts the YME1L/OMA1 balance leading to an OMA1-dependent rise in s-OPA1 and increased mitochondrial fission. Of note, mitoQC is also partly mediated by OMA1 as a reduction in the ΔΨm leads to a decline of ATP, which subsequently reduces YME1L activity and by doing so, drives fission via a build-up of s-OPA1. Interestingly, Parajuli et al. found that kidneys exposed to cold storage display an increase in OMA1 and suggested that a disturbed fusion/fission system might contribute to allograft rejection.

1.2.2.2 Mitochondrial fission

Mitochondrial fission is a highly regulated process, which can be divided into OMM and IMM fission. OMM fission is driven by DRP1, which accumulates on the OMM as a consequence of specific fission signals. The mechanisms behind IMM fission are only partly understood. Nonetheless, the IMM associated protein OPA1, was found to mediate IMM fission. Global knockout of Drp1 or Opa1 results in embryonic lethality which highlights the significance of a functioning fission system in the maintenance of cellular homeostasis.

OMM fission. Fission of the OMM is primarily dictated by DRP1. Unknown fission signals provoke the translocation of DPR1 from the cytoplasm to the OMM, where the proteins oligomerise and assemble into puncta. These mitochondria-bound
puncta merge, creating chain-like structures. (167) Ultimately, this leads to formation of a collar, which facilitates localised constriction of mitochondria. (167) Interestingly, the translocation of DRP1 is regulated post-translationally via differential phosphorylation: (168) DRP1 phosphorylation on serine 616 (S616) promotes mitochondrial accumulation and fission, while DRP1 serine 637 (S637) phosphorylation leads to DRP1 OMM detachment, and thus, opposes fission. (168)

To date, four DRP1 recruitment factors located on the OMM have been identified: mitochondrial dynamics proteins (MiD) of 49 kDa (MiD49) and 51 kDa (MiD51), fission 1 (FIS1) and mitochondrial fission factor (MFF). (169) All four proteins have been shown to bind DRP1, however, they are assumed to exert different downstream effects to regulate OMM fission. (169, 170) While MFF exerts only pro-fission functions, the roles of FIS1 and MiD49/51 remain to be defined. (171) This idea was derived from initial RNA interference (RNAi) studies, which showed that MFF RNAi strongly interfered with mitochondrial fission and triggered extreme mitochondrial elongation, while depletion of FIS1 resulted in less pronounced effects. (171-173) The latter observation may be due to the fact that MFF can functionally replace FIS1 but not vice versa. (173) Thus far little is known about the exact role and mechanism of action of MiD49/50 proteins. However, Palmer et al. suggested that both MiD protein knockdown and overexpression promotes mitochondrial fusion and not fission, thereby, highlighting the complexity of MiD biology. (174) In earlier studies it was reported that MiD protein overexpression specifically provokes the accumulation of functionally inactive, S637 phosphorylated DPR1 to promote fusion. (173) Recently it emerged that the outcome (fusion or fission) is far more complex and determined by tightly defined levels of cellular MiD proteins. (175)

The limited knowledge about the distinct roles of MFF, FIS1 and MiD49/51 and their impact on OMM fission dynamics highlights the importance of further detailed mechanistic studies.

1.2.3 Mitophagy

Mitophagy is a selective form of autophagy that facilitates the recognition and degradation of dysfunctional mitochondria, and is a vital element of the mitoQC machinery. (176) Mitophagy depends on the ΔΨM and is a central process to maintain homeostasis of the mitochondrial population. (176) Interference with the mitophagy system results in accumulation of malfunctioning mitochondria, which can be disease-causing or exacerbate pre-existing disease conditions. (176, 177) A well-known example for the former is Parkinson’s disease. (178, 179) This neurological disorder can be
caused by mutations or deletions in genes of the PTEN-induced putative kinase 1 (PINK1)/Parkin program – a sensor system which constitutively analyses the mitochondrial polarisation state.\(^{(178, 179)}\)

Mitophagy depends on two mechanisms: mitophagy induction (Section 1.2.3.1), meaning the detection and marking of damaged mitochondria; and, autophagosomal sequestration and subsequent lysosomal degradation (Section 1.2.3.2).\(^{(176)}\)

### 1.2.3.1 Mitophagy induction

There are two pathways by which mitophagy can be induced, a PINK1/Parkin-dependent pathway and a PINK1/Parkin-independent pathway, which makes use of mitophagy receptors.

**PINK1/Parkin-dependent pathway.** As previously mentioned, the induction of mitophagy is highly dependent on mitochondrial fission, and more specifically on the \( \Delta \Psi_M \) (Figure 1.3).\(^{(42)}\) The most-studied mechanism by which damaged mitochondria are detected and marked for degradation is the PINK1/Parkin pathway.\(^{(180)}\) PINK1 is a serine/threonine kinase, which acts as a mitochondrial stress sensor to identify malfunctioning mitochondria.\(^{(180)}\) Parkin is an E3-ubiquitin ligase which, if activated by PINK1, poly-ubiquitinates (polyUB) OMM proteins and marks dysfunctional mitochondria for degradation.\(^{(180)}\) Subsequently, ubiquitinated proteins on the OMM are recognised by mitophagy receptors which further mark damaged mitochondria for autophagic degradation.\(^{(180, 181)}\) The detailed mechanism behind the PINK1/Parkin pathway is as follows: In healthy mitochondria, an elevated \( \Delta \Psi_M \) drives the continuous import of PINK1 across the OMM by the translocase of the IMM (TIM)/-OMM (TOM) complex.\(^{(180)}\) In the mitochondrial intermembrane space, PINK1 is quickly cleaved by proteases and ultimately degraded via the cytosolic ubiquitin proteasome system.\(^{(180)}\) In damaged mitochondria, the \( \Delta \Psi_M \) is reduced and PINK1 accumulates at the OMM linked to the TOM complex where it is spared from degradation.\(^{(180)}\) Subsequent autophosphorylation of PINK1 triggers its kinase activity and leads to phosphorylation of OMM protein-bound ubiquitin (UB), giving rise to phospho-UB (pUB).\(^{(180)}\) pUB facilitates the recruitment and activation of Parkin which ubiquitinates additional OMM proteins. Such poly-ubiquitination generates new substrates for PINK1, thereby, generating a feed-forward loop for Parkin recruitment.\(^{(180)}\) Both, poly-UB (polyUB) chains and pUB can be recognised by specific mitophagy receptors, which mark damaged mitochondria for autophagic degradation.\(^{(180, 181)}\) Very interestingly, pUB of OMM proteins alone was shown to be sufficient to induce mitophagy.\(^{(181)}\)
Figure 1.3: Mitophagy. (A) Constant reduction in the $\Delta \Psi_M$ lead to PTEN-induced putative kinase 1 (PINK1) (pink) accumulation on the OMM, which provokes the accumulation of mitophagy receptors. These link damaged mitochondria to phagophores via the microtubule-associated proteins 1A/1B light chain 3 B (LC3-II) (red). Mitophagy receptors are: (i) OMM-associated receptors including B-cell lymphoma 2 19 kilodalton interacting protein 3 (BNIP3), its homolog BNIP3-ligand (BNIP3L/NIX) and FUN14 Domain Containing 1 (FUNDC1) (orange). (ii) Non-mitochondrial receptors including optineurin (OPTN), Ca$^{2+}$-binding and coiled-coil domain 2 (NDP52), tax-binding protein 1 (TAX1BP1), sequestosome 1 (p62/ SQSTM1) and neighbor of BRCA1 gene 1 (NBR1) (black) which all associate with ubiquitin (UB) (green). (iii) The phospholipid cardiolipin (blue). (B) Phagophores fuse and elongate to form autophagosomes. (C) Autophagosomes fuse with endolysosomes containing acidic hydrolases which degrade the engulfed cargo.

This gives rise to a model in which Parkin acts as an amplifier of PINK1 mitophagy signals. Of note, several lines of evidence indicate that the PINK1/Parkin-pathway directly impacts on mitochondrial architecture by promoting fission.(177) One mechanism includes PINK1/Parkin-mediated degradation of MFN1/2 which antagonises mitochondrial fusion.(182, 183) Another, newly published mechanism implicates PINK1-induced changes in the phosphorylation status of DRP1 and thus, orchestrates fission.(184) Of note, poly-ubiquitination of OMM proteins is highly regulated by deubiquitinating enzymes (DUB), which counteracts mitophagy by removing polyubiquitin.(177) Examples for DUBs are UPS8, UPS15 and UPS30.(177)
Mitophagy receptors (PINK1/Parkin-independent). Mitophagy receptors recognise and bind to mitochondria with a reduced $\Delta \Psi_M$, thereby, marking them for degradation. All mitophagy receptors share one structural entity: the microtubule-associated proteins 1A/1B light chain 3 B (LC3)-interacting region (LIR).(176) The LIR is the key element that enables the formation of a physical connection between damaged mitochondria and the phagophore (a cup-shaped autophagic membrane).(177) This link is formed through LIR binding to the phagophore-bound protein LC3-II and initiates the engulfment of damaged mitochondria. Of note, LC3 exists as three forms: pro-LC3, LC3-I and LC3-II.(176) Of the LC3 forms only LC3-II is conjugated to phosphatidylethanolamine (PE), thus, can be integrated into autophagosomal membranes.(176) The underlying mechanism of LC3 activation is as follows: The cysteine protease autophagy-related protein (ATG) 4 (ATG4) cleaves pro-LC3 and thereby, converts the cytosolic pro-LC3 to LC3-I.(176) When autophagy is induced, ATG3 and ATG7 conjugate PE to LC3-I in an UB-like reaction and give rise to LC3-II.(185) LC3-II is the only form able to associate with autophagosomal membranes.(185) Once integrated into the phagophore, LC3-II can associate with a variety of autophagy receptors and thereby enables non-selective or selective autophagy.(186)

Mitophagy receptors can be sub-divided into three categories based on their structure, localisation and mechanism of action: (i) non-mitochondrial receptors, (ii) OMM-associated receptors, and (iii) cardiolipin (CLN) (Figure 1.2): (177)

(i) Non-mitochondrial receptors. Non-mitochondrial mitophagy receptors are structurally characterised by an ubiquitin binding domain (UBD).(187) They bind poly-UB chains associated with proteins on the OMM and thus, link mitochondria with low $\Delta \Psi_M$ to phagophore-associated LC3-II.(188) To date, five different receptors have been identified: optineurin (OPTN), Ca$^{2+}$-binding and coiled-coil domain 2 (NDP52), tax-binding protein 1 (TAX1BP1), sequestosome 1 (p62/ SQSTM1) and neighbor of BRCA1 gene 1 (NBR1) (177) of which OPTN and NDP52 have emerged as key mitophagy receptors recruited by PINK1 (181). Of interest, OPTN and NDP52 can bind to pUB, and therefore can initiate autophagy independent of Parkin.(181) p62/SQSTM1, a widely used marker of general autophagic flux, and its functional homolog NBR1, bind and aggregate damaged mitochondria.(181)

(ii) OMM-associated receptors. OMM-associated receptors are the only mitophagy receptor family with a transmembrane domain (TMD), and therefore, constitutively localise to the OMM.(176) When transcriptionally induced, these receptors translocate to the OMM and mark mitochondria for autophagic degradation.(176) Like non-mitochondrial receptors, OMM-associated receptors act as anchors that bind LC3-II via their LIR and link mitochondria with a phagophore.(176) Well-studied examples are
B-cell lymphoma 2 19 kilodalton interacting protein 3 (BNIP3), its homolog BNIP3-ligand (BNIP3L/NIX) and FUN14 Domain Containing 1 (FUNDC1), which are all activated by hypoxia and are therefore regarded as central to induction of mitophagy during ischaemia. Of note, while BNIP3 and NIX are direct target genes of HIF1, FUNDC1 is activated through hypoxia-stimulated dephosphorylation. Interestingly, BNIP3 requires DRP1 activity to induce mitophagy and in addition, directly interacts with OPA1, thereby, opposing the pro-fusion activity of OPA1. These observations highlight the significance of mitochondrial fission to mitophagy.

(iii) Cardiolipin (CLN). The phospholipid CLN is a crucial component of the mitochondrial membranes, in particular, the IMM. CLN is not only central to formation and integrity of cristae but is also a key component required for the assembly of ETC complexes. Under physiological conditions, CLN mainly localises to the IMM where it is unable to bind LC3-II. However, reduction of the ΔΨm induces the redistribution of CLN to the OMM surface where it can bind to LC3-II to induce mitophagy. Of interest, CLN can also act as a pro-apoptotic factor if it is peroxidised on the OMM. This creates a scenario in which when mitophagy fails CLN induces apoptosis.

1.2.3.2 Autophagosomal degradation

Mitophagy receptors are vital structural components that establish a physical connection between damaged mitochondria and phagophores. Subsequently, phagophores fuse and eventually, engulf the LC3-II-associated mitochondria to form autophagosomes. These double-membrane structures merge with endolysosomes to mature to autolysosomes. Eventually, lysosomal hydrolases degrade the engulfed cargo.

1.2.4 Mitochondrial interplay with other cellular organelles

Friedman et al. suggested that ER-mitochondrial interplay might be crucial to define the exact sites of mitochondrial fission. These investigators proposed that potential fission sites are marked by the ER and thus, foster DPR1 assembly. Another organelle that interacts physically and functionally with mitochondria are lysosomes. Described as acidic degradation centres, lysosomes are required for degradation and recycling of dysfunctional organelles and macromolecules. Inhibition of the lysosomal program results in the accumulation of dysfunctional mitochondria and disturbance of cellular homeostasis. Conversely, it has emerged
that mitochondrial function is required for proper lysosomal activity. This was shown by Demers-Lamarche et al. who reported that disruption of mitochondrial function through, for example, deletion of *PINK1*, *OPA1* or the inhibition of the ETC, impairs lysosomal function leading to an accumulation of enlarged endolysosomal vesicles. (199) This, together with the observation that both mitochondrial as well as lysosomal biogenesis are regulated by the same TF, TFEB, indicates a strong functional link between the two. (198)

Collectively, the interplay of mitochondria with other cellular organelles emphasises the importance of thinking beyond mitochondria as isolated organelles in order to better understand the highly complex mechanism(s) behind mitochondrial dynamism and thereby, to maximise the therapeutic potential.

### 1.3 Cellular bioenergetics

To meet the cellular bioenergetic demand, ATP is generated via two major pathways: glycolysis and OXPHOS. Both, glycolysis and the tricarboxylic acid (TCA) cycle produce NADH and therefore, are highly dependent on NAD⁺ availability. To gain a better understanding of cellular bioenergetics, the next Section focuses on the basic mechanistic principles underlying glycolysis and OXPHOS as well as considering the NAD⁺ synthesis pathways.

#### 1.3.1 Glycolysis

Glycolysis is carried out in the cytosol and converts glucose to pyruvate (aerobic; O₂-dependent) or lactate (anaerobic; O₂-independent). (200) Aerobic glycolysis is a key metabolic process which fuels OXPHOS through pyruvate production, thereby, linking the two metabolic pathways. (201) Anaerobic glycolysis facilitates ATP production when oxygen is low, for example, during intense exercise or acute ischaemia, and is a short-term solution to prevent an ATP shunt. (202) Both pathways yield two ATP/glucose and are therefore not particularly efficient. (201)

#### 1.3.2 Oxidative phosphorylation

The majority of cellular ATP is produced in the mitochondrial matrix by a process known as OXPHOS. The mechanism behind OXPHOS is O₂-dependent and relies on chemiosmotic coupling through the following mechanism: The transport of electrons (e⁻) along the ETC gives rise to a proton (H⁺) gradient across the IMM, which is used by the
F1/F0-ATP synthase to generate ATP. As previously mentioned, one substrate that fuels OXPHOS is pyruvate, which is produced via glycolysis. Pyruvate enters the mitochondria, is oxidised to acetyl-CoA and condenses with oxaloacetate to form citrate, the first substrate of the TCA cycle. Additional substrates for OXPHOS are glutamine and fatty acids (e.g. palmitate). In mitochondria, glutamine is converted to glutamate, which is then turned over to α-ketoglutarate (α-KG) and as such enters the TCA cycle. Fatty acids can be taken up by peroxisomes and mitochondria, where these are oxidised to acetyl-CoA, which eventually enters the TCA cycle as citrate. In the TCA cycle, NADH is formed which is an e' donor required for ETC activity. OXPHOS yields 31.45 ATP/glucose, 12 ATP/glutamine and 112.9 ATP/palmitate, which explains why the majority of tissues use OXPHOS to generate sufficient ATP.

**Architecture and function of the ETC.** The ETC is localised to the IMM and is comprised of the following components: NADH-dehydrogenase (complex I), succinate dehydrogenase (complex II; SDH), coenzyme Q/ubiquinone (Q or CoQ₁₀), Cyt C, cytochrome c reductase (complex III; bc₁ complex), and cytochrome c oxidase (complex IV) (Figure 1.4).
The electron transfer between the ETC complexes is carried out by the lipophilic, membrane-soluble Q and Cyt C. There are two points in which e$^-$ can enter the ETC: via (i) complex I or via (ii) Q-reducing enzymes such as complex II, glycerol-3-phosphate dehydrogenase (GPDH) or electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO).(2)

(i) At the first entry point, NADH transfers an e$^-$ to complex I, which is subsequently used to reduce Q to ubiquinol (QH$_2$).(2) Consequently, complex III utilises QH$_2$ to reduce Cyt C in the mitochondrial intermembrane space. Cyt C is used by complex IV to reduce molecular oxygen (electron acceptor), which reacts to H$_2$O. A total of 10 protons are translocated from the IMS to the mitochondrial matrix for every NADH molecule oxidised. Complex II (succinate dehydrogenase; converts succinate to fumarate) is an additional entry point for electrons. Adenine nucleotide translocase (ANT) localises to the mitochondrial inner membrane and is required for ADP/ATP exchange. Uncoupling proteins (UCP) are proton transporters which return protons from the mitochondrial matrix to the IMS and thereby uncouple oxidative phosphorylation (proton leak). Figure is adapted from Sazanov et al., 2015 (2).

(ii) The second way through which e$^-$ can enter the ETC is through Q-reducing enzymes.(208) Similar to complex I, Q-reducing enzymes have the ability to reduce Q to...
QH$_2$ and thus, supply complex III with QH$_2$. However, there is a major difference: At none of the alternative entry points are H$^+$ pumped to the P side. Hence, in the case of complex II which converts succinate to fumarate using flavine adenine nucleotide (FAD) as a prosthetic group (tightly-bound redox co-factor), only 6 H$^+$ are shuttled from the N- to the P-side for every succinate oxidised. The catalysed reactions are summarised below:

**Complex I:** NADH + H$^+$ + Q + 4 H$^+$$_{in}$ → NAD$^+$ + QH$_2$ + 4 H$^+$$_{out}$  
**Complex II:** Succinate + FAD + Q → Fumarate + FADH$_2$ + QH$_2$  
**Complex III:** QH$_2$ + 2 Cyt C$^{3+}$ + 2 H$^+$$_{in}$ → Q + 2 Cyt C$^{2+}$ + 4 H$^+$$_{out}$  
**Complex IV:** 1/2 O$_2$ + 2 Cyt C$^{2+}$ + 4 H$^+$$_{in}$ → 2 H$_2$O + 2 Cyt C$^{3+}$ + 2 H$^+$$_{out}$

Of note, complex II is also a key enzyme of the TCA cycle and is highly regulated by the Q/QH$_2$ ratio in the IMM, thereby, connecting OXPHOS with the TCA cycle.

**Proton-motive force.** As mentioned above, the flow of e$^-$ along the ETC is coupled to a transfer of H$^+$ across the IMM. This build-up of H$^+$ in the IMS induces an electrochemical gradient which exerts a proton-motive force (Δp) measured in millivolts (mV). Typically, Δp is ~200 - 220mV and acts as driving force for the F1/F0-ATP synthase (complex V)-mediated rotary catalysis to synthesise ATP (2.7 H$^+$/ ATP). Of note, Δp consists of two components: (1) an electrical element, the ΔΨ$_M$ and, (2) a chemical element, the pH gradient (ΔpH). In contrast to ΔΨ$_M$, which is measured in mV, ΔpH has to be converted mathematically. This is based on the observation that at 37°C a ΔpH of 1 unit corresponds to a transmembrane potential of 60mV. Hence, the relationship between ΔpH, ΔΨ$_M$ and Δp can be described as:

$\Delta p = \Delta \Psi_M - 60 \Delta pH$

In living cells, mitochondrial ΔpH values are ~0.5-1, which indicates that the contribution to Δp is as little as 30-60 mV. Therefore, the majority of Δp (80-85%) is generated by ΔΨ$_M$. Interestingly, apart from the electrical contribution, ΔpH serves another decisive purpose as it regulates the catalytic efficiency of the F1/F0-ATP synthase. Thus Δp not only promotes complex V activity but also a low pH within the cristae, an ultimate consequence of H$^+$ accumulation. This highlights the importance of both components of Δp to mitochondrial respiration.

**Proton leak.** Mitochondrial coupling efficiency during OXPHOS is not 100%, which indicates that some of the energy is lost as heat. Such uncoupling is
predominantly caused by proton leak which allows H\(^+\) to cross the IMM and return to the mitochondrial matrix from the IMS.(215) This process is independent of the F1/F0-ATP synthase and can be experimentally shown as residual respiration rate in the presence of the F1/F0-ATP synthase inhibitor oligomycin.(215) There are two forms of proton leak: (i) the basal leak; and, (ii) the inducible leak.

(i) Basal leak. The basal leak accounts for ~20-50% of the total proton leak.(216) It is tightly linked to the metabolic rate, thus it varies in a cell type-specific manner.(216) The exact mechanisms behind the basal leak are not fully understood, particularly, since the IMM per se is ion-impermeable.(216) Nonetheless, it has emerged that there is a positive correlation between the basal proton leak and the abundance of adenine nucleotide translocases (ANTs) 1-4 (ANT1-4).(216) ANT1-4 facilitate the exchange of cytosolic ADP with mitochondrial ATP, and therefore, play a pivotal role in regulation of F1/F0-ATP synthase activity (Figure 1.4).(217)

(ii) Inducible leak. The inducible proton leak is controlled by specific integral IMM proteins including uncoupling proteins (UCPs) 1-3 (UCP1-3).(216) UCP1, for instance, localises to the BAT and drives adaptive heat generation under non-shivering conditions.(218) The expression of UCP1 is regulated by caloric intake (starvation or overeating) as well as surrounding temperature (cold or heat exposure) and thus controls metabolic efficiency.(218) In addition to thermogenesis, the proton leak exerts another vital function in the management of reactive oxygen species (ROS).(216, 219) This is of great importance as mitochondria are the prime source of cellular ROS, which can induce mutations in mitochondrial as well as nuclear DNA and thus, damage cellular components.(219, 220) Complex I and III are the predominant source of mitochondrial ROS.(216) In addition, complex II has been shown to be an important contributor to ROS.(221) During OXPHOS, single e\(^-\) leak and partially reduce O\(_2\) to form superoxide (O\(_2^.-\)).(222) O\(_2^.-\) is subsequently converted to hydrogen peroxide (H\(_2\)O\(_2\)) by superoxide dismutase 2 (SOD2).(222) Very interestingly, Galloway et al. were the first to suggest that mitochondrial uncoupling is tightly linked to the fusion/ fission machinery.(223) They found that inhibition of fission through a dominant negative (DN) form of DRP1 provoked an increase in oxygen consumption rate (OCR) and proton leak in vitro.(223) Subsequently, these investigators conducted an in vivo study in which doxycycline-inducible DN DRP1 mutant mice were treated with streptozotocin (STZ) to induce hyperglycaemia (223) and the kidneys analysed. The data showed that inhibition of fission protected STZ-treated mice from organ damage, most likely due to a proton leak-mediated reduction in mitochondrial ROS.(223)
1.3.3 NAD⁺ metabolism

NAD⁺ is a potent activator and NADH is an inhibitor of enzymes of the TCA cycle; changes in the NAD⁺/NADH ratio strongly affect mitochondrial function, in particular, OXPHOS.(224) Therefore, it is important to maintain an optimal NAD⁺/NADH ratio (Figure 1.5).

![Figure 1.5: Regulation of the NAD⁺/NADH ratio.](image)

The cellular NAD⁺/NADH ratio is a vital factor in the regulation of cellular energy metabolism (glycolysis and OXPHOS). NAD⁺ and NADH are not able to cross the mitochondrial membrane, thus, cytosolic/nuclear and mitochondrial NAD⁺/NADH pools are separate entities. Nonetheless, the cytosolic/nuclear and mitochondrial ATP pools are tightly connected via NAD⁺/NADH shuttles (via the glycerol-3-phosphate shuttle and malate/aspartate shuttle). Multiple, tightly-regulated processes such as glycolysis, TCA cycle and the ETC (complex I) help to maintain an optimal NAD⁺/NADH ratio in both compartments: NAD⁺ is required for the turnover of glucose to pyruvate (by glycolysis). Pyruvate subsequently enters mitochondria (via the pyruvate transporter) and is enzymatically converted to acetyl-CoA, the first substrate of the TCA cycle. The TCA cycle requires NAD⁺ as a co-factor and gives rise to NADH, an electron donor which fuels the ETC (via complex I). Cytosolic/nuclear and mitochondrial NAD biosynthesis is driven by multiple pathways which are regulated by the nutritional state and oxygen levels in the cell. Nicotinamide (NAM) is converted to NMN by NAM phosphoribosyltransferase (NAMPT). NMN mononucleotide (NMN) can cross the mitochondrial membranes and enter the mitochondrial matrix or remain in the cytosol, where it is converted to NAD⁺ by NMN adenylyltransferase (NMNAT) 3 or 1, respectively.
1.3.3.1 NAD⁺ biosynthesis

To better understand the implications of boosting NAD⁺ metabolism to enhance cellular bioenergetics, the following Section focuses on NAD⁺ biosynthesis. NAD⁺ can be produced through two pathways: (i) via de novo synthesis from amino acids; or, (ii) through the salvage pathways, which make use of intermediates such as nicotinic acid (NA).

(i) De novo synthesis. The first, rate-limiting step of de novo NAD⁺ biosynthesis requires the essential amino acid L-tryptophan, which originates from dietary intake (Figure 1.6). In this step tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO) catalyse the oxidative cleavage of L-tryptophan to N-formylkynurenine. TDO and IDO differ in their tissue distribution as TDO is predominantly expressed in liver and brain, whereas, IDO expression appears to be ubiquitously (227, 228). This initial step is followed by four enzymatic reactions in which N-formylkynurenine is converted to α-amino-β-carboxymuconate-ε-semialdehyde (ACMS). This unstable intermediate can then either be transformed to α-amino-β-muconate-ε-semialdehyde via enzymatic conversion mediated by ACMS decarboxylase (ACMSD) or undergo spontaneous, non-enzymatic cyclisation, which leads to the NAD⁺ precursor quinolinic acid.

In the second rate-limiting step quinolinate phosphoribosyltransferase (QPRT), which is mainly expressed in liver and kidney, adds 5-phospho-α-d-ribose 1-diphosphate to quinolic acid, forming nicotinic acid (NA) mononucleotide (NAMN). In a subsequent enzymatic reaction, NAMN is turned into NA adenine dinucleotide (NAAD) by NAM mononucleotide (NMN) adenylyltransferase (NMNAT), which uses adenine monophosphate (AMP) as a co-substrate. Three isoforms (NMNAT1-3) have been identified in humans, which differ in their tissue expression as well cellular distribution. NMNAT1, which is a nuclear enzyme, shows universal expression with increased levels in skeletal muscle, heart, kidney as well as liver and to a lesser extent in the brain. NMNAT2 is highly expressed in the brain and resides in the cytoplasm as well as the Golgi apparatus. NMNAT3 shows highest expression in lung, spleen and kidney and is expressed in mitochondria and the cytoplasm. It remains widely unclear why the NMNAT isoforms are so highly compartmentalised (nucleus, cytoplasm, mitochondria). To address the question whether they contribute to the formation of different NAD⁺ pools, Cambronne et al. recently generated a fluorescent biosensor which binds NAD⁺ and thus, allows its visualisation. Using a knockdown strategy for the three NMNAT isoforms, the group showed that NMNAT1 contributes to cytoplasmic NAD⁺ levels and that depletion of...
NMNAT2 also affects mitochondrial NAD$^+$ levels. Their findings suggest a substantial contribution of NMNAT isozymes to the cellular NAD$^+$ pool independent of their localisation. This hypothesis is also supported by observations in vivo as Nmnat3$^{-/-}$ mice do not show changes in mitochondrial NAD$^+$ levels.(237)

In the last step of de novo NAD$^+$ biosynthesis, NAD$^+$ synthase (NADS) amidates NAAD to NAD$^+$ in an ATP-dependent manner with glutamine as an amide donor.(238) The human NADS1 enzyme is also called NADsyn1 and is predominantly expressed in the small intestine, liver and kidney.(238)

Notably, in a ground breaking study, Tran et al. identified the transcriptional co-activator PGC1α, which is a master regulator of mitochondrial biogenesis, as key to the regulation of expression of enzymes involved in de novo synthesis of NAD$^+$.(64) The
group showed that in vivo manipulation of PGC1α expression (Pgc1α−/− versus renal-specific, inducible Pgc1α mice) correlated with changes in expression of enzymes of the de novo synthesis pathway and thus, NAD⁺ levels. Moreover, using an IRI model the group showed that increasing NAD⁺ levels enhances OXPHOS and thereby, acts nephroprotective.

(ii) Salvage pathways. As mentioned earlier, there is also an alternative way to synthesise NAD⁺, which makes use of the three dietary NAD⁺ precursor vitamins NA (also known as niacin or Vitamin B₃), NAM, nicotinic acid riboside (NAR) and nictotinamide riboside (NR). These metabolic pathways are referred to as salvage pathways and are presumed to be the leading sources of NAD⁺ (Figure 1.7).

![Figure 1.7: NAD⁺ biosynthesis via salvage pathways.](image-url)

NAD⁺ can be synthesised via the salvage pathway which uses the following precursors as substrate: nicotinic acid (NA), nicotinamide (NAM) and nicotinamide riboside (NR). The synthesis of nicotinic acid mononucleotide (NAMN) from NA (via NA phosphoribosyltransferase (NAPT)) is referred to as the Preiss-Handler pathway and gives rise to nicotinic acid mononucleotide (NAMN). NAMN can also originate from de novo NAD⁺ biosynthesis in which quinolinic acid is converted to NAMN by quinolinate phosphoribosyl-transferase (QPRT). NAMN is subsequently transformed to nicotinic acid adenine dinucleotide (NAAD) by nicotinamide mononucleotide acetyltransferase (NMMAT). NAD synthase consequently amidates NAAD to nicotinamide adenine dinucleotide (NAD). An additional salvage pathway uses nicotinamide (NAM) as a substrate. NAM is converted to NAM mononucleotide (NMN) by NAM phosphoribosyltransferase (NAMPT). NMN can also be generated by NR kinase (NRK) which uses NR as a substrate. NMN is subsequently converted to NAD by NMMAT. Riekelt H. Houtkooper, Carles Cantó, Ronald J. Wanders, Johan Auwerx, The Secret Life of NAD⁺: An Old Metabolite Controlling New Metabolic Signalling Pathways, Endocrine Reviews, Volume 31, Issue 2, 1 April 2010, Pages 194–223 by permission of Oxford University Press.
Of the three NAD* precursors, NAM appears to be the main contributor to NAD* production in mouse and man.(240, 241) The enzyme responsible for NAM conversion to NMN is the NAM phosphoribosyltransferase (NAMPT).(239) NAMPT is widely expressed with increased levels in kidney and heart as well as liver, and the lowest levels in the brain.(242, 243) In transformed human embryonic kidney 293 (HEK293T) cells, NAMPT predominantly resides in the cytosol, however, it can also be found in the nucleus and mitochondria.(244) Yang et al. were the first to show that NAMPT expression is induced by nutrient depletion, hypoxia and genotoxic stress and thus, protects cells from poly-ADP-ribose polymerase (PARP)-1 mediated apoptosis.(244)

The second salvage pathway, which contributes to cellular NAD* synthesis, is the Preiss-Handler pathway. In the first step of this pathway, NA and 5-phospho-α-d-ribose 1-diphosphate are catalytically transformed to NAMN by the enzyme NA phosphoribosyltransferase (NAPT).(245) Subsequently, NAMN is channelled into the de novo synthesis pathway, giving rise to NAD* as final product. The highest expression of NAPT has been reported in the small intestine, kidney, heart, liver and stomach and similar to NAMPT, it appears to be very low in the brain.(243) Of note, NAPT predominantly localises to the nucleus and cytosol but not to mitochondria.(246, 247)

The last salvage pathway listed in this paragraph is catalysed by NR kinase (NRK, also named NMRK), which phosphorylates and converts NAR and NR to NAMN and NMN, respectively.(239) Both products are then further transformed by NMNAT enzymes, which show a rare dual substrate specificity.(239) In mammals, two NRK isoforms have been found (NRK1 and NRK2), which are both assumed to be functional redundant.(248) Of note, NRK1 is widely expressed in various tissues with peak levels in the kidney whereas NRK2 seems to predominantly reside in the muscle.(249) Based on the observation that NRKs primarily localise to the cytoplasm, it is presumed that the NRK1/2 product NMN may be able to cross the mitochondrial membrane and thereby, contribute to NMNAT3-mediated NAD* synthesis.(246, 250)

1.3.3.2 NAD* turnover

Apart from NAD* turnover through glycolysis or the TCA cycle, there are three major enzyme families which require NAD* as a cofactor: (i) PARPs, (ii) CD38/CD157 enzymes and (iii) SIRTs.(251-253)

(i) PARPs. PARPs localise to the nucleus and are involved in the regulation of DNA repair, metabolism and transcriptional regulation.(251) One example is PARP-1, which is regarded as a nuclear enzyme with primary functions in DNA repair and chromatin remodelling (254), however, it has also been shown to localise to
mitochondria. (255) Although its exact functions remain controversial, it is generally accepted that PARP-1 hyperactivation promotes mitochondrial dysfunction as indicated by reduced $\Delta \Psi M$ as well as respiration. (254-257) Martin et al. found that PARP-1 expression increased in PTECs in a rat model of IRI (in particular the injury-sensitive S3 segment) and this exacerbated tubular cell injury via ATP depletion, which could be alleviated by PARP-1 inhibition. (258) Interestingly, while PARP-1 mediated NAD$^+$ depletion may be one mechanism of how PARP-1 impairs cellular bioenergetics (255, 259), the enzyme has also been shown to affect metabolic pathways including glycolysis more directly by poly(ADP-ribosyl)ation of e.g. glyceraldehyde-3-phosphate dehydrogenase (260). Notably, mitochondrial PARP-1 also induces, the translocation of the apoptosis-inducing factor (AIF) from the mitochondria to the nucleus, and thus, leads to the induction of programmed cell death. (261-263)

(ii) CD38/CD157-type enzymes. CD38/CD157-type enzymes are ectoenzymes (localise to the cell surface), which catalyse NAD$^+$ to cyclic ADP ribose (cADPR) and NAADP which are implicated in Ca$^{2+}$ signalling. (252) Both PARPs and CD38/CD157 are regarded as major NAD$^+$ consuming enzymes. (264)

(iii) SIRTs. The third family of NAD$^+$-consuming enzymes are the SIRTs, which localise to the nucleus, cytosol and mitochondria where they have been shown to regulate a variety of biological processes such as energy metabolism, ROS management and gene transcription. (1, 265) SIRTs are functionally characterised as NAD$^+$-dependent KDACs due to their ability to remove a multitude of PTMs including acetylation, malonylation, glutarylation and succinylation from K residues. (1, 265, 266) PTMs have emerged as key protein modifications which have major implications on cellular function ranging from the regulation of major metabolic enzymes to stem cell pluripotency. (266-268) Due to their importance in the regulation of cellular bioenergetics, the next Section will focus on PTMs and their regulation through the SIRTs.

1.4 Post-translational modifications

To maintain metabolic homeostasis, organisms have developed adaptive mechanisms to fine-tune the efficiency of cellular ATP production based on substrate supply and energetic demand. As previously discussed (Section 1.2.1.2), metabolic changes induced by, for example, exercise, trigger specific transcriptional programs (e.g. via PGC1α), which directly impact on mitochondrial metabolism and biogenesis. Although transcriptional induction is one approach to adapt bioenergetically, it is regarded as a mechanism for long-term adjustment, as gene transcription can take several minutes. (269, 270) This would mean that by the time metabolic genes are turned
over, the metabolite pools would be depleted and cells would run the risk of entering an ATP crisis leading to apoptosis. Therefore, organisms have developed an additional, more rapid mechanism for bioenergetic adaptation through adjusting the efficiency and capacity of metabolic enzymes. This is mediated by allosteric regulation and/or PTMs and allows an immediate response (within seconds). Typical examples of PTMs that influence mitochondrial enzyme activities/levels are phosphorylation, ubiquitinylation and K-acylations such as K-acetylation (KAc), -succinylation (KSucc), -alonylation (KMal) or -glutarylation (KGlut). In recent years, K-acylations have emerged as key regulatory modifications that control cellular metabolism. This idea arose from the observation that the K-acylation status is controlled by cellular concentrations of various acyl-CoAs such as acetyl-/malonyl-/succinyl-/glutaryl-CoA. Acyl-CoAs are generated and consumed by the TCA cycle, and therefore, directly link mitochondrial metabolism to metabolic flux. In fact, changes in nutrient availability provoke variations in the levels of acyl-CoAs including acetyl- and succinyl-CoA. Excessive caloric intake through, for instance, a high fat diet (HFD), provokes an increase in acetyl- and succinyl-CoA. This in turn brings about elevated levels of mitochondrial KAc and KSucc, which are known to impact on enzyme activities. The mechanisms through which K-acylations modulate cellular metabolism lie in the disruption of ionic interactions, particularly, though the elimination of the positive charge (+1) on K. Of note, acetyl-CoA as well as malonyl-, -succinyl and -glutaryl-CoA differ in their size and chemical properties: While acetyl-CoA is not charged and contains two carbons, malonyl-, -succinyl and -glutaryl-CoA contain an acid group, which is negatively charged at physiological pH. In addition, the latter three are larger than acetyl-CoA and comprise three, four and five carbons, respectively. Based on the charge difference, KAc ultimately renders K neutral (from +1 to 0), while the acidic modifications KSucc, KMal and KGlut, result in a change from +1 to -1. In particular, the dramatic change from a positively charged, unmodified lysine to a negatively charged, acidic modification (KSucc, KMal and KGlut) has the potential to disrupt any pre-existing ionic bonds with negatively charged moieties and thereby, influence protein structure and function.

Of note, KAc can be formed enzymatically by K acetyl transferases (KATs) or by non-enzymatic addition of acetyl-CoA. In contrast, no KGlut, KMal or KSucc transferases have been identified which is why it is thought that conjugation of acidic acyl-CoAs occurs non-enzymatically. Under basic conditions as acyl-CoAs are found in the mitochondrial matrix (pH 7.9), they are highly reactive. This combined with their increased abundance in the mitochondria, generates an optimal milieu for non-enzymatic K-acetylation. The removal of K-acylations is mediated by specific K...
deacylases. (266) The enzymes responsible for K-deacylation are commonly known as KDACs (formerly referred to as histone deacetylases (HDACs)). (272) A KDAC family that recently emerged as central regulators of K-acylation status is the SIRT family of NAD⁺-dependent class III KDACs. (274, 275)

1.5 The Sirtuins

The SIRTs are a highly conserved protein family, which can be found in organisms ranging from bacteria through yeast to humans. (276-278) In particular eukaryotic SIRTs show strong phylogenetic similarities in the preserved ~260 amino acid (AA) catalytic core domain. (1) The sequence homology of the catalytic core is of significance, as it is assumed that this facilitates formation of a channel in which NAD⁺ reacts with its substrate. (279) Of interest, based on the phylogenetic classification, the SIRTs can be further divided into four sub-classes, which mirror their enzymatic activities: (278) Class I sirtuins (SIRT1-3) are strong deacetylases; class II sirtuins (SIRT4) possess weak ADP-ribosyl transferase activity; and, class III sirtuins (SIRT5) have weak deacetylase activity; SIRT5 is described as de-malonylase/glutarylase/succinylase. (1) Class IV sirtuins (SIRT6/7) possess ADP-ribosyltransferase and deacetylase activity (SIRT6) (1) as well as recently discovered, desuccinylase activity (SIRT7) (280) (Figure 1.8).

![Figure 1.8: Classification of the SIRT family. Class I (Blue): SIRT1-3, Class II (Green): SIRT4, Class IV (Yellow): SIRT5, Class V (Red): SIRT6 and SIRT7. Size of the proteins is given in kDA. Figure adapted from Hirschey et al., 2011.(1) ](#)

The variation in substrate preference is, at least in part, determined by the size of the substrate-binding site. This hypothesis was proposed by Schuetz et al. who analysed the crystal structure of SIRT5 and revealed an enlarged substrate-binding site (281) which would also explain the ability of SIRT5 to remove large K modifications such as malonylation, glutarylation and succinylation.
The mammalian sirtuin family consists of seven members (SIRT1-7), which differ in their subcellular localisation: nuclear (SIRT1-3 and 5-6) (282-284), nucleolar (SIRT7) (282), mitochondrial (SIRT3-5) (285) and cytosolic (SIRT1-3, 5) (286-288). SIRTs target a large number of proteins throughout the cell and thereby, regulate a variety of processes including metabolism, the cell cycle, chromatin structure and transcription.(289) The SIRTs themselves are regulated on two levels: (i) enzyme activity; and, (ii) transcription.

(i) Regulation of SIRT enzyme activity. Changes in nutrient availability, excess or depletion, result in a decrease or increase in cellular NAD⁺, respectively, which directly impacts on SIRT activity.(290) To quantify basal NAD⁺ levels in various subcellular compartments, Cambronne et al. developed a NAD⁺ biosensor and showed variations in local NAD⁺ concentrations.(236) The reported levels of NAD⁺ in nucleus, cytosol and mitochondria were 106, 109 and 230μM, respectively.(236) Interestingly, although the SIRTs require NAD⁺ as a co-factor, fluctuations in NAD⁺ levels do not affect all SIRTs equally.(291) This is due to variations in the K_M values of SIRTs for NAD⁺: 95μM (SIRT1), 83μM (SIRT2), 880μM (SIRT3), 35μM (SIRT4), 980μM (SIRT5) and 26μM (SIRT6).(291) Given that the NAD⁺ concentrations in the nucleus, cytosol and mitochondria are 106, 109 and 230μM, respectively, (236) it appears that the SIRTs with very low K_M values (SIRT4 and 6) may be relatively unresponsive to NAD⁺ fluctuations, while others including SIRT1, 3 and 5 might compete for NAD⁺.

(ii) Regulation of SIRT transcription. Little is known about the transcriptional regulation of the SIRTs. However, it has been reported that PGC1α and TFEB can regulate SIRT expression. PGC1α drives the transcription of SIRT3 (292) and 5 (293) (via PPARα and ERRα) as well as enzymes involved in NAD⁺ synthesis (e.g. NAMPT, NMNAT1, IDO2, etc.) (64), thereby, directly promoting mitochondrial metabolism. Of interest, PGC1α (127) itself as well as its activator SIRT1 (141), are direct targets of the nutrient-sensitive transcription factor TFEB, giving rise to an intricate network that directly links nutrient availability to mitochondrial function.

Dysregulation of SIRT activities is believed to influence a wide range of pathologies including CVD, kidney disease, diabetes and cancer.(253) In fact, mitochondrial SIRT3 and 5 were recently identified as key enzymes to cardiac and renal health.(6)

1.5.1 Mitochondrial sirtuins

Three of the seven sirtuins (SIRT3, 4 and 5) are predominantly located in the mitochondria, and are referred to as mitochondrial SIRTs.(6) Based on their subcellular
localisation, mitochondrial SIRTs are regarded as central stress sensors that modulate mitochondrial enzyme activities when stimulated. Several lines of evidence indicate that all three SIRTs are involved in the regulation of fundamental metabolic pathways including FAO (294-296), ketogenesis (296, 297) and glycolysis/gluconeogenesis (288). Additionally, it has emerged that SIRT3 (298, 299) and 5 (300) negatively regulate autophagy, a process which is highly activated during caloric restriction (CR) and through which they counteract cell death induced by excessive autophagic flux. The following paragraphs will briefly discuss the mitochondrial SIRTs (SIRT3 and 5), to assess their potential for as therapeutic targets to treat mitochondrial-associated metabolic dysfunction.

1.5.1.1 SIRT3

The K deacetylase SIRT3, is the most studied mitochondrial sirtuin. SIRT3 is highly enriched in metabolically-active tissues such as kidney, brain, heart, BAT and liver. When Sirt3⁻/⁻ mice were originally characterised, they were described as phenotypically normal, however, they displayed global mitochondrial hyperacetylation. Subsequent studies found that SIRT3 ablation results in a significant reduction of cellular ATP (~50%) in heart, kidney and liver under basal conditions. The reason for this was explained by Hirschey et al. who showed that Sirt3⁻/⁻ mice exhibit a defect in FAO leading to disturbed mitochondrial bioenergetics. The group also showed that Sirt3⁻/⁻ mice lack metabolic flexibility during CR and fail to switch from a glucocentric to an adipocentric metabolism. Additional phenotypic characterisation revealed that Sirt3⁻/⁻ mice develop age-associated cardiac hypertrophy as well as multi-organ fibrosis and are susceptible to developing metabolic syndrome due to bioenergetic dysfunction.

**SIRT3-regulated pathways.** When glucose levels drop, cells switch metabolically to find nutrients that can be used to sustain metabolic homeostasis. As previously discussed, CR is a potent stimulus that increases SIRT3 expression and activity. In subsequent consecutive deacetylation reactions, SIRT3 activates enzymes of numerous metabolic pathways to promote aerobic metabolism and maintain cellular ATP levels. Specific enzymes targeted by SIRT3 catalyse central reactions of metabolic pathways including (i) FAO, (ii) ketogenesis and (iii) amino acid metabolism. In addition, SIRT3 activity has been shown to (iv) reduce cellular ROS levels and (v) control mitochondrial architecture.

(i) FAO. A central mitochondrial FAO enzyme activated by SIRT3 is long-chain acyl-CoA dehydrogenase (LCAD). High fat diets or SIRT3 ablation results in LCAD...
hyperacetylation resulting in reduced enzymatic activity and defective FAO.(307) An
additional enzyme activated by SIRT3 is the trifunctional enzyme, subunit A (HADHA),
an enzyme central to mitochondrial FAO.(308)

(ii) Ketogenesis. During starvation, free acetate is released into the blood stream
and subsequently converted to acetyl-CoA which can either enter the Krebs cycle to
promote ATP production, or can be used for ketone body production.(309) The
enzymatic conversion of acetate to acetyl-CoA is mediated by acetyl-coA synthethase 2
(ACS2), which is targeted and activated by SIRT3.(310) Further studies found that SIRT3
deaetyltes 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), the rate-limiting
enzyme in ketogenesis and thus, increases its catalytic activity.(297) This metabolic
pathway is of great importance as ketone bodies are an essential energy source,
replacing glucose in various tissues, particularly the brain.(311) Sirt3⁻/⁻ mice failed to
activate HMGCS2 resulting in reduced serum levels of ketone bodies compared to wild-
type mice.(297)

(iii) Amino acid metabolism. Prolonged starvation is a potent activator of
autophagy, which releases free amino acids to supply cells with an additional energy
source.(312) Interestingly, SIRT3 was also reported to increase the activity of glutamate
dehydrogenase (GDH), an enzyme central to amino acid catabolism during
starvation.(313) GDH catalyses the conversion of glutamate to α-ketoglutarate (α-KG), a
metabolite that can enter the TCA cycle to fuel ATP production.(313) Furthermore,
Hallows et al. reported that SIRT3 can activate ornithine transcarbamoylase (OTC), a
key enzyme of the urea cycle and promotes detoxification and clearance of ammonia
during CR.(314)

(iv) ROS management. Beyond the regulation of metabolic enzymes, SIRT3
activity has also been linked to ROS management: SIRT3 deacetylates and activates the
transcription factor forkhead box O3a (FoxO3a), which subsequently increases the
expression of superoxide dismutase 2 (SOD2) and catalase.(315, 316) A second
proposed mechanism through which SIRT3 moderates cellular oxidative stress is via
activation of isocitrate dehydrogenase 2 (IDH2).(317) IDH2 reduces isocitrate to α-KG,
thereby, increasing the levels of NADPH, a redox co-factor essential for ROS elimination
through generation of reduced glutathione.(317)

(v) Mitochondrial architecture. Although the impact of SIRT3 on mitochondrial
dynamism remains enigmatic, it has emerged that SIRT3 can act as a pro-fusion
molecule. One study indicated that SIRT3 deacetylates and activates OPA1 and thereby,
protects cardiomyocytes from doxycycline (DOX)-induced cell death.(318)

SIRT3 in disease. As mentioned earlier, SIRT3 is the most studied mitochondrial
SIRT both in vitro and in vivo.(319) Multiple renal injury models in Sirt3⁻/⁻ mice such as
palmitate, cisplatin (320), sepsis (321) or harmful environments that reduce SIRT3 expression in wild-type mice such as angiotensin II (AngII) infusion (322), aggravated organ damage. Injury could be partially rescued by artificial overexpression (323, 324) or activation of SIRT3 via honokiol (324) treatment, highlighting a putative protective role of SIRT3 in the kidney. Interestingly, the beneficial effects of SIRT3 activation are not restricted to the kidney. Pillai et al. showed that honokiol-mediated SIRT3 activation also reverses pressure-mediated cardiac hypertrophy in a mouse model.(325)

In conclusion, the current findings suggest that activation of SIRT3 may be a putative pharmacological strategy to treat mitochondrial dysfunction-associated diseases.

1.5.1.2 SIRT5

SIRT5 is a weak K-deacetylase (313), however, it displays strong K-desuccinylase (326), -demalonylase (326) and -deglutarylase (327) activities. Similar to SIRT3, SIRT5 expression is highest in heart, liver, kidney and brain.(328) When initially characterised, Sirt5
def mice were described as phenotypically normal without any obvious metabolic defect under basal conditions, although, they displayed a global increase of protein KSucc and KMal.(329) Subsequent studies, however, showed that like Sirt3
def mice, Sirt5
def mice present with reduced cardiac ATP levels due to defective fatty acid metabolism.(330) As a consequence, SIRT5-null mice develop cardiac hypertrophy with age.(330) Additional studies showed that ablation of SIRT5 dramatically impairs metabolic flexibility as previously described for Sirt3
def mice.(296, 328) In brief, it emerged that SIRT5 targets and activates key enzymes of FAO, ketogenesis and ammonia detoxification.(296, 328) As a result Sirt5
def mice fail to adapt to a low glucose environment and, display reduced ketogenic capacity and hyperammonaemia during fasting.(296, 328)

SIRT5-regulated pathways. SIRT5 activity and expression is induced by metabolic stresses such as hypoxia.(293, 331, 332) Intriguingly, SIRT5 appears to control the same metabolic pathways as SIRT3 including (i) FAO, (ii) ketogenesis, (iii) amino acid metabolism, (iv) ROS management as well as (v) mitochondrial architecture. SIRT5 was also found to regulate (vi) glycolysis.

(i) FAO. Sirt5
def mice display reduced cardiac ATP levels as a consequence of defective FAO.(330) Differential acylation analysis showed that mitochondrial trifunctional enzyme, subunit alpha (HADHA) and very long-chain acyl-CoA dehydrogenase (VLCAD) (333), two enzymes key to FAO, are SIRT5 targets. As (1) HADHA and VLCAD are essential enzymes for mitochondrial FAO and (2) fatty acids are
the major source of energy production in the heart, this finding offers an explanation for why Sirt5−/− mice develop cardiac hypertrophy with age.(330)

(ii) Ketogenesis. HMGCS2 is the rate-limiting enzyme, which controls conversion of acetoacetyl-CoA and acetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).(296) HMGCS2 was found to be a primary target of SIRT5. Mass spectrometric analysis of mitochondrial extracts from murine Sirt5−/− livers showed that compared to all other mitochondrial proteins analysed, HMGCS2 contains the most KSucc sites as well as the most KSucc sites targeted by SIRT5.(296) In addition, it emerged that some of the KSucc sites are located in the substrate binding pocket resulting in a dramatic reduction in enzyme activity when succinylated.(296) This explains how SIRT5-mediated K-desuccinylation facilitates HMGCS2 activation and thus, drives ketogenesis, a mechanism key to cell survival during CR.(296)

(iii) Amino acid metabolism. Polletta et al. showed that SIRT5 desuccinylates and inhibits glutaminase (GLS), thereby, controlling glutamine metabolism.(300) GLS catalyses the conversion of glutamine to glutamate and ammonia which is known to induce autophagy.(300) These investigators also found that the SIRT5 inhibition-mediated increase in ammonia results in the accumulation of the mitophagy receptor BNIP3, and promotes mitophagy.(300) Of note, SIRT5 also deacetylates carbamoyl phosphate synthetase 1 (CPS1), an enzyme that catalyses the initial step of the urea cycle, and so promotes ammonia detoxification during starvation.(328) Taken together, these findings clarify the causes of hyperammonemia (328) and increased mitophagy (300) observed upon SIRT5 ablation.

(iv) ROS management. Interestingly, SIRT5 was reported to desuccinylate and activate the cytosolic SOD1 (334), which reduces cellular oxidative stress and thereby, promotes lipid metabolism. An additional SIRT5 K-desuccinylation target is the succinate dehydrogenase, subunit A (SDHA), an important component of ETC complex II.(335) K-desuccinylation of SDHA has been described as both activating (336) and inhibitory (335). Complex II is regarded an important source of mitochondrial ROS (221), indicating that reducing its activity may be beneficial to decrease cellular oxidative stress under certain conditions. Notably, Boylston et al. have shown that Sirt5−/− increases cellular ROS and aggravates cardiomyocyte injury during ischaemia, which could be ameliorated by complex II inhibition.(337)

(v) Mitochondrial architecture. Very recently, Guedouari et al. demonstrated that in the context of nutrient deprivation, SIRT5 stimulates mitochondrial elongation and protects mitochondria from autophagic degradation and apoptosis.(338) The group showed that loss of SIRT5 increased the protein levels of the pro-fission marker DRP1 and its mitochondrial receptors FIS1 and MiD51, leading to increased
It remains to be seen whether SIRT5 directly regulates mitochondrial dynamics/mitophagy or whether the increased mitophagy seen in Sirt5−/− cells under basal conditions, is a result of metabolic dysfunction.(338)

(vi) Glycolysis. Nishida et al. showed that glucose metabolism is a major SIRT5 target.(288) SIRT5-mediated K-demalonylation facilitated the activation of essential glycolytic enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aldolase B (ALDOB) to increase cellular glycolytic capacity.(288) Conversely, SIRT5 ablation reduced glycolytic capacity and ATP synthesis, indicating the importance of SIRT5 for the regulation of glycolysis.(288)

SIRT5 in disease. As yet, few injury models have been carried out aimed at dissecting the role of SIRT5 in disease. Zhu et al. were the first to show that intermittent hypoxia, a treatment regime associated with increased stress tolerance, leads to increased cardiac SIRT5 protein expression.(332) Later, Boylsten et al. conducted a cardiac ischaemia model in mice and showed that SIRT5 augments injury by inhibiting oxygenation-induced mitochondrial ROS production, most likely in a complex II-dependent manner.(337) In a newly published cardiac pressure overload model, Hershberger et al. showed increased damage in Sirt5−/− hearts due to the hypersuccinylation-induced reduction in FAO.(339) The group also showed that ablation of SIRT5 impairs glucose oxidation by reducing the activity of TCA cycle enzymes, which contributes to disease pathogenesis.(339) As previously mentioned, SIRT5 has been shown to promote mitochondrial fusion in a MiD51/DRP1-dependent manner.(338) Of interest, Perry et al. recently reported that ablation of DRP1 ameliorated IRI-induced damage, possibly, through maintaining mitochondrial architecture and thereby, function.(52) As SIRT5 is highly expressed in the kidney (328), and SIRT5 regulates a large number of metabolic processes including FAO and mitochondrial structure, it may be hypothesised that SIRT5-deficiency would aggravate experimental renal injury.

In conclusion, the current findings suggest that SIRT5 may be a promising target to support mitochondrial function in a nutrient-deprived environment such as occurs in CR or ischaemia (acute and chronic). Further studies, including in vivo animal disease models, are necessary to gain a better understanding of SIRT5 function and assess its potential as a drug target to treat mitochondrial dysfunction in the heart and kidney.

1.5.2 SIRT SNPs and the link to human disease

SIRT3. Rose et al. were the first to suggest that a SIRT3 polymorphism may enhance human longevity.(340) Subsequent investigations by Bellizzi et al. revealed that this specific intronic polymorphism localises to a variable number of tandem repeat
(VNTR) region resulting in an increase of SIRT3 protein expression.\(^{(341)}\) In addition, Hirschey et al. identified a single nucleotide polymorphism (SNP) in SIRT3 leading to a reduction of SIRT3 activity which increased the susceptibility to developing type two Diabetes Mellitus (T2DM) and metabolic syndrome.\(^{(305)}\) These findings support the notion that SIRT3 expression and activity may be crucial to health and longevity. Yin et al. found five DNA sequence variants in patients with myocardial infarction which reduced SIRT3 promoter activity suggesting that resulting reduction in SIRT3 levels may increase susceptibility to cardiac infarction.\(^{(342)}\)

**SIRT5.** Glorioso et al. identified a polymorphism in the human SIRT5 promoter which reduced gene expression and was associated with older molecular age of the brain based on specific age-related biosignatures.\(^{(343)}\) Furthermore, it was reported that polymorphisms in SIRT5 result in a predisposition to diabetic nephropathy in T2DM patients (344), enlarged carotid plaques in patients with atherosclerosis (345) and can modulate human lifespan (346). In addition, Chen et al. identified five SIRT5 gene promoter variants (SNPs and DNA sequence variants) which reduced SIRT5 promoter activity by decreasing TF binding, in patients with acute myocardial infarction.\(^{(347)}\) Although the group did not quantify SIRT5 levels, their findings indicated that reduced SIRT5 promoter activity may result in lower SIRT5 expression thereby, increase the susceptibility to develop a myocardial infarct.\(^{(347)}\)

**PGC1α.** In addition to the disease-associated polymorphisms found in the SIRTs, a polymorphism in the human PGC1α gene has been identified which is associated with insulin resistance as well as an increased risk of developing T2DM.\(^{(348, 349)}\) The same variant has also been linked to an increased risk of microalbuminuria in patients with T2DM.\(^{(350)}\) However, these studies are purely correlative and it remains unclear whether this variant actually impairs PGC1α function and thus, contributes to T2DM formation. Current evidence strengthens the link between mitochondrial dysfunction and insulin resistance \(^{(351)}\) which may arise from or be a consequence of a reduction of OXPHOS genes in patients with T2DM (352). One reason for this may be the decline of PGC1α mRNA expression seen in diabetic patients (353), which potentially leads to reduced SIRT3 and 5 as well as dysregulation of mitochondrial biogenesis. However, it should be stressed that without experimental proof no functional deductions can be made.
1.6 Aims and objectives

Acute and chronic kidney diseases are a major global health concern and are associated with high levels of morbidity and mortality due to the lack of effective therapies.(354, 355) Interestingly, accumulating evidence has identified mitochondrial dysfunction as a major contributor to the pathogenesis of AKI as well as CKD.(356) In particular, excessive mitochondrial fragmentation results in metabolic dysfunction and in addition, induces apoptosis due to Cyt C release.(357) Recent studies have shown that the NAD$^+\text{-dependent enzyme SIRT5, is essential to prevent mitochondrial fragmentation during starvation (338) and therefore, might be a promising target to ameliorate kidney disease.}

The overall goal of this PhD project was to dissect the role of SIRT5 in cellular metabolism in the kidney under basal conditions as well as during acute and chronic renal disease with a particular interest in mitochondrial dysfunction as indicated by structural and functional changes of the organelle. The three major aims of the present study were:

Aim 1. Determine the expression of SIRT5 in human and murine kidneys and assess the impact of AKI and CKD on expression levels. These studies addressed three objectives to: (i) elucidate the pattern of SIRT5 expression in the human kidney and correlate this with mitochondrial content; (ii) examine age-related changes in SIRT5 expression and mitochondrial content; and, (iii) determine the effect of acute and chronic kidney disease on SIRT5 expression.

Aim 2. Determine the regulation of SIRT5 expression and SIRT5 function in hPTECs in vitro. These studies addressed three objectives to: (i) develop an in vitro model which mimics in vivo renal ischaemia (combined oxygen/nutrient deprivation (OND)); (ii) determine whether in human proximal tubular epithelial cells (hPTECs) SIRT5 expression is affected by metabolic stress (induced by hypoxia and nutrient deprivation) and dissect the underlying regulatory mechanisms; and, (iii) assess whether SIRT5 is required for normal mitochondrial function in hPTECs and whether SIRT5 depletion (by RNAi) exacerbates mitochondrial dysfunction in OND.

Aim 3. Determine the role of SIRT5 in AKI and CKD in vivo. These studies addressed three objectives to: (i) assess the impact of a Sirt5$^\text{-/-}$ on normal renal function and determine the effect of SIRT5 ablation on disease pathogenesis in (ii) an acute and (iii) a chronic mouse model of kidney injury.
Chapter 2

General Materials and Methods
This Chapter contains only general material and methods, which are relevant to more than one Results Chapter. Specific methods are described in detail in the relevant Chapters.

2.1 Chemicals

Unless otherwise indicated, all chemicals were obtained from Sigma®.

2.2 Mouse breeding, maintenance and renal injury models

For all experimental procedures male wild-type (WT) or Sirt5⁻/⁻ mice on a C57BL/6J background (10-12 weeks) were used. Sirt5⁻/⁻ mice, which were originally on a mixed background (C57BL/6J and 129SV), were backcrossed for 5 generations with C57BL/6J mice by the Auwerx group. Mice were bred and raised in the Biological Services Unit, UCL Royal Free Campus. Mice were housed in groups of 3-5 and reared under specific pathogen-free (SPF)-conditions in individually ventilated cages (IVCs) with a diurnal 12h light and dark cycle. All animals had free access to water/standard mouse chow diet (RM3 Expanded; Special Diets Services, Cat# 801066). Mice were housed and handled in accordance with good animal practice as defined by the Federation of European Laboratory Animal Science Associations (FELASA). All procedures were carried out under Home Office licenses (Personal license: I98BBAB4D; Project license: P6377F606).

2.2.1 Unilateral ischaemia and reperfusion injury

All surgical procedures were performed under aseptic conditions. Mice underwent clamping and release of the renal artery to induce IRI. Contralateral (CL) kidneys served as control. Mice were anaesthetised with 2% isoflurane (Merial) and placed on a heating pad (37°C) to prevent hypothermia. After the depth of anaesthesia was confirmed by a loss of reflexes (toe pinch), the anterior abdominal skin was shaved and wiped with Videne® Antiseptic Solution (Ecolab). Analgesia was provided by subcutaneous (s.c.) injection of Carprofen (5mg/kg; Norbrook®). A midline laparotomy was conducted via an incision of the avascular linea alba. To induce IRI, the left kidney was exposed and the renal artery was clamped with a vascular clamp (S&T®, B-1 V) for 30 or 40min. During the ischaemic period, a sterile gauze moistened with saline was placed over the incision site to prevent the abdominal cavity from drying out. At the end of this period, the clamp was removed and the linea alba and then skin were closed separately using sterile sutures: The muscular layer was closed with a continuous running suture and the skin was closed with a horizontal interrupted mattress suture. The
wound was sanitised with Videne® Antiseptic Solution and 1ml warm (37°C) isotonic sodium chloride solution was injected subcutaneously. Mice were placed in a heat box and monitored until they recovered consciousness. The following day (24h post IRI), mice were culled by cervical dislocation and blood collected by cardiac exsanguination. Blood was centrifuged at 8000xg to remove solid components and sera stored at -80°C until further analysis. Tissues were either flash frozen in liquid N₂ and stored at -80°C or fixed in 4% formaldehyde (TAAB Laboratories, Cat# F017/2) in PBS (Gibco®, Cat# 14190144) supplemented with calcium (0.9mM) and magnesium (0.49mM).

2.2.2 Bilateral ischaemia and reperfusion injury

Mice underwent either (i) uninephrectomy followed by clamping of the renal artery of the remaining kidney to induce IRI or (ii) sham surgery. All procedures up to the midline laparotomy were carried out as described in 2.2.1. (i) IRI: Mice in the IRI group underwent uninephrectomy prior to ischemic injury. The right kidney was exposed by blunt dissection, the renal hilum and renal vessels were ligated once with a suture (Vicryl®, 4-0) and the kidney removed. To induce IRI, the left kidney was exposed and the renal artery was clamped with a vascular clamp (S&T®, B-1 V) for 30min. During the ischaemic period, a sterile gauze moistened with saline was placed over the incision site to prevent the abdominal cavity from drying out. At the end of this period, the clamp was removed and the linea alba and then skin were closed separately using sterile sutures, the wound was sanitised with Videne® Antiseptic Solution (Ecolab) and 1ml warm (37°C) isotonic sodium chloride solution was injected (s.c.). Mice were placed in a heat box and monitored until they recovered consciousness. (ii) Sham surgery: Mice were subjected to the same surgery as for IRI without uninephrectomy or clamping of the renal artery. After the 30min ischaemia-equivalent period the abdomen was closed with two sutures, 1ml saline was injected (s.c.) and, mice were placed in a heat box for observation until full recovery from anaesthesia. Kidneys were harvested 48h post-surgery (IRI or sham). Mice were sacrificed and tissues harvested as described in Section 2.2.1.

2.2.3 Folic acid nephropathy

Mice received one intraperitoneal (i.p.) injection of either folic acid (240µg/g body weight; Sigma Cat# F7876) dissolved in 0.3M NaHCO₃ (Sigma, Cat# S5761; vehicle) (20mg/ml) or vehicle control only. After 13d, mice were transferred into metabolic cages for 18h for urine collection. The following day, mice were culled by cervical dislocation and blood was collected by cardiac exsanguination. Urine and blood were centrifuged at
8000xg to remove solid components and stored at -80°C until further analysis. Tissue were either flash frozen in liquid N$_2$ and stored at -80°C or fixed in 4% formaldehyde (TAAB Laboratories, Cat# F017/2) in PBS (Gibco®, Cat# 14190144) supplemented with calcium (0.9mM) and magnesium (0.49mM).

2.3 Molecular biological analyses

2.3.1 Kidney samples and immunostaining

Human tissues were obtained from normal human kidneys anatomically unsuitable for transplantation collected under ethical approval (Ethics Number 05/Q0508/6) or from archived blocks (PKD Charity Biobank) (total number of kidneys: n=21; age range: 2 months - 78 years). Murine tissues were obtained from male C57BL/6J mice (10-12 weeks) in concordance with the Home Office (Project license: P6377F606). Tissues were fixed in 4% formaldehyde in PBS, embedded in paraffin and 3μm sections cut at UCL IQ Path, UCL Institute of Neurology.

2.3.1.1 Immunohistochemistry

Sections (3μm) were deparaffinised through sequential steps of xylene (2x3min), graded ethanols (100%, 90%, 70% and 50%; 3min each) and a final rehydration step in water (10min). Heat-induced antigen retrieval (microwave) was achieved by immersing the samples in boiling 1mM EDTA (Sigma®, Cat# E6635) pH 8, for 10min. Subsequently, the samples were cooled under running tap water for 10min and a hydrophobic barrier was applied around the sections using a PAP pen (ImmEdge Pen, Vector Laboratories, Cat# H-4000). After three washes (5min each) in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) sections were blocked in 10% normal goat serum (NGS; Sigma®, Cat# G9023) and 1% bovine serum albumin (BSA; Calbiochem™, Cat# 126575) in TBS for 1h at room temperature (RT) to prevent non-specific antibody binding. Sections were then incubated with primary antibodies or control IgGs at equivalent concentrations (antibodies listed in Chapter 3, Table 3.1 and Chapter 5, Table 5.1). Antibodies and IgG controls were diluted in TBS supplemented with 5% NGS and incubated in a humidified chamber overnight at 4°C. Sections were washed twice with TBST (5min each) to remove excess antibody. Endogenous peroxidase activity was blocked by 15min incubation in 0.3% H$_2$O$_2$ followed by two TBST washes (5min each). For immunodetection, the DAKO EnVision+/HRP reagent (anti-rabbit, Cat# K400211-2; or anti-mouse, Cat# K400111-2) was applied according to the manufacturer's protocol. These reagents make use of the 3,3’-diaminobenzidine (DAB) chromogen (DAKO, Cat#
K3468) which is oxidised by the horseradish peroxidase (HRP), producing a brown stain. Sections were incubated at RT for 2min. The reaction was terminated by incubating the sections in water for 5min. Sections were dehydrated through graded ethanols (50%, 70%, 90% and twice 100%; 3min each) followed by two xylene dehydration steps (3min each). Finally sections were mounted with toluene-free mounting medium (DAKO, Cat# CS70530-2) and stored at RT for analysis. Images were collected on a Nikon ECLIPSE Ci-L microscope (Nikon Digital Sight D5-Fi2 camera) and analysed using ImageJ v1.52 (NIH).

2.3.1.2 Immunofluorescence

For immunofluorescence (IF) detection of protein expression, sections were processed as in 2.3.1.1 up to creating a hydrophobic barrier using a PAP pen. Sections were washed three times in TBST (5min each) and incubated with freshly prepared 50mM NH₄Cl for 15min to quench free aldehyde groups followed by two additional TBST washes (5min each). Sections were blocked in 10% NGS and 1% BSA in TBS for 1h at RT then incubated with primary antibodies or control IgGs at equivalent concentrations (antibodies listed in Chapter 3, Table 3.1 and Chapter 5, Table 5.1). All primary antibodies were diluted in TBS supplemented with 5% NGS and incubated overnight in the dark at 4°C in a humidified chamber. Sections were washed three times with TBST (5min each) to remove excess antibody and incubated with secondary fluorescence-tagged antibodies (antibodies listed in Chapter 3: Table 3.1 and Chapter 5: Table 5.1) diluted 1:500 in TBS supplemented with 5% NGS, for 1h in the dark at RT. After three washes with TBST (5min each) sections were incubated with DAPI (0.1µg/ml) for 5min at RT to visualise nuclei. Following three TBST wash steps (5min each) sections were mounted with FluorSave™ mounting medium (Millipore, Cat# 345789), coverslips were sealed using nail polish and the slides stored at 4°C for analysis. Tissues were imaged with a Leica TCS SP8 confocal microscope (Leica HyD photon counter) and analysed with ImageJ v1.52 (NIH).

2.3.1.3 Quantification of IHC

All sections were analysed in a blinded manner to minimise observer bias. Images were taken with a Nikon ECLIPSE Ci-L microscope (Nikon Digital Sight D5-Fi2 camera) at a 20x magnification. Renal cortex and medulla of each sample were screened thoroughly across the section. A NIH ImageJ plug-in was applied to achieve a concise, semiautomatic quantification of positively stained areas. This plug-in [threshold colour
Gene expression analyses by RT-qPCR

Total RNA was extracted using the RNeasy® Mini kit (Qiagen, Cat# 74104) with an RNase-free DNAse I (Qiagen, Cat# 79254) digestion step to remove genomic DNA. Cells in 6-well plates were washed twice with chilled PBS (Gibco™, Cat# 14190-094) and lysed in 350µl buffer RLT (Qiagen RNeasy® Mini kit) supplemented with β-mercaptoethanol (β-ME; Sigma, Cat# M-3148) and transferred to 1.5ml tubes. After homogenisation by vortexing, RNA was isolated according to the manufacturer's protocol. RNA concentration was quantified on a NanoDrop 8000 (Labtech) spectrophotometer (for samples with A260/280 ratios >2.0) and either stored at -80°C or used immediately for cDNA syntheses. The High-Capacity cDNA™ Reverse Transcription Kit (Applied Biosystems, Cat# 4368814) was used for reverse transcription using 2µg total RNA of each sample according to the manufacturer’s instructions. All quantitative (q)-PCR reactions (LightCycler® 96 System, Roche) were performed in triplicate with an input of 1µl 1:5 diluted cDNA in a total reaction volume of 20µl. The 2x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, Cat# PB20.15-20) reaction mix was used, with SYBR Green as a fluorescent reporter. All primer pairs used in this study were either designed with Primer 3 or selected from previous publications and were obtained from Sigma®. All primers were tested for specificity using the NCBI Primer-BLAST tool. Primers used are listed in the Chapter 4, Table 4.1 and Chapter 5, Table 5.2.

For all qPCRs the following conditions were applied: The initial denaturation and activation of the HS Taq DNA Polymerase occurred at 95°C for 10min followed by 40 cycles of: 10s at 95°C (denaturation), 10s at 60°C (annealing) and 10s at 72°C (extension). The melting curve analysis entailed one cycle of 10s at 95°C, 60s at 65°C and a final step of 97°C for 1s. The ΔΔCt method was used to determine levels of gene expression.
2.3.3 Western blot analyses

hPTECs in 6-well plates were lysed on ice using 150µl chilled RIPA buffer (Sigma®, Cat# R0278-50ML) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Cat# 78440) and pepstatin A (Sigma®, Cat# P5318-5MG). Kidney tissues were homogenised and lysed in the same RIPA buffer (20µl/mg tissue), using a TissueLyser LT (Qiagen) according to the manufacturer’s protocol. Lysates were centrifuged at 15,800xg at 4°C for 15min and the supernatant transferred to new pre-cooled 1.5ml tubes. Protein concentration was quantified using the RC DC™ Protein Assay Kit (Bio-Rad, Cat# 5000121) with BSA as a standard (7 standards ranging from 0.2-1.4mg/ml). The assay was carried out in a 96-well plate (Corning®, Cat# 3599) format and absorption was measured at 650nm (Biochrom EZ Read 400, Cambridge, UK). Samples (10-25µg) were denatured by addition of 4x Laemmli buffer (Bio-Rad Cat# 161-0747; 277.8mM Tris-HCl, pH 6.8, 44.4% (v/v) glycerol, 4.4% lithium dodecyl sulfate (LDS), 0.02% bromophenol blue) supplemented with β-ME (final concentration 355mM) and heating for 5min at 95°C. For electrophoretic separation of the proteins, 8 or 12% Tris-based polyacrylamide gels were used and run in an aqueous Tris-glycine buffer (5mM Tris, 50mM glycine, 0.02% (w/v) SDS, pH 8.3) for 2h at 100V. PVDF membranes (Roche, Cat# 03010040001; 0.20 µm pore size) were activated in methanol for 3min at RT and immersed in transfer buffer (25mM Tris-base, 190mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) for 5min. Protein transfer onto the PVDF membrane was carried out for 3h (350mA, on ice) using a Mini Trans-Blot® Cell system (Bio Rad; Cat# 1703930). Membranes were blocked with 5% fat-free, skimmed milk (Marvel) in TBST (blocking solution) for 1h at RT with gentle agitation to prevent nonspecific antibody binding. Membranes were washed three times for 10min with TBST and incubated with gentle agitation overnight at 4°C with the respective primary antibodies (antibodies are listed Chapter 4, Table 4.3 and Chapter 5, Table 5.3) diluted in blocking solution.

Membranes were washed three times with TBST (10min each) and incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies in blocking solution for 1h at RT. Mouse-derived primary antibodies were detected with HRP-tagged goat-anti-mouse immunoglobulins (IgGs) (DAKO, Cat# P0447, polyclonal, 1:2000), rabbit-derived antibodies were detected with HRP-tagged goat-anti-rabbit IgGs (DAKO, Cat# P0448, polyclonal, 1:2000) and goat-derived antibodies were detected with HRP-tagged rabbit-anti-goat IgGs (DAKO, Cat# P0449, polyclonal, 1:2000). Membranes were washed three times (10min each) with TBST under gentle agitation. Excess fluid was drained off, membranes covered with Immobilon Western HRP Substrate (Merck,
Cat# WBKLS0500) and imaged using a BioSpectrum® 810 Imaging System (UVP). In some cases, membranes were stripped with the Restore™ PLUS Western Blot Stripping Buffer (Thermo, Cat# 46430) for 15min at RT followed by three 10min washes with TBST and re-probed with another primary antibody followed by secondary antibodies and imaging as described above. ImageJ v1.52 (NIH) was used for densitometric analyses.

2.3.4 Transmission electron microscopy

hPTEC pellets (Chapter 4, Section 4.3.8) or murine kidneys (de-capsulated and cut transversely) were fixed in 5ml of 2.5% glutaraldehyde buffered with 100mM sodium cacodylate (Sigma®, Cat# C4945, pH 7.2) for 24h at RT followed by secondary fixation in 1.0% osmium tetroxide (Agar Scientific, Cat# AGR1016). Samples were dehydrated in graded ethanols, transferred to propylene oxide (Agar Scientific, Cat# AGR1080) and then infiltrated and embedded in Agar 100 epoxy resin (Agar Scientific, Cat# AGR1031). Polymerisation was carried out at 60°C for 48h. Ultrathin sections (90nm) were cut using a Diatome diamond knife on a Leica EM UC7 ultramicrotome. Sections were collected on copper grids and stained with alcoholic uranyl acetate (Agar Scientific, Cat# AGR1260A) and lead citrate (Agar Scientific, Cat# AGR1210). The samples were examined with a JEOL 1400 transmission electron microscope and digital images were recorded using an AMT XR80 digital camera.

2.3.5 Statistical analyses

For statistical analyses GraphPad Prism 7.0 software was used. All data are presented as mean ± S.D. and a p-value of <0.05 was considered statistically significant. The p-values are denoted in the figures as follows: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. For n=2 groups, differences between groups were assessed using a Mann-Whitney U test. Where n>2 groups, differences between groups were measured using a Kruskal-Wallis one-way analysis of variance (ANOVA) followed by a Dunn’s test to correct for multiple comparisons or a two-way ANOVA in conjunction with Tukey’s post hoc test to correct for multiple comparisons. For in vivo experiments (Chapter 3 and 5) and when human tissue was used, 6-12 animals/individuals were analysed in each experimental group. For in vitro experiments (Chapter 4), experiments were carried out in triplicates to hexuplicates (technical replicate) and repeated 3-5 times (biological replicates).
Chapter 3

SIRT5 is expressed in murine as well as human kidneys and its expression is affected by acute and chronic injury
3.1 Introduction

Accumulating evidence has identified the NAD+-dependent K-deacylase SIRT5 and its family member SIRT3, as central regulators of cellular bioenergetics by targeting key metabolic enzymes. Both SIRTs are ubiquitously expressed in mice with the highest levels in organs with increased metabolic requirements including the kidneys. Interestingly, mRNA transcript analyses in human tissues showed that this seems to also apply to human kidneys suggesting mouse-human translatability. Although mRNA expression levels are an indicator of protein expression levels, a linear relationship between mRNA and protein levels cannot be assumed and has to be confirmed by, for example, WB. However, to define the localisation of SIRT5 and 3 expression in the kidneys, which may be key to defining SIRT function along the nephron, IHC/IF analyses are required. The transcriptional co-activator PGC1α is a major regulator of SIRT5 and 3 gene expression and also a master regulator of mitochondrial biogenesis. PGC1α is highly expressed in the human kidney, but, similar to SIRT5 and 3, the distribution along the nephron is unknown.

The SIRT family has been linked to longevity and polymorphisms in the SIRT5 promoter leading to reduced expression have been associated with increased brain molecular age in humans. Ageing is associated with a decline in renal function and is therefore, a major risk factor for the development of kidney failure. Growing evidence highlights mitochondrial dysfunction and the bioenergetic consequences as a significant contributor to AKI and CKD. While de Cavanagh et al. revealed that ageing in rats is associated with a reduction in mitochondrial number and bioenergetic output in PTs, to our knowledge, it has not been shown that the same is true for the human kidney. Furthermore, it is not established whether advanced age also results in a decline of SIRT5 expression in human kidneys and whether this may be a contributor to the age-related increase in susceptibility to AKI. As previously mentioned, SIRT5 expression is regulated by PGC1α through PPARα and ERRα. Interestingly, Lee et al. have shown that renal ageing in mice is associated with a reduction in PGC1α, however it remains to be tested whether this is also the case in human kidneys and whether this has any implications for SIRT5 expression levels.

While the PGC1α-PPARα/ERRα axis is one identified mechanism by which SIRT5 transcription is regulated under basal conditions, there is very limited information on the transcriptional network driving SIRT5 transcription under metabolic stress. Zhu et al. were the first to report that cardiac SIRT5 levels increased in rats exposed to intermittent hypoxia in vivo suggesting that SIRT5 expression might be stimulated to facilitate metabolic adaption and protect hearts from injury. This finding is
consistent with the observation that Sirt5 deletion increased cardiac susceptibility to ischaemic injury in mice (337). As yet, it is not clear whether renal SIRT5 expression is regulated by pathological insults known to cause renal injury such as ischaemia or nephrotoxic agents. Thus, it remains to be tested whether SIRT5 expression in the kidneys is affected by acute or chronic injury.

3.2 Specific aims

The aims of the studies presented in this Chapter were therefore to: (i) assess the protein levels of SIRT5, 3 and their transcriptional co-activator PGC1α in the human kidney and determine their expression pattern along the human nephron; (ii) evaluate whether SIRT5 and PGC1α levels decline with age; and, (iii) measure SIRT5 protein levels in pathological murine kidney samples to test whether SIRT5 levels are affected by acute and/or chronic injury conditions.

3.3 Methods

3.3.1 Mouse breeding, maintenance and renal injury models

The murine renal tissues used in this Chapter were archived sections from previous Home Office-approved studies. Sections of kidneys exposed to bilateral IRI were provided by Prof. Alan Salama (unpublished). Sections from folic acid nephropathy (FAN) study (365) were provided by Prof. Jill Norman. Of note, for the IRI study mice with a C57BL/6J background were used while in the FAN study mice with a mixed background were used (365).

3.3.2 Immunohistochemistry and immunofluorescence

The detailed protocol for IHC staining is given in Chapter 2, Section 2.3.1.1 and 2.3.1.2. The antibodies used are listed below:

Table 3.1: Antibodies and fluorescence stains.

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<th>Application (Dilution)</th>
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<td>Abcam</td>
<td>ab9566</td>
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<td>Type</td>
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<td>Application</td>
</tr>
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<td>Santa Cruz</td>
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**Control IgG**

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**Murine studies**

**Primary antibodies**

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<th>Application</th>
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<td>IgG Rabbit pc R&amp;D Systems</td>
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<td>R&amp;D Systems</td>
<td>AB-105-C</td>
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<td>pc</td>
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<td>K400211-2</td>
</tr>
</tbody>
</table>

| Lectin staining                                           | Fluorescein-tagged LTL                | Lotus tetragonolobus | Vector | FL-1321 | IF (1:200) |

**Key:** H+L: Heavy and light chains, HRP: Horseradish peroxidase, IF: Immunofluorescence, IHC: Immunohistochemistry, LTL: Lotus tetragonolobus lectin, mc: monoclonal, pc: polyclonal, * equivalent concentrations of IgG to the relevant primary antibody were used.
3.4 Results

3.4.1 PGC1α, SIRT5 and SIRT3 localise to mitochondria-rich nephron segments in the human kidney

PGC1α, SIRT5 and SIRT3 are expressed in the human kidney. To examine the expression of PGC1α, SIRT3 and SIRT5 in the human renal cortex and medulla, IHC staining was performed on adult normal human kidney (NHKs; age range: 57-68 years) specimens. As shown in Figure 3.1A, PGC1α was highly expressed in the renal cortex, but showed little expression in the renal medulla. The intensity of the staining varied between renal tubules, in both the cortex and medulla, indicating differences in tubular PGC1α expression along the nephron. In the renal cortex, the highest expression appeared in large tubules with cuboidal epithelium (most likely PTs), and comparably lower expression in smaller tubules with cuboidal epithelium (most likely DTs).

![Figure 3.1: Immunostaining of human renal cortex and medulla for PGC1α, SIRT5 and SIRT3](image)

Of note, no PGC1α⁺ cells were observed in the glomeruli. In the medulla, PGC1α levels were higher in tubules with smaller cuboidal epithelium (most likely thick ascending limb...
(TAL) of Henle’s loop (LoH)) and in comparison, lower in tubules with large cuboidal epithelium (most likely collecting duct (CD)).

IHC staining for SIRT5 revealed strong protein expression in the renal cortex as well as in the medulla, with increased levels in the latter (Figure 3.1B). Similar to PGC1α, SIRT5 staining exhibited a heterogeneous expression pattern in the different nephron segments. In the renal cortex SIRT5 levels were highest in small tubules with cuboidal epithelia (most likely DTs) and in comparison showed reduced levels in large tubules with cuboidal epithelium (most likely PTs). SIRT5 was not expressed in glomerular cells. In the renal medulla, SIRT5 levels peaked in small tubules with cuboidal epithelia (most likely TALs), with comparably lower and heterogeneous expression in large tubules with cuboidal epithelia (most likely CDs). SIRT5 was absent in small tubules. These could be either thin descending limbs (TDL) of the LoH (squamous epithelial cells) or the vasa recta (VR) (endothelial cells).

Staining for SIRT3 showed that it mainly localised to the renal medulla although there was some expression in the renal cortex (Figure 3.1C). Interestingly, the level of SIRT3 protein expression was variable in both the cortex and medulla and showed the same expression pattern as described for SIRT5, particularly, in the renal cortex, which indicated differences in expression in the nephron segments. Similar to PGC1α and SIRT5, glomerular cells did not show any staining for SIRT3.

Taken together, these findings indicated that PGC1α, SIRT5 and 3 are expressed in the human kidney, with distinct expression patterns in the different nephron segments. Based on the location and structure of the tubules it appears that all three proteins are enriched in nephron segments with increased mitochondrial mass such as PTs, DTs and TALs (34).

**PGC1α, SIRT5 and SIRT3 are enriched in PTs, DTs and TALs.** To establish more precisely the cell type-specific expression patterns along the human nephron (i) PGC1α, (ii) SIRT5 and (iii) SIRT3 were co-stained with segment-specific markers: Aquaporin-1 (AQP1) for PT in the renal cortex as well as the TDL of LoH and the VR of the renal medulla. Tamm-Horsfall-protein (THP) for the cortical/medullary TAL of LoH. Calbindin-D-28k (CBD) for DTs in the cortex and AQP2 for cortical/medullary collecting ducts (CD).
Figure 3.2: Co-localisation of PGC1α and segment-specific markers in the human kidney. Normal human kidneys (NHKs) were immunofluorescently co-stained for PGC1α (green) and segment-specific markers (red) for cortical (A-D) and medullary (E-G) nephron segments. Renal cortex: (A) Aquaporin-1 (AQP1), a marker for proximal tubules. (B) Tamm-Horsfall-protein (THP), marker for the thick ascending limb (TAL) of Henle’s loop (LoH). (C) Calbindin-D-28k (CBD), a marker for distal tubules (DT). (D) AQP2, a marker for collecting ducts (CDs). Renal medulla: (E) AQP1, a marker of thin descending limbs (TDLs) of the LoH (full arrow) and the vasa recta (VR) (hollow arrow). (F) THP, marker for the TAL of the LoH. (G) AQP2, a marker for CDs. DAPI (blue) was used to visualise nuclei. Scale bar: 100μm. Micrographs shown are representative images from 2 different kidneys (age 57-68 years). One section/kidney was examined.
(i) PGC1α

Renal cortex. IF co-localisation revealed that PGC1α predominantly localised to the AQP1+ PTs (Figure 3.2A) and at a slightly lower level, to the CBD+ DTs (Figure 3.2C). Additionally, PGC1α was found in cortical THP+ TALs (Figure 3.2B), however, there was very little expression in cortical AQP2+ CDs (Figure 3.2D).

Renal medulla. Compared to the cortex, PGC1α levels appeared low in the medulla of human kidney (Figure 3.2E-G). The strongest staining localised to THP+ TALs (Figure 3.2F), while the AQP1+ TDLs (Figure 3.2E, filled arrow) and AQP2+ CDs (Figure 3.2G), showed very little PGC1α expression. AQP1+ VR was negative for PGC1α (Figure 3.2E, hollow arrow).

(ii) SIRT5

Renal cortex. As shown in Figure 3.3A-D, SIRT5 was highly expressed in the human renal cortex with strongest expression in the CBD+ DTs (Figure 3.3C). SIRT5 was also strongly expressed in the AQP1+ PTs (Figure 3.3A) and the THP+ TALs (Figure 3.3B) although this was lower than in DTs. SIRT5 was also expressed in AQP2+ CDs (Figure 3.3D). Interestingly, within the CDs expression appeared heterogeneous, possibly representing different levels of expression in the principal cells and mitochondria-rich intercalated cells type A and B (366).

Renal medulla. In the renal medulla, SIRT5 expression was highest in THP+ TALs (Figure 3.3F). However, SIRT5 was also present in AQP2+ medullary CDs (Figure 3.3G), in a pattern similar to cortical CDs (Figure 3.3C). There was no SIRT5 expression in AQP1+ TDL (Figure 3.3E, filled arrow) or in AQP1+ VR (Figure 3.3E, hollow arrow).

(iii) SIRT3.

Renal cortex. SIRT3 was primarily expressed in CBD+ DTs (Figure 3.4C) and THP+ TALs (Figure 3.4B), with comparably lower expression in AQP1+ PTs (Figure 3.4A).

Renal medulla. SIRT3 exhibited a similar expression pattern to SIRT5 with highest expression in THP+ TALs (Figure 3.4E) and no apparent staining in AQP1+ TDLs and VR (Figure 3.4D).
Figure 3.3: Co-localisation of SIRT5 and segment-specific markers in the human kidney. Normal human kidneys (NHKs) were immunofluorescently co-stained for SIRT5 (green) and segment-specific markers (red) for cortical (A-D) and medullary (E-G) nephron segments. **Renal cortex:** (A) Aquaporin-1 (AQP1), a marker for proximal tubules. (B) Tamm-Horsfall-protein (THP), marker for the thick ascending limb (TAL) of Henle’s loop (LoH). (C) Calbindin-D-28k (CBD), a marker for distal tubules. (D) AQP2, a marker for collecting ducts (CDs). **Renal medulla:** (E) AQP1, a marker of thin descending limbs (TDLs) of the LoH (full arrow) and the vasa recta (VR) (hollow arrow). (F) THP, marker for the TAL. (G) AQP2, a marker for CDs. Intercalated cell (full arrow). DAPI (blue) was used to visualise nuclei. Scale bar: 100μm. Micrographs shown are representative images from 2 different kidneys (age 57-68 years). One section/kidney was examined.
Figure 3.4: Co-localisation analysis of SIRT3 in the human kidney. Normal human kidneys (NHKs) were co-stained for SIRT3 (red) and segment-specific markers (red) for cortical (A-C) and medullary (D-E) nephron segments by immunofluorescence. **Renal cortex:** (A) Aquaporin-1 (AQP1), a marker for proximal tubules (PTs). (B) Tamm-Horsfall-protein (THP), marker for the thick ascending limb (TAL) of the loop of Henle (LoH). (C) Calbindin-D-28k (CBD), a marker for distal tubules (DTs). **Renal medulla:** (D) AQP1, a marker of thin descending limbs (TDLs) of the loop of Henle (LoH) (full arrow) and the vasa recta (VR) (hollow arrow). (E) THP, marker for the TAL of the LoH. 4’,6-diamidino-2-phenylindole (DAPI) (blue) was used to visualise nuclei. Scale bar: 100 μm. Micrographs shown are representative images from 2 different kidneys (age range 57-68 years). One section/kidney was examined.

Collectively, these data suggested that the protein levels of PGC1α and its target genes SIRT5 and SIRT3, are enriched in PTs, DTs and TALs, which are known to be rich in mitochondria (34). This is consistent with the known functions of PGC1α and SIRT5/3 in promoting mitochondrial biogenesis and bioenergetics, respectively, either directly and/or indirectly. Interestingly and somewhat unexpectedly, PGC1α and SIRT5/3 showed some differences in staining intensities in mitochondria-rich nephron segments: On one hand, PGC1α appeared highest in PTs and with a slight reduction in DTs and
TALs while on the other hand, SIRT5 and SIRT3 expression seemed highest in DTs and TALs, with reduced SIRT5 and little SIRT3 expression in PTs.

**SIRT5 localises to the nuclei of human DTs.** SIRT5 was originally described as a mitochondrial sirtuin (367), but has also been found in the cytosol and nucleus (284). As yet, it has not been determined whether in the human kidney, SIRT5 is able to translocate from the cytosol to the nucleus. As SIRT5 expression peaked in DTs, confocal microscopic analysis was performed using samples immunofluorescently co-stained for SIRT5 and CBD. A series of 40 Z-stack confocal images was taken and reconstructed into a 3-dimensional (3D) image (Figure 3.5). This showed that SIRT5 co-localised with DAPI, thereby, confirming nuclear localisation.

![Figure 3.5: Nuclear localisation of SIRT5 in the distal tubule of the human kidney.](image)

**Figure 3.5:** Nuclear localisation of SIRT5 in the distal tubule of the human kidney. Immunofluorescence co-staining of SIRT5 (magenta), CBD (cyan) and DAPI (green) followed by Z-stack analysis revealed that SIRT5 localises to the nuclei of DT epithelial cells (DTECs). Micrograph showing a maximum intensity Z-projection. Lower panel shows horizontal plane and right panel shows vertical plane at the position of the yellow cross. D: DT, G: glomerulus and P: PT. Scale bar: 20μm. ImageJ was used for image processing (pseudo colour and maximum intensity Z-projection). Micrograph shown is a representative image from 2 different kidneys (age range 57-68 years). One section/ kidney was examined.

In summary, the data indicated that SIRT5 may localise to DT nuclei suggesting that SIRT5 may also target nuclear proteins.
3.4.2 Mitochondrial abundance decreases with age in the human kidney

In human kidneys, DTs have higher mitochondrial content than PTs. It is generally accepted that PTs, DTs and TALs are the cortical nephron segments with the highest mitochondrial density. Interestingly, Hall et al. showed that in murine kidneys, PTECs exhibit reduced mitochondrial mass relative to DTECs. To test, whether this also applies to human kidneys, IF co-staining of the mitochondrial marker TOM20 with AQP1 and CBD was performed (Figure 3.6). TOM20 strongly localised to the CBD+ DTs (Figure 3.6B) while AQP1+ PTs exhibited little co-staining (Figure 3.6A). These observations suggested that in both murine and human kidneys, the mitochondrial mass in DTs is greater than that in PTs.

Figure 3.6: Co-localisation of TOM20 with AQP1 and CBD in the human renal cortex. Normal human kidneys were co-stained for the mitochondrial marker translocase of outer membrane 20 (TOM20; green) and either (A) the proximal tubule (PT) marker, Aquaporin-1 (AQP1; red) or (B) the distal tubule (DT) marker, Calbindin-D-28k (CBD; red) by immunofluorescence. DAPI (blue) was used to visualise nuclei. Scale bar: 100μm.

Figure 3.3 showed that SIRT5 levels are increased in tubular segments with increased mitochondrial content: DTs, TALs and PTs. To confirm this finding and assess whether SIRT5 levels are higher in cells with increased mitochondrial content, human renal tissues were co-stained for SIRT5 and TOM20 by IF. As shown in Figure 3.7, SIRT5 levels strongly correlated with TOM20 in both the renal cortex and medulla, indicating that SIRT5 is enriched in tubular segments with increased mitochondrial mass. Of note, as shown in Figure 3.3G, SIRT5 expression appeared heterogeneous in medullary CDs (Figure 3.7B). This may be due to fact that CDs contain both principal cells and mitochondria-rich intercalated cells type A and B, which differ in their mitochondrial
content (366) and suggests that SIRT5 levels are higher in intercalated cells, which are high in mitochondria mass compared to principal cells.

Figure 3.7: Co-localisation of SIRT5 with TOM20 in the human kidney. Normal human kidneys were co-stained for SIRT5 and the mitochondrial marker translocase of outer membrane 20 (TOM20). Fluorescence image showing (A) renal cortex and (B) renal medulla by immunofluorescence. Thick ascending limb (hollow arrow) and intercalated cells of the collecting duct (full arrow). DAPI (blue) was used to visualise nuclei. Scale bar: 100μm.

Ageing is associated with a reduction of mitochondrial mass in the human renal cortex. Declining mitochondriogenesis is a widely accepted feature of ageing.(368) To address whether this also applies to human kidneys, normal human kidney (NHK) sections (age range 2 months - 78 years) were stained for the mitochondrial marker TOM20, followed by quantification of the positively stained areas in the cortex and medulla. As shown in Figure 3.8A, TOM20+ staining declined with age in the renal cortex but not in the medulla. To quantify the percent mitochondrial mass reduction and correlate this with age, TOM20-stained NHKs were divided into three age groups with n≥3 in each group: Group 1 (0-29 years), Group 2 (40-57 years) and Group 3 (61-78 years). In addition, NHKs were subdivided into renal cortex and medulla. TOM20+ stained areas in the renal cortex and medulla were quantified (Figure 3.8B). Quantification confirmed the qualitative observations that ageing is associated with a decline in mitochondrial mass in the renal cortex from ~20% TOM20 positive-stained area in Group 1 to ~13% in Group 2, (p<0.05 versus Group 1) and ~9% in Group 3, (p<0.001 versus Group 1) (Figure 3.8B). In contrast, there was no statistically significant age-associated change in medullary mitochondrial content (Figure 3.8B).
These data indicated that in humans, age-related changes in mitochondrial mass occur primarily in cortical nephron segments.

3.4.3 Ageing does not affect SIRT5 expression in the human kidneys

Renal ageing in humans is not associated with a decline in PGC1α or SIRT5 protein levels. Based on the observation that cortical nephron segments showed an age-dependent decline in mitochondrial content, it was of interest to examine whether this may be due to reduced expression of the master regulator of mitochondrial
biogenesis, PGC1α. Recently, it has emerged that ageing might also lead to a reduction SIRT5 expression.(369) However, the expression of SIRT5, which has been shown to maintain mitochondrial mass (338), has not been analysed in aged human kidneys.

PGC1α- and SIRT5-stained NHKs were split into the three age groups with n≥3 in each group: group 1 (0-29 years), group 2 (40-57 years) and group 3 (61-78 years) (Figure 3.9A,B). For both molecules, the renal cortex and medulla were analysed separately. Interestingly, quantification analyses showed that, although there was a slight trend to reduced PGC1α as well as reduced SIRT5 in the renal cortices, no significant age-induced changes could be detected in either the cortex or the medulla.

**Figure 3.9:** Quantitative analyses of PGC1α and SIRT5 expression in normal human kidneys. Cortical and medullary regions of human kidneys were stained for PGC1α and SIRT5 expression by IHC. (A) Quantification of PGC1α expression in the renal cortex (left) and medulla (right). (B) Quantification of SIRT5 expression in renal cortex (left) and medulla (right). Between 10 and 20 pictures of each IHC-stained kidney (cortex and medulla) were taken and the total positively stained area in each visual field was quantified. Data are mean ± SD. A Kruskal-Wallis One-Way ANOVA was carried out followed by Dunn’s post hoc test to normalise for multiple comparisons.

### 3.4.4 SIRT5 levels are regulated by acute and chronic renal injury

Renal protein expression of several members of the sirtuin family including SIRT3 have been shown to be reduced under acute and chronic injury conditions and this promotes disease progression.(321, 370) So far, whether renal SIRT5 expression is affected by AKI or CKD has not been examined and therefore, remains to be tested.
SIRT5 expression increases after ischaemic AKI in mice. To test whether renal SIRT5 levels are affected by acute injury conditions, mouse kidney sections (C57BL/6J background) from a previous IRI study by Prof. Alan Salama, UCL Department of Renal Medicine (unpublished) were screened by IHC. Ischaemia provoked an increase in SIRT5 levels in cortical nephron segments compared to the sham-treated group (Figure 3.10A).

Subsequent immunofluorescence co-labelling of SIRT5 and Lotus Tetragonolobus Lectin (LTL), a PT marker, revealed increased SIRT5 levels in PTECs (Figure 3.10B).

Tubular SIRT5 expression decreases in nephrotoxic CKD in mice. Next, renal SIRT5 levels were assessed in chronic injury in mice. Kidney sections from a previous 14d FAN study (365) were screened by IHC. As shown in Figure 3.11A, FA-
treated mice showed reduced cortical and medullary SIRT5 protein levels compared to the vehicle-treated group. It is worth mentioning that the SIRT5 staining pattern in the renal cortex of the vehicle-treated group was slightly different to that seen in the sham-treated group from the IRI study (Figure 3.11A compared to Figure 3.10A). The overall staining in the vehicle-treated kidneys was fainter and with less pronounced difference between the cortical tubules. This might be due the fact that the FA-treated mice had a mixed genetic background whereas the AKI study used C57BL/6 mice. It is also possible that staining was affected by long term (~5 years) storage of cut sections (FAN study) versus freshly cut sections (IRI study).

![Figure 3.11: SIRT5 expression decreased in chronic kidney disease.](image)

In summary, the data showed that SIRT5 protein levels increased in PTECs in response to renal ischaemia (model of AKI) and decreased after FA treatment (model of CKD) in murine kidneys.
3.5 Discussion

The NAD⁺-dependent metabolic stress sensors SIRT5 and 3 have emerged as central regulators of cellular bioenergetics.(294, 305, 326, 329) While it has been shown that SIRT5 and 3 are expressed in murine (302, 330) and human kidneys (358, 359), at least on an mRNA level, their tissue distribution in humans is unknown. The same applies to the transcriptional co-activator PGC1α, which drives SIRT5 and 3 expression (293).

This first part of this Chapter sought therefore, to determine the distinct expression patterns of SIRT5, 3 and PGC1α along the human nephron.

In the renal cortex, SIRT5 protein expression peaks in mitochondria-rich DTs. IHC showed that SIRT5 and SIRT3 were present in both the cortex and the medulla with some enrichment in the renal medulla (Figure 3.1B,C). Based on the structure and location of the tubules it was found that in the cortex SIRT5 and SIRT3 expression was highest in DTs and lower in PTs. In the medulla, the highest levels of both enzymes were detected in TALs with little expression in CDs. Intriguingly, SIRT5 and 3 seemed to be absent from glomerular cells (cortex) and the VR (medulla). PGC1α expression was highest in tubules which were thought to be PTs with little expression in DTs (cortex) and therefore showed the exact opposite pattern to SIRT5 and 3 (Figure 3.1A). This was surprising as PGC1α has been shown to be a transcriptional regulator of SIRT5 and 3.(293) PGC1α also appeared to be absent in glomeruli. IF co-staining of SIRT5, SIRT3 and PGC1α with segment-specific markers revealed that in the medulla, SIRT5 and SIRT3 primarily localised to the TALs while in the renal cortex, SIRT5 and 3 were highest in DTs and TALs (Figures 3.3, 3.4), which are all nephron segments with high mitochondrial content.(32) PGC1α expression peaked in PTs, although it was also expressed in DTs and TALs (Figure 3.2). The SIRT5 data are in agreement with a recent study by Chiba et al. which reported that SIRT5 expression is increased in DTs and furthermore, found high levels in PTs and CDs in the murine kidney.(371) However, it should be pointed out that these investigators did not quantify SIRT5 protein levels directly but assessed the levels of β-galactosidase (by IF) as a measure of SIRT5 expression in Sirt5⁻/⁻ mice. For this, a lacZ reporter cassette, which codes for β-galactosidase, was integrated into the mutant allele in Sirt5⁻/⁻ mice. Although increased levels of β-galactosidase are a consequence of enhanced Sirt5 promoter activity, this approach does not take into account secondary effects (e.g. translation efficiencies may be different between lacZ and Sirt5). In addition, the IF data presented in that study only showed high magnification images to show the respective tubular segment of interest (e.g. DTs or CDs) and it was not possible to get an overview of the whole tissue to assess differences in tubular expression. Notably, Ransick et al. recently published a searchable
database called Kidney Cell Explorer (https://cello.shinyapps.io/kidneycellexplorer/), which entails single cell RNAseq data derived from murine renal cells isolated from three different regions (cortex, outer medulla and inner medulla).(372) By using this tool, it emerged that Sirt5 levels peak in DTECs, epithelial cells of the TAL and mitochondria-rich intercalated cells of the CDs. This finding is in particularly interesting as it fits with our observation in human kidneys (Figure 3.3.) suggesting mouse-human translatability. Nonetheless, although mRNA levels are an indicator of protein expression, SIRT5 protein levels would have to be measured (by e.g. IF for co-localisation) to be certain.(360)

Interestingly, it emerged that in DTs, SIRT5 also localises to the nuclei (Figure 3.5). This finding may be explained by the fact that there are four SIRT5 isoforms, which are most likely all detected by the polyclonal anybody used in this study: SIRT5iso1-4.(358) SIRT5iso1-3 contain a mitochondrial targeting signal (MTS) (358) and
therefore, one would expect increased levels in the mitochondria-rich tubules whereas SIRT5\textsuperscript{iso4} lacks the MTS and predominantly localised to the cytosol. Interestingly, Matsushita et al. showed that in HEK293 cells only SIRT5\textsuperscript{iso1} but not SIRT5\textsuperscript{iso2} also localises to the nuclei and revealed that this is determined by a C-terminal GPCG motif, which inhibits the proteasomal degradation of SIRT5\textsuperscript{iso1} in the cytosol after mitochondrial export.\textsuperscript{(373)} It remains to be tested whether other isoforms can also translocate to the nucleus. While all four isoforms were found to be expressed in the human kidney at the mRNA level\textsuperscript{(358)}, it is unknown where exactly the individual isoforms are expressed along the human nephron. However, to our knowledge, there no commercially-available isoform-specific antibodies which would be required to dissect potential tubule- as well as organelle-specific localisation. However, one approach would be to screen human kidney sections by fluorescence \textit{in situ} hybridization (FISH). This approach would allow detection of mRNA expression and co-localise these with markers of the specific nephron segments, thus, could be used to identify tubular-specific mRNA expression patterns of the isoforms. Intriguingly, it has been shown that the four SIRT5 isoforms differ in their activities as K de-succinylases (SIRT5\textsuperscript{iso1} has strongest activity).\textsuperscript{(358)} This suggests that the different isoforms might play different functional roles, possibly in an organ/tissue-dependent manner. Further studies are required to determine whether there are also differences in their activities as K de-malonylases/ de-glutarylases/ de-acetylases.

As previously discussed, SIRT5 was found in the nuclei of DTs (Figure 3.5). This, together with the fact that succinylation\textsuperscript{(280, 374)}, glutarylation\textsuperscript{(375)} and malonylation\textsuperscript{(374)} of amino acids have been found in histones suggests that histone PTMs might be a new potential connection between metabolism and histone biology. This is consistent with the finding by Park et al. who screened liver tissue from Sirt5\textsuperscript{-/-} mice for K-succinylation sites and showed that Sirt5 deletion caused a significant increase in histone K-succinylation suggesting that SIRT5 may also target histones.\textsuperscript{(335)} This is a particularly interesting finding as it hints that SIRT5 is potentially not only a regulator of metabolic enzymes but may also act as epigenetic eraser and impact on chromatin structure.

Taken together, the data indicated that although SIRT5, SIRT3 and PGC1α expression peaks in mitochondria-dense tubular segments (DTs, TALs and PTs), the distinct expression patterns of SIRT5 and SIRT3 (enriched in DTs and TALs) did not correlate with PGC1α expression (enriched in PTs). Furthermore, it emerged that in DTs, SIRT5 localised to the nuclei hinting that SIRT5 function may be more complex and not only restricted to metabolic regulation but rather involve a multitude of pathways. Further investigations including the functional characterisation/ localisation of the different SIRT5
In the human kidney, DTs exceed PTs in mitochondrial density. Hall et al. (45) and others (59) have shown that in mice, DTs exceed PTs in mitochondrial content. The present study showed that a similar pattern is observed in the human kidney (Figure 3.6). However, this observation raised the question of why mitochondrial mass did not show a positive correlation with the expression of PGC1α, the master regulator of mitochondrial biogenesis (61). A plausible explanation for this phenomenon was recently offered by McWilliams et al. who suggested that in PTs, mitochondria are subject to rapid turnover.(59) Using a genetic mouse model that allows the visualisation of mitolysosomes (mitochondria incorporated into lysosomes), the group revealed that although DTs exceed PTs in mitochondrial mass, PTs exhibit a marked elevation of mitolysosomes relative to DTs, indicating high mitochondrial turnover.(59) This finding would rationalise the increased protein levels of PGC1α found in PTs as elevated mitophagic flux necessitates higher mitochondriogenesis to keep cellular bioenergetics in balance.(376) It is however, very difficult to determine whether rapid mitochondrial turnover also occurs in human PTs. While it would be possible to establish the extent of general autophagic flux, there are, to our knowledge, no tools available to visualise mitochondrial turnover in tissue samples. However, mitochondrial turnover could be analysed in vitro by applying reporter genes such as mito-QC (377) or the Mtophagy dye (378). Although there are a number of human PTEC lines available, to date no human DTEC lines have been created which could be used for comparative analysis. Of note, Robin Felder’s group have reported generation of a human DTEC line (379), however, they used antibodies against THP and dolichos biflorus agglutinin (DBA) for cell isolation. THP is a specific marker for TALs (380) and DAB is a non-specific marker that stains both connecting tubules as well as collecting ducts (381, 382). Therefore, to test whether there are any differences in mitochondrial turnover between PTs and DTs, one major future goal would be to generate a human distal tubular cell line.

In this Chapter, it was also shown that SIRT5 levels correlate with mitochondrial mass (Figure 3.7). SIRT5 protein levels were the highest in DTs and TALs and comparably low in PTs (Figure 3.3). As discussed in the previous paragraph on SIRT5 localisation along the nephron, this finding is not surprising given that most of the SIRT5 isoforms expressed in the kidney contain a MTS and therefore, localise to mitochondria (358) so would be expected to be highest in those cells with the highest mitochondrial content.
As previously mentioned in Section 3.1, ageing is a major risk factor for the development of kidney disease (362) and this may be partly driven by age-associated reduction in mitochondrial mass and the bioenergetic consequences (356, 363). SIRT5 has been shown to promote mitochondrial function (333, 338) in mice and conversely, reduced levels of SIRT5 have been linked to increased brain molecular age in humans (343). SIRT5 expression is regulated by PGC1α (293), a transcriptional co-activator which drives mitochondrial biogenesis (61), and furthermore, has been shown to decline with age in murine kidneys (364). To our knowledge, it has not yet been established whether age affects mitochondrial mass or results in a decline of SIRT5 or PGC1α expression in the human kidney. Therefore, the work in the second part of this Chapter tested the hypothesis that mitochondrial mass (measured by TOM20 expression levels), SIRT5 and PGC1α protein levels decline with age.

Mitochondrial content declines with age in human kidneys. The present study showed that in humans, renal mitochondrial mass declines with age (Figure 3.8), potentially offering an explanation for the elevated risk of AKI associated with age (362). Interestingly, the data suggested that the renal expression of PGC1α and SIRT5, both highly involved in mitochondrial metabolism, was not affected by age (Figure 3.9A,B). At first this observation appeared surprising, particularly, as a positive correlation between a reduction in mitochondrial mass and the expression of PGC1α, the master regulator of mitochondriogenesis, might have been expected.(123) However, bearing in mind that PGC1α acts as a molecular switch by targeting specific TFs and NHRs (61), it may be that ageing is associated with a decline in PGC1α targets such as NRFs, PPARs or ERRs in the kidney. Although this has not been shown in human kidneys, a recent study by Chung et al. in rats and mice found that increased age is associated with a decline in PPARα, which promotes fibrosis, a central characteristic of the ageing kidney.(383) Interestingly, the group reported that, similar to our finding, PGC1α levels were not affected by age, at least in rats (murine kidneys were not screened for PGC1α).(383) In contrast, Lee et al. reported that old mice display lower renal PGC1α protein levels compared to young mice (364) suggesting that mechanisms associated with renal ageing may vary in different rodent species (rat versus mouse) although they may have a common denominator, mitochondrial dysfunction (363). Notably, neither of these two studies quantified mitochondrial mass. Therefore, no link between reduced levels of PPARα (in rats and mice) and/or PGC1α (in mice) and mitochondrial mass reduction was established. To test whether a reduction in PGC1α targets could also be a putative explanation for the observation of age-related decline in mitochondrial mass in human kidneys, further analyses need to be carried out. These may include analyses of the
expression of TFs and NHRs associated with mitochondriogenesis (e.g. PPARα and ERRα) and correlate these with age. In addition, it should be emphasised that while the quantification of protein levels on tissue sections allows the analysis of cortical/medullary areas as well as distinct regions within both tissue compartments (i.e. glomeruli versus tubules in the cortex) there are limitations to this approach. Key disadvantages are: (i) NHKs were harvested on different days/years at different institutions by different operators leading to differences in tissue handling and processing; (ii) slight differences in section thickness have a major impact on staining intensity, although, this should be controlled by using a larger number of samples, (iii) subtle differences in protein expression may be below the level of detection. Only one marker (TOM20) was analysed to determine mitochondrial mass in NHKs. Additional analyses such as transmission electron microscopy (TEM) analysis, mitochondrial enzyme activity assays (384) and WB analyses should be conducted to confirm the age-induced decline in mitochondrial mass in the human kidney and to dissect the underlying mechanisms. As the assessment of mitochondrial mass in NHKs was not a central component the project no further analyses were carried out but it remains an area for further investigation.

Several members of the Sirtuin family including SIRT1, 3 and 6, have been shown to be renoprotective in acute and chronic renal disease models while their ablation has been shown to exacerbate injury.(77) Prior to this study, the role of SIRT5 in renal disease had not been explored. A study by Zhu et al. showed that SIRT5 levels increase in murine hearts upon hypoxic stress in vivo and suggested that this may be a mechanisms that facilitates cardioprotection.(332) Renal ischaemia (in ischaemic AKI or as a consequence of capillary rarefaction in CKD) (385) equally gives rise to a hypoxic tissue environment hinting that SIRT5 gene expression may be affected. Thus the third part of this Chapter tested the hypothesis that SIRT5 protein levels change in AKI and in CKD.

**SIRT5 levels are affected by acute and chronic renal injury.** IHC analyses of murine kidneys revealed that IRI increased SIRT5 protein expression in renal tubules, with the strongest increase in the cortex (Figure 3.10). Furthermore, IF co-labelling of SIRT5 with the PTEC marker LTL, showed a strong increase in LTL+ PTs. These data indicated that Sirt5 gene expression is stimulated by acute ischaemia (30min). Of note, Wang et al. reported that SIRT5 mRNA expression increased in human alveolar epithelial cells exposed to cigarette smoke extract (CSE). Furthermore, the group showed that this increase is key to protect the cells from apoptosis by de-acetylation and thus, activation of FoxO3, a TF which recently has been shown to prevent ischaemic AKI-to-CKD
progression (386). The data therefore suggested that the ischaemia-induced increase in SIRT5 levels might be aimed to protect tubular cells from IRI. However, to determine whether the ischaemia-induced increase in SIRT5 levels is indeed renoprotective, a renal ischaemia study in vivo using Sirt5 knockout (Sirt5−/−) mice would need to be performed.

Buler et al. revealed Sirt5 gene expression is regulated by PGC1α in MEFs and identified PPARα and ERRα (both these TFs are PGC1α targets) binding sites in the human SIRT5 promoter.(293) This together with the fact that renal PGC1α levels were found to decline in multiple murine models of CKD as well as in human CKD (62, 63), suggested that SIRT5 protein levels may also decline in murine and human CKD tissue. Analysis of tissue from a murine FAN model (365) showed a reduction of SIRT5 protein in murine kidneys subject to chronic injury (Figure 3.11A). Perhaps surprisingly it was noticed that the SIRT5 staining pattern in the renal cortex of the vehicle-treated group from the FAN study (Figure 3.11A) differed slightly from the sham group in the IRI study (Figure 3.10A) with the staining appearing more diffuse and a less pronounced variation between the different tubule segments. It is suggested that this could be either strain differences (IRI study: C57BL6/J versus FA study: mixed background) or the age of the tissue blocks and sections (IRI study: blocks stored for 1 year and sections freshly cut versus FAN study: sections stored for 5 years).

3.6 Conclusion

In conclusion, the data presented in this Chapter showed that SIRT5, 3 and PGC1α are expressed in human kidneys and identified their expression patterns along the nephron. Furthermore, they exposed that SIRT5 protein levels are increased in mitochondria-rich nephron segments including DT, TALs and PTs. In addition, it was shown that SIRT5 localised to the nuclei in human DTs indicating that SIRT5 may also function as epigenetic eraser in these cells. The impact of ageing on mitochondrial mass, SIRT5 and PGC1α levels in the human kidney was also investigated. While increased age was associated with reduced mitochondrial mass, no significant reduction in SIRT5 or PGC1α could be detected. Finally, the regulation of SIRT5 levels in acute and/or chronic injury in murine kidneys was assessed. This showed that ischaemic AKI (IRI) increased SIRT5 expression, while nephrotoxic CKD (FAN) reduced SIRT5 levels.
Chapter 4

SIRT5 depletion exacerbates OND-induced mitochondrial dysfunction in hPTECs
4.1 Introduction

Renal PTECs display high metabolic turnover (32) due to their active reabsorption/excretion processes driven by a basolateral Na⁺/K⁺-ATPase and they are highly dependent on oxidative phosphorylation for ATP generation (3). Ischaemia, as a result of insufficient blood supply to the kidneys, deprives tubular cells of oxygen and nutrients, and is a major cause of acute and chronic damage. (387, 388) PTECs are extremely susceptible to ischaemic injury and extended periods of ischaemia can induce irreversible pathophysiological changes. (389) Early diagnosis and intervention are vital to limit disease progression and to prevent acute organ failure. A leading cause of IRI is renal hypoperfusion during surgery (390) suggesting that at-risk individuals would benefit from pre-emptive treatment to prevent or ameliorate AKI. Current treatments are primarily supportive, and there is a pressing unmet medical need for new therapeutic strategies.

The investigation of IRI necessitates the use of in vivo models to better understand the underlying pathophysiological mechanisms that drive disease progression. However, in order to determine whether the observations in rodents can be translated to humans, it is also important to conduct in vitro experiments using human cells. A common model to mimic renal ischaemia in vitro, is to expose renal cells to hypoxia (1% O₂), however, reduced blood flow to the kidneys also deprives cells of nutrients adding another metabolic stressor to the equation. This is why, in recent years, an increasing number of in vitro studies have implemented hypoxia and nutrient deprivation. This is key because both stimuli have a dramatic impact on the cellular bioenergetic profile: While hypoxia drives energy metabolism towards anaerobic glycolysis (391), nutrient starvation stimulates autophagy, a key survival mechanism that facilitates the release of micronutrients by degrading cellular components and helps to remove damaged organelles (e.g. mitochondria via mitophagy) (392). At present there is very limited knowledge on how oxygen and nutrient deprivation (OND) in vitro impacts on mitophagy in human PTECs (hPTECs) and whether this reflects the findings in vivo.

The SIRT family of NAD⁺-dependent KDACs are important cellular stress sensors that regulate energy metabolism and promote mitochondrial function in organs with high metabolic demand, e.g. heart and kidneys. (339) Multiple lines of evidence have shown that the K-deacetylases SIRT1 and 3, play important roles in the kidney and their activation during metabolic stress is regarded as a compensatory mechanism to avert mitochondrial dysfunction and protect cells from damage. (320, 321, 393) More recently SIRT5 has emerged as a central regulator of cellular energy metabolism. Unlike SIRT1 and 3, SIRT5 displays strong K-desuccinylase/-demalonylase/-deglutarylase activity with only very weak K-deacetylase activity. (326, 327, 394) As discussed in Chapter 3
(Section 3.5), SIRT5 primarily localises to mitochondria, where it regulates metabolic pathways including FAO, the Krebs cycle and ketogenesis (296, 337) but also localises to the cytosol where it has been shown to boost glycolysis (288) and to the nucleus (Figure 3.5). SIRT5 has been found to enhance cardiac energy metabolism in vivo (330) and has been implicated as a vital regulator of mitochondrial structure in MEFs in vitro. (338) Zhu et al. showed that intermittent hypoxia, a treatment regimen associated with increased stress tolerance, leads to increased cardiac SIRT5 expression in rats, suggesting a protective role for SIRT5 during ischaemia. (332) While this has been confirmed in the heart (337) and brain (395), it is unknown whether this is also the case in the kidneys. The in vivo data discussed in Chapter 3 (Figure 3.10) revealed that IRI stimulates SIRT5 expression in murine PTECs and therefore, hints that SIRT5 may also exert a protective function in the kidneys. Nonetheless, it has not been determined whether the findings in murine renal cells can be translated to human PTECs (hPTECs).

Accumulating evidence has identified mitochondrial dysfunction as a major contributor to AKI. (3) Mitochondria are highly dynamic organelles required for energy production and undergo constant fission and fusion to maintain quality control (42) and to meet metabolic requirements (44). Mitochondrial structure and function are closely-linked: fission is required for degradation of dysfunctional mitochondria (mitophagy) and is associated with impaired energy metabolism, whereas fusion protects mitochondria from mitophagy and boosts ATP generation. (42, 43) Mitochondrial dynamics are tightly regulated by nuclear-encoded GTPases (fission and fusion proteins). (396) Fission is driven by DRP1, which is primarily cytosolic, but on phosphorylation (S616) DRP1 translocates to the mitochondrial membrane, where it binds to its receptors (MFF, FIS1 and/or MiD49/51), accumulates and induces mitochondrial fragmentation. (173) Fusion is mediated by MFN1/2 and OPA1 anchor proteins that promote fusion of the mitochondrial outer and inner membranes, respectively. (396) As previously discussed in Chapter 1 (Section 1.2.2.1), OPA1 exists as five isoforms, two long isoforms (L1 and L2) and three short isoforms (S1, S2 and S3) all of which are important for mitochondrial form and function. (159, 397) Proteolysis of I-OPA1 decreases mitochondrial inner membrane fusion competency and thereby, provokes mitochondrial fragmentation. (159) Interestingly, s-OPA1 has been linked to cristae formation and maintenance of mitochondrial bioenergetics (397) highlighting that s-OPA1 is not only a product of I-OPA1 degradation but serves an important physiological function. The peptidases OMA1 (ATP-independent) and YME1L (ATP-dependent) can both degrade I-OPA1 to s-OPA1, however, only OMA1 can convert all I-OPA1 to s-OPA1 thereby promoting fission (Figure 4.1). (158) In healthy mitochondria, ATP levels are high which leads to activation of YME1L and subsequent degradation of OMA1 to promote fusion. (158) Decline in ATP
levels and the $\Delta \Psi_M$ impair YME1L activity and activate OMA1, which subsequently degrades YME1L to promote fission.(158)

In an *in vivo* study, Xiao et al. showed that renal ischaemic insults stimulate l-OPA1 degradation in PTECs via a mechanism that involves OMA1.(398) These investigators also showed that ablation of OMA1 averts l-OPA1 proteolysis and thereby, rescues the pathophysiologic mitochondrial ultrastructural changes which drive IRI in mice.(398) While this mechanism has been shown *in vivo* in mice, it is not clear whether it also applies to human cells (hPTECs *in vitro*).

Excessive mitochondrial fragmentation (46, 399) and swelling (400) in renal tubules are early and important features of injury. Recently, Perry et al. showed that maintaining mitochondrial structure following IRI in mice preserved renal function, indicating a potential therapeutic approach in AKI.(52) Two tightly-linked elements, the $\Delta \Psi_M$ and the ATP pool are central determinants of mitochondrial architecture.(45, 46)

During ischaemia, $\Delta \Psi_M$ and ATP levels decline which drives mitochondrial

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**Figure 4.1: l-OPA1 proteolysis by OMA1 causes mitochondrial fragmentation.** Long (l)-OPA1 conveys mitochondrial fusion competence. The peptidases OMA1 (ATP-independent; S1 cleavage site) and YME1L (ATP-dependent; S2 cleavage site) cleave l-OPA1 which results in s-OPA1 formation. This is important for fusion/fission dynamics and facilitates mitoQC. OMA1 and YME1L undergo mutual degradation which is key to the l-OPA1/s-OPA1 equilibrium. Decreased $\Delta \Psi_M$ or oxidative damage activate OMA1 which consequently degrades l-OPA1 as well as YME1L. This disturbs the l-OPA1/s-OPA1 and OMA1/YME1L equilibria. OMA1 ultimately undergoes autocatalysis. Loss of l-OPA1 reduces fusion competency and impairs cristae formation, which drives fission and increases mitochondrial susceptibility to degradation (mitophagy). Under acute injury conditions, mitochondria recover. However under chronic injury conditions, mitochondria cannot recover and stimulate cell death. Republished with permission of Journal of Cell Science from *OPA1 processing in cell death and disease – the long and short of it*, MacVicar and Langner, 129, 2297-2306, (2016); permission conveyed through Copyright Clearance Centre, Inc.(157)
fragmentation. Of interest, the ischaemia-induced decline of the ΔΨₘ has been shown to reverse F1/F0-ATP synthase activity from synthesis to hydrolysis of ATP. This mechanism, which is aimed to increase the ΔΨₘ and preserve mitochondrial structure during low-oxygen conditions, requires a metabolic switch from OXPHOS to glycolysis to supply sufficient ATP. However, renal tubular cells differ in their capacity to switch to anaerobic metabolism resulting in large variations in the extent of cellular injury along the nephron. As previously mentioned, the nephron segment primarily affected by ischaemia is the PT. Interestingly, although PTECs primarily depend on OXPHOS it should be emphasised that these cells can undergo a metabolic switch during ischaemia both in vivo and in vitro. Boosting glycolytic ATP production in PTECs might therefore be a putative therapeutic strategy to preserve mitochondrial structure and alleviate ischaemic injury. As discussed above, SIRT5 regulates both mitochondrial as well as glycolytic energy metabolism and is involved in the regulation of mitochondrial structure. However, it is not clear what role SIRT5 plays in hPTECs and what effect SIRT5 depletion has on ischaemia-induced mitochondrial dysfunction.

4.2 Specific aims

The aims of the studies presented in this Chapter were: (i) To characterise the OND model and assess whether it represents a more in vivo-like model of renal ischaemia in vitro; (ii) determine whether SIRT5 gene and protein expression are regulated by ischaemia in vitro in hPTECs; and, (iii) Investigate the function of SIRT5 in hPTECs during ischaemia in the context of metabolic/mitochondrial dysfunction using a RNAi-mediated knockdown strategy.

4.3 Methods

4.3.1 Cells and cell culture

The human proximal tubular epithelial cell line, human kidney clone 8 (HKC-8) used for these experiments were originally purchased from ATCC (deposited by L. Racusen (403)). Cells were raised from archived cryogenic stocks and grown in complete medium (CM): Dulbecco’s-modified Eagle’s medium: Ham’s F12 medium (1:1; DMEM:F12; Gibco™, GlutaMAX™, Cat# 313310-28) supplemented with 5% foetal bovine serum (FBS; Seralab International, Cat# EU-000-F, Lot# A201009) and cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Cells were passaged at 90% confluency using 0.05% Trypsin-EDTA (Gibco™, Cat# 25300062) and were not passaged more than 10 times. Cultures were tested for mycoplasma contamination.
(LookOut® Mycoplasma PCR Detection Kit, Sigma®, Cat# MP0035-1KT) and were negative.

4.3.2 In vitro simulation of ischaemia

HKC-8 cells were seeded into 6-well plates (Corning®, Cat# 3516) at a density of 6x10^5 cells/well. After 24h cells were 90% confluent and were used for experiments. To mimic in vivo ischaemia in vitro a combined oxygen and nutrient-deprivation (OND) model was developed, which implements both aspects of ischaemia i.e. hypoxia and nutrient starvation. In order to differentiate between the impact of (i) oxygen deprivation (OD; hypoxia) and (ii) nutrient deprivation (ND; starvation), HKC-8 cells were cultured in CM or in starvation medium (Hank's balanced salt solution (HBSS); Gibco™, Cat# 24020-117) supplemented with 10mM HEPES (Gibco™, Cat# 15630-049, pH 7.4) under either normoxia (21% O_2, 5% CO_2, 74% N_2) or hypoxia (1% O_2, 5% CO_2, 94% N_2). Control cells were kept in an incubator under normal culture conditions (normoxia). To induce hypoxia, cells were transferred into a modular incubator chamber (Billups-Rothenberg; Cat# MIC-101) and a 10cm petri dish filled with water was placed beneath the cells to ensure air humidification. The chamber was filled with the low oxygen gas through a gas exchange port A (flow rate: 10 litre/min). Port B was kept open to facilitate gas exchange. After 20min, both ports (A and B) were closed and the chamber was transferred to an incubator (37°C). Initially, to investigate the effects of hypoxia/normoxia with and without starvation, cells were harvested after 24h. For subsequent experiments, OND was compared to normoxia/CM and cells harvested after 6h. Additional treatments included CoCl_2 (100µM, Sigma, Cat# C8661-25G, Lot# SLBS3342V) to investigate the HIF pathway (404) and TORIN-1 (100nM, MCE MedChem, Cat# HY-12003, Lot# 19026) to specifically target the mTOR pathway (405). CoCl_2- and TORIN-1-treated cells were harvested 24h post-treatment. Experiments were carried out in triplicate (technical replicates) and repeated three to four times (biological replicates).

4.3.3 SIRT5 knockdown using siRNA

SIRT5 expression was reduced by RNA interference (RNAi) using the SMARTpool: ON-TARGETplus system (Dharmacon). HKC-8 were transfected with either the small interfering RNA (siRNA) pool against SIRT5 (L-013448-01-0005, 50nM) or the control siRNA pool (ON-TARGETplus Non-targeting Pool, D-001810-10-05, 50nM). Dharmafect-1 (Dharmacon, Cat# T-2001-02) was used as transfection reagent and transfection was carried out according to the manufacturer’s protocol. To reduce the
toxicity of the transfection reagent, culture medium was exchanged 24h post-transfection. Knockdown efficiency was determined by WB and 72h post-transfection was chosen as optimal time-point with >85% knockdown.

For WB, Fluorescence-activated cell sorting (FACS) and mitochondrial structure analyses (confocal and electron microscopy (EM)) HKC-8 cells were seeded and transfected in T25 flasks (Corning®, Cat# 430639; 1.5x10⁶ cells/flask). The following day, transfection reagent-containing medium was removed and replaced with CM. Forty-eight hours post-transfection, cells were re-plated into 6-well plates (WB and FACS analyses; 6x10⁵ cells/well), 8-well chamber slides (Nunc™ Lab-Tek™ II Chamber Slide System™, Cat# 154534; 5x10⁴ cells/chamber) (confocal microscopy), 10cm petri dishes (Corning®, Cat# 430167; 4.5x10⁶ cells/flask) (TEM) or 8-well Seahorse XFp plates (Agilent, Cat# 103025-100; 2.8x10⁴ cells/well) (respirometry).

For ATP quantification, cells were seeded into 96-well white clear bottom plates (Corning®, Cat# CLS3610-48EA; 1.8x10⁴ cells/well), transfection medium was replaced with CM 24h post-transfection and the assay was carried out 72h post-transfection without re-plating.

4.3.4 **TFEB and TFE3 knockdown using siRNA**

TFEB and TFE3 expression were reduced by RNAi using the SMARTpool: ON-TARGETplus system (Dharmacon). HKC-8 cells were plated in 12-well plates at a density of 2x10⁵ cell per well and transfected the following day with either the siRNA pool against **TFEB** (L-009798-00-0005, 50nM) or **TFE3** (L-009363-00-0005, 50nM) or the control siRNA pool (ON-TARGETplus Non-targeting Pool, D-001810-10-05, 50nM). Dharmafect-1 (Dharmacon, Cat# T-2001-02) was used as transfection reagent and transfection was carried out according to the manufacturer’s protocol. To reduce the toxicity of the transfection reagent, culture medium was replaced with CM 24h post-transfection. Forty-eight hours post transfection, hPTECs were stimulated with TORIN-1 (100nM) and harvested after 24h.

4.3.5 **Assessment of the mitochondrial membrane potential**

HKC-8 cells (control and SIRT5 RNAi-treated) were detached from 6-well plates (Corning®, Cat# 3516) using TrypLE™ Express Enzyme (Gibco™, Cat# 12604013). Trypsin was inactivated with 0.75ml HEPES-buffered DMEM (Gibco™, Cat# 1063029) supplemented with 1mM sodium pyruvate (Sigma®, Cat# S8636-100ML) (FACS medium). Cells were centrifuged for 5min at 400xg and the pellet was resuspended in
1ml FACS medium. Cells were counted in a haemocytometer, diluted to $4 \times 10^5$ cells/ml and the suspensions transferred into 2ml Eppendorf tubes (1m/tube in two tubes each). To assess background fluorescence intensities, 10µM Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added to one tube of each group (control and SIRT5 RNAi) and incubated for 20min in the dark at 37°C. FCCP is a potent uncoupling agent which results in rapid dissipation of the mitochondrial membrane potential and therefore, prevents mitochondrial accumulation of Tetramethylrhodamine, methyl ester (TMRM), a cationic and fluorescent dye. The fluorescence intensities measured in the FCCP-treated cells are therefore regarded as non-specific. After FCCP incubation, TMRM was added to all four tubes (control and SIRT5 siRNA-treated cells; with and without FCCP) at a final concentration of 25nM and incubated for exactly 30min in the dark at 37°C. TMRM fluorescence intensities of all four samples were measured using a MoxiGo cytometer (Orflo Technologies) and data were analysed with the FlowJo™ software tool (BD Biosciences). The background fluorescence intensities, as determined by FCCP treatment prior to TMRM incubation, were subtracted from the TMRM fluorescence intensities in the respective groups (control and SIRT5 RNAi).

4.3.6 Assessment of mitochondrial morphology

HKC-8 (control and SIRT5 RNAi-treated) were seeded into 8-well chamber slides at a density of $5 \times 10^4$ cells/well. The following day, cells were washed with CM to remove floating cells and incubated with 100nM Mitotracker™ Red CMXRos (Invitrogen, Cat# M7512) in CM for 45min at 37°C. To remove excess, non-mitochondrial, Mitotracker™ Red CMXRos, cells were washed with PBS and incubated in CM for 30min at 37°C. After two PBS washes to remove CM, cells were fixed with 4% formaldehyde in PBS (supplemented with calcium (0.9mM) and magnesium (0.49mM)) for 15min at 37°C and subsequently, washed twice with PBS (5min each) to remove excess formaldehyde. Cells were permeabilised with 0.1% Triton X-100 (Sigma®, Cat# T-8787) in PBS for 10min at room temperature (RT), washed twice with PBS (5min each) and incubated with 4’,6-diamidino-2-phenylindole (DAPI) (0.1µg/ml; Sigma®, Cat# D5942) for 5min at RT to visualise nuclei. Following two PBS washes (5min each), the chambers were removed and cells mounted with FluorSave™ mounting medium (Millipore, Cat# 345789). Coverslips were sealed using nail varnish and slides stored in the dark at 4°C until images were taken on a Leica TCS SP8 confocal microscope (Leica HyD photon counter).

For semi-automated assessment of mitochondrial morphology, the Mitochondrial Network Analysis (MiNA) toolset generated by Valente et al. (406) was applied. This tool
implements existing ImageJ (NIH) plug-ins and allows analysis of mitochondrial structure through binary conversion of the original images. In total, 42 control RNAi- and 51 SIRT5 RNAi-treated cells from three independent knockdown experiments were analysed.

4.3.7 ATP quantification

The ATPlite Luminescence Assay System (Perkin Elmer, Cat# 6016941) was used to determine the impact of SIRT5 RNAi on cellular energy metabolism. HKC-8 were seeded into white clear bottom 96-well plates (Corning®; 1.8x10^4 cells/well), transfected the following day and analysed 72h post-transfection. To differentiate between mitochondrial and glycolytic ATP generation, cells were either incubated with 25mM 2-Deoxy-D-glucose (2-DG; Sigma®, Cat# D8375) in glucose-free DMEM (Gibco™, Cat# 11966-025) supplemented with 1mM pyruvate and 5% FBS for 1h or 2µM oligomycin A (Sigma®, Cat# 75371) in 25mM glucose-containing DMEM (Gibco™, Cat# 31966-021) supplemented with 5% FBS for 25min, respectively. The ATP assay was carried out according to the manufacturer’s protocol and luminescence was measured. To determine the ATP levels per cell, the cell number/well was quantified with the CyQUANT® Cell Proliferation Assay kit (Life Technologies, Cat# C7026). This fluorescence-based assay determines cell number based on DNA quantity. Both, luminescence (ATP quantification) and fluorescence (DNA quantification) were measured with a Mithras LB 940 Multimode plate reader (Berthold Technologies).

4.3.8 Assessment of mitochondrial structure by electron microscopy

HKC-8 (control and SIRT5 RNAi) were seeded in 10cm petri dishes (Corning®, Cat# 430167) at a density of 4.5x10^6 cells/plate. The following day, cells underwent either 6h of OND or were cultured in CM under normoxic conditions (control; protocol described in Section 4.3.2). Cells were washed with PBS, detached from the culture plates using 3ml 0.05% Trypsin-EDTA (Gibco™, Cat# 25300062) and suspended in 7ml CM for trypsin inactivation. Cells were centrifuged for 5min at 400xg, supernatants removed and the pellets washed with PBS. After an additional centrifugation step for 5min at 400xg, supernatants were aspirated and cells were fixed in 5ml 2.5% glutaraldehyde buffered with 100mM sodium cacodylate (Sigma®, Cat# C4945, pH 7.2) for 24h at RT. The detailed protocol, which describes sample processing and imaging is given in Chapter 2 (Section 2.3.4).
To quantify changes in mitochondrial morphology, mitochondrial diameter was measured using ImageJ. In total, between 162 and 273 mitochondria from >10 cells/treatment group were analysed.

### 4.3.9 Assessment of mitochondrial function by respirometry

The impact of SIRT5 siRNA knockdown on mitochondrial bioenergetics in HKC-8 cells was assessed using a Seahorse XF Cell Mito Stress Test on a Seahorse XFp Analyzer (Agilent). Two readouts were quantified: (1) the oxygen consumption rate (OCR) which gives insight in mitochondrial OXPHOS; and, (2) the extracellular acidification rate (ECAR), which indicates the ability of cells to switch to anaerobic glycolysis for ATP production when OXPHOS is inhibited. Cells (control and SIRT5 RNAi) were seeded in Seahorse XFp 8-well plates at a density of 2.8x10^4 cells/well in CM and grown overnight. The next day, cells were either used for analysis (ATP rate assay) or washed twice with HBSS and subjected to 6h of OND (Mitochondrial stress test). Cells were washed twice with assay medium (Agilent, Seahorse XF DMEM medium, pH 7.4, Cat# 103575-100; supplemented with 10mM glucose (Sigma, Cat# G8270), 1mM pyruvate (Gibco, Cat# 11360070) and 2mM L-glutamine (Gibco; Cat# 25030081)) and incubated for 1h in a humidified chamber without CO2. This step, also referred to as “outgassing”, is necessary for accurate ECAR measurements. The assay involves sequential injections of toxins into the assay medium, which result in manipulation of components of the ETC. The continuous measurement of the OCR as well as ECAR of SIRT5 RNAi versus control RNAi-treated cells shows the impact of reduced SIRT5 levels on mitochondrial respiration.

**Mitochondrial stress test.** The following parameters are measured during the assay by injecting various toxins into the medium: Initially, the basal cellular respiration (1) is quantified. Subsequently, oligomycin A (2μM, Sigma®, Cat# 75351), a mitochondrial F1/F0-ATP-synthase inhibitor, is injected, which decreases mitochondrial respiration by reducing electron flow along the ETC. This gives insight in the proton leak and thus, non-ATP-linked oxygen consumption (2). Next, maximal cellular respiration (3) is quantified by injecting FCCP (0.75μM, Sigma®, Cat# C2920), a mitochondrial uncoupling agent which disrupts ATP synthesis by transporting protons across the inner mitochondrial membrane. Finally, mitochondrial respiration is completely eliminated by application of rotenone (1μM, Sigma®, Cat# R8875), a complex I inhibitor and antimycin A (1μM, Sigma®, Cat# A8674), a complex III inhibitor. This allows the measurement of non-mitochondrial respiration (4). OCR and ECAR were measured every 3min for a total of 2h.
**ATP rate assay.** To determine ATP production rates, the following parameters are measured during the assay by injecting various toxins into the medium: Basal respiration (1) and respiration after injection of oligomycin A (2μM) (2) as well as rotenone (1μM) together with antimycin A (1μM) (3). OCR and ECAR were measured every 3min for a total of 1.5h.

In both assays, OCR and ECAR were normalised to total cell number/well using the CyQUANT® Cell Proliferation Assay kit (Life Technologies, Cat# C7026). ATP production rate was calculated using the XF Real-Time ATP Rate Assay Report Generator (Seahorse).

### 4.3.10 Mitophagy detection

The Mitophagy detection kit (Dojindo, Cat# MD01-10) was applied according to the manufacturer’s instructions. Transfected cells (control and SIRT5 RNAi) were loaded with a pH-dependent, fluorescent probe (Mtphagy dye), exposed to 6h of normoxia in CM or OND. Lysosomes were then labelled using the Lyso dye (Dojindo). The fluorescence intensity of the Mtphagy dye increases in an acidic environment, thus, the combination of Mtphagy and Lyso dyes allows specific detection of lysosome-engulfed mitochondria. Cells were imaged on a Leica TCS SP8 confocal microscope with Leica HyD photon counters and images processed with ImageJ v1.52 or ImagePro 10 (Media Cybernetics). Integrated optical density (IOD) of Mtphagy dye was assessed using ImagePro 10.

### 4.3.11 Gene expression analyses by RT-qPCR

The detailed protocol for RNA extraction, cDNA synthesis and RT-PCR are described in Chapter 2, Section 2.3.2. Three housekeeping genes: β2-microglobulin (B2M), β-actin (ACTIN) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were tested of which the latter was chosen as an internal control for all qPCR analyses. The primers used in the studies in this Chapter are listed below and were obtained from Sigma®:

**Table 4.1 PCR primers for quantitative gene expression analyses**

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<th>Gene</th>
<th>Direction (5’→3’)</th>
<th>Primer sequences</th>
<th>Amplicon size (bp)</th>
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<td>ACTIN</td>
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<td></td>
<td>Antisense</td>
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<td>Antisense</td>
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<td>B2M</td>
<td>AGATGAGTATGCCTGCCGTGT</td>
<td>GCTTACATGTCCTGATCCACTTA</td>
<td>78</td>
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<td>BNIP3</td>
<td>CACAAGATACCAACAGGGCTTTCT</td>
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<td>GLUT1</td>
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<td>HPRT1</td>
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<td>LDHA</td>
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<td>PINK1</td>
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<td>SIRT1</td>
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<td>SQSTM1 (p62)</td>
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<td>Thompson et al., 2003 (415)</td>
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<td>TFEB</td>
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<td>GTGGGCAAAACTTGTTC</td>
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<td>TFE3</td>
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4.3.12 Quantification of mtDNA by qPCR

The Qiagen DNeasy® Blood & Tissue kit (Qiagen, Cat# 69504) was used to isolate total DNA from HKC-8 cells. Cells in 12-well plates were washed twice with ice-cold PBS and lysed with 200µl buffer ATL supplemented with proteinase K (Qiagen) on ice. Lysates were transferred into 1.5ml Eppendorf tubes, incubated at 56°C for 5min and homogenised by vortexing to ensure complete lysis. DNA was extracted as described in the manufacturer’s protocol and quantified with a NanoDrop 8000 (Labtech) spectrophotometer. Samples were diluted to a final concentration of 5ng/µl and stored at -20°C. All qPCRs (LightCycler® 96 System, Roche) were performed in triplicate with an input of 1µl DNA (5ng/µl) in a total reaction volume of 20µl. The 2x qPCRBO SyGreen Mix Lo-ROX (PCR Biosystems, Cat# PB20.15-20) reaction mix was used, with SYBR Green as a fluorescent reporter. To quantify mtDNA, primers located in the mitochondrial
D-loop were used and were normalised to the amount of nuclear DNA (nDNA) as measured by the relative amount of the single-copy nuclear gene β2-microglobulin (B2M). The primers (Table 4.2) were as described by Bai and Wong (2005) (417) and were obtained from Sigma®.

Table 4.2: PCR primers for mtDNA quantification

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<th>Amplicon size (bp)</th>
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<td>Antisense</td>
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<td>Antisense</td>
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The qPCR was run under the same conditions as described in Chapter 2, Section 2.3.2 and the ΔΔCt method was used to determine mtDNA levels.

4.3.13 Analysis of mitochondria/ER contacts by IF

HKC-8 cells (control and SIRT5 RNAi-treated) were seeded into 8-well chamber slides at a density of 5x10⁴ cells/well. The following day, cells were washed with HBSS or CM to remove detached cells and exposed to either 6h OND or control conditions, respectively. After treatment, cells were washed with PBS and fixed with 4% formaldehyde in PBS (supplemented with calcium (0.9mM) and magnesium (0.49mM)) for 15min at 37°C. Subsequently, cells were washed twice with PBS (5min each) to remove excess formaldehyde. Cells were permeabilised with 0.1% Triton X-100 (Sigma®, Cat# T-8787) in PBS for 10min at RT, washed twice with PBS (5min each) and were incubated with freshly prepared 50mM NH₄Cl for 15min to quench free aldehyde groups followed by two TBST washes (5 min each). Cells were blocked in 10% NGS and 1% BSA in TBS for 1h at RT. Cells were then incubated with the primary antibodies to detect mitochondria as well as the ER (Table 4.3). All primary antibodies were diluted in TBS supplemented with 5% NGS and incubated overnight in the dark at 4°C in humidified chamber. Cells were washed three times with TBST (5min each) to remove excess antibody and incubated for 1h in the dark at RT with secondary fluorescently-tagged antibodies (Table 4.3) diluted 1:500 in TBS supplemented with 5% NGS. After three washes with TBST (5min each) cells were incubated with DAPI (0.1µg/ml) for 5min at RT to visualise nuclei. Following three TBST washes (5min each), the chambers were removed and slides were mounted with FluorSave™ mounting medium (Millipore,
Coverslips were sealed using nail polish and the slides were stored at 4°C for analysis. Cells were imaged with a Leica TCS SP8 confocal microscope (Leica HyD photon counter) and ImageJ (NIH) as well as ImagePro 10 (Media Cybernetics) was used for image analyses.

Table 4.3: Antibodies for immunofluorescence analyses.

<table>
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<tr>
<th>Name</th>
<th>Species</th>
<th>Clonality (clone)</th>
<th>Provider</th>
<th>Cat#</th>
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<td>mc (F-10)</td>
<td>Santa Cruz</td>
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<td>Endoplasmic reticulum marker</td>
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Key: H+L: Heavy and light chains, IF: Immunofluorescence, mc: monoclonal, pc: polyclonal

4.3.14 Live-cell calcium measurements

HKC-8 cells (control and SIRT5 RNAi-treated) were seeded into 8-well chamber slides (Ibidi; Cat# 80826) at a density of 5x10^4 cells/well. The following day, cells were washed with HBSS or CM to remove detached cells and exposed to either 6h OND or control conditions, respectively. After treatment, cells were washed with HBSS and incubated with 100nM Mitotracker™ Deep Red FM (Invitrogen, Cat# M22426), 4µM Fluo4-AM (Biotium, Cat# 50018) as well as 1µM Rhod2-AM (Abcam, Cat# ab142780) in HBSS for 20min at room temperature. To increase the solubility of Rhod2-AM, the surfactant Pluronic™ F-127 (Sigma, Cat# P2443-250G; stock solution: 20% w/v in DMSO) was added to a final concentration of 0.02% w/v. Cells were washed with HBSS and transferred to a CO₂ incubator at 37°C for exactly 30min for de-esterification of the Ca²⁺ dyes. Live-cell images were taken on a TCS SP8 confocal microscope (Leica) equipped with a 37°C incubation chamber.

4.3.15 Western blot analyses

The detailed protocol for cell sample preparation and WB analysis is described in Chapter 2, Section 2.3.3. β-actin (referred to as actin) and α-tubulin (referred to as
tubulin) were used as loading controls. The antibodies used in the studies described in this Chapter are listed in Table 4.4:

Table 4.4: Antibodies for Western blot analyses

<table>
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<tr>
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<th>Species</th>
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**Key:** mc: monoclonal, pc: polyclonal
4.3.16 Citrate synthase activity assay

HKC-8 cells were lysed in 0.1% Triton X-100 in PBS (without calcium and magnesium) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoScientific) and pepstatin A (Sigma®). Protein concentration was determined (DC™ Assay; Bio-Rad), with BSA as a standard. Citrate synthase (CS) activity was measured according to Srere et al.(418) Briefly, acetyl Co-enzyme A (100µM, Sigma®, Cat# A2056), oxalacetic acid (100µM, Sigma®, Cat# O4126) and 5,5′-dithiobis(2-nitrobenzoic acid) (200µM, Sigma, Cat# D8130) were combined in Tris·HCl buffer (100mM, pH 8.0) and the rate of 5-thio-2-nitrobenzoic acid formation was measured at 30°C using a plate reader (412nm; BioTek® Synergy HT). CS activities were normalised to protein concentration/sample.
4.4 Results

4.4.1 OND causes mitochondrial dysfunction in hPTECs

**Starvation, hypoxia and OND stimulate mitophagy.** Renal ischaemia in vivo caused by reduced blood flow to the organ manifests in hypoxia and starvation due to oxygen and nutrient deprivation, respectively. To characterise the impact of starvation, hypoxia and combined OND on hPTECs, RT-qPCRs were carried out to screen for expression of mRNA levels of markers involved in cellular glucose metabolism (as an indication of a glycolytic switch and a hypoxic response) and autophagy/mitophagy (a measure of nutrient starvation) (Figure 4.2).

**Figure 4.2: qPCR gene expression analyses of the OND model.** hPTECs were exposed to hypoxia (1% O₂) or normoxia (21% O₂) with or without nutrient depletion (HBSS) for 24h. (A,B) **Hypoxia:** Bar graphs showing (A) lactate dehydrogenase A (LDHA) and (B) glucose transporter 1 (GLUT1) mRNA expression. (C-E) **Mitophagy:** Bar graphs showing (C) B-cell lymphoma 2 19 kilodalton interacting protein 3 (BNIP3), (D) autophagy receptor p62 and (E) PTEN-induced putative kinase 1 (PINK1). Data are from three independent experiments with n=3 replicates/group. Data are mean ± SD. To determine statistical significance of changes in mRNA expression a Two-Way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. Data were normalised to hypoxanthine phosphoribosyltransferase 1 (HPRT1). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Induction of hypoxia was confirmed by measurement of mRNA levels of the hypoxia-inducible factor 1α (HIF1α) target genes lactate dehydrogenase A (LDHA) and glucose transporter 1 (GLUT1), both are glycolytic enzymes; and, the mitophagy receptor...
B-cell lymphoma 2 19 kilodalton interacting protein 3 (BNIP3). As shown in Figure 4.2A-C, hypoxia and OND significantly increased the expression of all three markers (hypoxia: LDHA: p<0.0001; GLUT1 and BNIP3: p<0.0001 and OND: LDHA: p<0.001; GLUT1 and BNIP3: p<0.0001). Interestingly, while GLUT1 and BNIP3 expression were significantly higher in hPTECs exposed to OND compared to hypoxia alone (GLUT1: p<0.0001; BNIP3: p<0.05), LDHA mRNA levels were significantly lower in OND- relative to hypoxia-treated cells (p<0.05). Of note, while starvation alone did not impact on LDHA and BNIP3 mRNA levels, GLUT1 levels increased relative to the normoxia/CM control (p<0.01). This finding was not unexpected as GLUT1 has also been shown to be a TFEB target gene, a TF which is a key inducer of autophagy during nutrient starvation (138).

Induction of autophagy/mitophagy was assessed by screening for mRNA levels of the autophagy receptor p62 and the mitochondrial stress sensor PTEN-induced putative kinase 1 (PINK1), which is known to drive mitophagy (180). As shown in Figure 4.2D, starvation and OND significantly increased the p62 mRNA levels compared to the normoxia/CM control (both: p<0.0001) while hypoxia alone had no significant effect on p62 mRNA expression. To assess the induction of mitophagy qPCR was carried out to screen for PINK1 mRNA expression (Figure 4.2E). The qPCR analysis revealed that PINK1 expression increased upon both hypoxia and OND (both: p<0.001) while starvation alone did not significantly impact on PINK1 mRNA levels relative to the normoxia control.

In summary, while both hypoxia and OND increased expression of glycolytic enzymes and PINK1 as a marker of mitophagy, the mRNA data suggested that there may be differences in the induction of autophagy in response to the two stimuli.

Subsequently, it was assessed whether putative differences in the induction of autophagy in response to hypoxia or OND individually or in combination could be confirmed at the protein level. Western blot (WB) analyses were performed to screen for the autophagosomal linker protein LC3-II, its precursor LC3-I (a shift from LC3-I to LC3-II indicates autophagy induction) and the autophagy receptor p62 (a decrease in p62 levels indicates increased autophagic flux) (Figure 4.3A). As shown in Figure 4.3B, starvation (p<0.0001), hypoxia (p<0.001) and OND (p<0.0001) all resulted in reduced LC3-I protein levels suggesting its conversion to LC3-II as a consequence of autophagy induction. However, quantification of LC3-II protein levels only showed an increase in HBSS- (p<0.0001) and OND-treated cells (p<0.05) with only a marginal, non-significant increase in cells exposed to hypoxia alone when compared to the normoxia/CM control (Figure 4.3C). These findings were also reflected in the LC3-II/LC3-I ratio (Figure 4.3D).
which showed that autophagy was significantly higher in OND- compared to hypoxia-treated cells (p<0.0001).

**Figure 4.3: OND induces autophagy.** hPTECs were exposed to hypoxia (1% O\(_2\)) or normoxia (21% O\(_2\)) with or without nutrient depletion (HBSS) for 24h. (A) WB showing protein levels of HIF1α and the autophagy markers LC3 and p62 and actin. (B-E) Scatter plots showing densitometry results of LC3-I (B), LC3-II (C), LC3-II/LC3-I ratio (D) and the autophagy receptor p62 (E). Data are from three independent experiments with n=3 replicates/group. Data are mean ± SD. To determine statistical significance a Two-Way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. Data were normalised to actin. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. ImageJ was used for densitometric analyses.

As an indication of autophagic flux, p62 protein levels were quantified. As shown in Figure 4.3E, p62 protein levels significantly declined in HBSS- and OND-treated cells with no significant change in hypoxia-treated cells compared to the normoxia/CM control (HBSS: p<0.01; OND: p<0.001). Accordingly, when compared to hypoxia, OND treatment showed significantly decreased p62 protein levels (Figure 4.3E; p<0.01) suggesting increased autophagic flux in OND- versus hypoxia-treated cells.

Taken together, the data indicated that OND promotes autophagy and increases the autophagic flux and therefore, may be a better *in vitro* model to mimic renal ischaemia.

**Starvation, hypoxia and OND stimulate mitochondrial mass reduction.** Renal ischaemia causes mitochondrial fragmentation (46)(Yoshii, 2017 #289) and degradation (422) *in vivo*. To assess whether mitochondrial mass is affected by HBSS, hypoxia or OND, protein levels of the mitochondrial marker translocation outer membrane 20 (TOM20) and the pro-fusion protein OPA1 were assessed by WB (Figure
4.4A). As shown in Figure 4.4B, TOM20 protein levels declined to the same extent in HBSS, hypoxia and OND treatment groups indicating equal induction of mitophagy (p<0.001). This is not surprising given that both stimuli (hypoxia and starvation) have individually been shown to induce mitophagy.\(^{(423)}\) Interestingly, when OPA1 levels were measured and the data from n=3 experiments pooled, there was no significant change in total OPA1 levels in any of the treatment groups (Figure 4.4C). This was surprising given the visible changes in the larger isoforms seen in the WB (Figure 4.4A).

![Figure 4.4: HBSS, hypoxia and OND induce mitophagy.](image)

**Figure 4.4: HBSS, hypoxia and OND induce mitophagy.** hPTECs were exposed to hypoxia (1% \(O_2\)) or normoxia (21% \(O_2\)) with or without nutrient depletion (HBSS) for 24h. (A) WB showing protein levels of the mitochondrial markers optic atrophy 1 (OPA1) and translocase of outer membrane 20 (TOM20) as well as the loading controls tubulin and actin (B+C) Scatter plots showing densitometry results of TOM20 (B) and OPA1 (C). All data are from three independent experiments with n=3 replicates/group. Data are mean ± SD. To determine statistical significance Two-Way ANOVA was carried out followed by Tukey's post hoc test to normalise for multiple comparisons. Data were normalised to actin. ***p<0.001. ImageJ was used for densitometric analyses.

**OND but not hypoxia-only drives l-OPA1 proteolysis.** As discussed earlier (Section 4.1), OPA1 exists as five isoforms, two long (L1 and L2) and three short (S1, S2 and S3), all of which are important for mitochondrial form and function.\(^{(159, 397)}\) Proteolytic degradation of l-OPA1 by the peptidase OMA1, decreases mitochondrial inner membrane fusion competency and thereby, provokes mitochondrial fragmentation \(^{(159)}\), a pathophysiological feature of IRI in tubular cells *in vivo* \(^{(398)}\).

As a proof a principle experiment for l-OPA1 degradation, hPTECs were treated with carbonyl cyanide m-chlorophenyl hydrazine (CCCP; 5µM; 24h), an uncoupling agent, which induces l-OPA1 degradation due to dissipation of the \(\Delta\Psi_M\) \(^{(162)}\). As shown in Figure 4.5A, l-OPA1 levels declined upon CCCP treatment indicating l-OPA1 proteolysis.

To address whether HBSS, hypoxia or OND treatment induce l-OPA1 degradation, l- and s-OPA1 protein levels in hPTECs were determined using WB (Figure 4.5B). As shown in Figure 4.5C, l-OPA1 levels only declined in HBSS- and OND-treated cells (HBSS: p<0.0001; OND: p<0.001) with no change in the hypoxia-treated
group relative to the normoxia/CM control. When compared to hypoxia alone, OND treatment significantly reduced l-OPA1 protein levels \((p<0.001)\). This finding is particularly interesting as it suggested that the combination of starvation and hypoxia may be fundamental to generating an environment which more accurately mimics \textit{in vivo} renal ischaemia in PTECs.

![Figure 4.5: OND stimulates l-OPA1 degradation. (A)](image)

\(hPTECs\) were treated with the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazine (CCCP; 5µM) or DMSO for 24h. WB showing protein levels of long (L1 and L2) and short (S1, S2 and S3) optic atrophy 1 (OPA1) isoforms. (B) \(hPTECs\) were exposed to hypoxia \((1\% \text{ O}_2)\) or normoxia \((21\% \text{ O}_2)\) with or without nutrient depletion (HBSS) for 24h. WB showing protein levels of OPA1. (C-E) Scatter plots showing densitometry results of l-OPA1 (C), s-OPA1 (D) and l/s-OPA1 ratio (E). Data are from three independent experiments with n=3 replicates/group. Data are means ± SD. To determine statistical significance a Two-Way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. Data were normalised to tubulin. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) and ****\(p<0.0001\). ImageJ was used for densitometric analyses.

Next, s-OPA1 expression was assessed. As shown in Figure 4.5D, s-OPA1 levels increased upon HBSS and OND treatment (both \(p<0.01\)) relative to the normoxia/CM control (Figure 4.5D) suggesting a conversion from l- to s-OPA1. However, in the hypoxia-treated group, s-OPA1 levels were unchanged compared to the normoxia/CM control (Figure 4.5D). As a consequence, OND-treated cells showed significantly higher levels of s-OPA1 compared to hypoxia-treated cells \((p<0.05)\).

**OND but not hypoxia causes a decline in YME1L.** As previously discussed (Section 4.1), the peptidases OMA1 (ATP-independent) and YME1L (ATP-dependent), are two major l-OPA1-degrading enzymes, which have been shown to undergo reciprocal degradation, thereby, regulating mitochondrial dynamics.(158)
Figure 4.6: OND stimulates YME1L degradation. (A) hPTECs were treated with the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazine (CCCP; 5µM) or DMSO (0.05% v/v) for 24h. WB showing protein levels of YME1L. (B) hPTECs were exposed to hypoxia (1% O₂) or normoxia (21% O₂) with or without nutrient depletion (HBSS) for 24h. WB showing protein levels of YME1L. Tubulin served as a loading control.

Renal ischaemia causes a reduction in the ΔΨᵢ in PTs (424) and has been shown to provoke l-OPA1 degradation (398). To assess whether ΔΨᵢ dissipation may drive l-OPA1 degradation by decreasing YME1L protein levels, hPTECs were stimulated with CCCP (5µM; 24h) and analysed by WB. As shown in Figure 4.6A, CCCP treatment reduced YME1L protein expression compared to DMSO treatment control. Next, it was tested whether the OND-induced decline in l-OPA1 levels (Figure 4.5C) may be associated with a reduction in YME1L. It was shown that YME1L protein levels declined in OND-treated cells relative to the normoxia/CM control. Interestingly, YME1L levels appeared higher in hypoxia-only treated cells (Figure 4.6B). This finding is in line with the observation that hypoxia-only does not significantly stimulate l-OPA1 degradation compared to OND treatment (Figure 4.5C). Of note, at the time the data in Figure 4.6 were generated another batch of the anti-OMA1 antibody had been obtained which did not detect a specific band with the expected molecular weight, thus, is was not possible to assess OMA1 expression and only YME1L protein levels were analysed.

Taken together, given that l-OPA1 degradation, which drives mitochondrial fragmentation, may be a key pathophysiologic factor driving mitochondrial pathologies in renal ischaemia in vivo (398) these data suggested that OND is a more representative in vitro model of in vivo ischaemia than hypoxia alone.

4.4.2 OND stimulates SIRT5 expression in hPTECs in vitro.

OND and hypoxia increase SIRT5 mRNA and protein levels. In Chapter 3 it was shown that IRI causes an increase in SIRT5 protein levels in murine PTECs (Figure 3.10). To test whether SIRT5 is also upregulated in hPTECs in response to ischaemic injury in vitro and dissect the independent and combined effects of hypoxia and starvation, hPTECs were either exposed to hypoxia or normoxia either with or without nutrient deprivation (Figure 4.7).
As shown in Figure 4.7A, mRNA expression analysis revealed an increase in SIRT5 levels in response to hypoxia (p<0.001) and OND (p<0.0001) with no statistically significant change with HBSS treatment compared to the normoxia/CM control. Notably, when compared to hypoxia alone, OND treatment provoked a significantly larger increase in SIRT5 mRNA (p<0.01) indicating an additive effect of hypoxia and starvation.

To put the SIRT5 mRNA expression data in context with the mitochondria-associated sirtuins SIRT3 and 1 and assess whether these are all equally regulated by hypoxia, qPCR expression analyses were carried out (Figure 4.7B and C). Interestingly, SIRT3 and 1 mRNA expression were differently affected by hypoxia and starvation: SIRT3 expression only increased with hypoxia (p<0.01), with no significant change after HBSS or OND treatment relative to the normoxia/CM control while SIRT1 expression increased during starvation (p<0.0001) and OND (p<0.0001), with no change in response to hypoxia. Notably, SIRT1 mRNA levels were significantly lower in OND-compared to HBSS-treated cells (p<0.01) suggesting a suppressive effect of hypoxia on SIRT1 mRNA expression (Figure 4.7C).
Finally it was tested whether the SIRT5 mRNA expression data was reflected in the protein levels as measured by WB (Figure 4.7D). In line with the mRNA data, it was shown that both hypoxia and OND significantly increased SIRT5 protein levels (both: p<0.05) while starvation had only a small, non-significant effect on SIRT5 protein expression (p>0.05) compared to the normoxia/CM control (Figure 4.7E).

In conclusion, the data suggested that SIRT5 expression (mRNA and protein) is regulated by OND, with hypoxia as the primary driver.

**OND-induced increase in SIRT5 mRNA levels is independent of HIF1α.**

HIF1α is a key TF which facilitates metabolic adaptation during low oxygen conditions.(425) Under normoxic conditions, prolyl hydroxylase domain proteins (PHDs) continuously hydroxylate two prolyl residues located within the oxygen-dependent degradation domain of HIF1α, thereby, marking the TF for degradation. During hypoxia, PHDs are inhibited resulting in an accumulation of HIF1α.(425) To test whether hypoxia-induced SIRT5 and 3 mRNA upregulation is mediated by HIF1α, hPTECs were stimulated with CoCl$_2$ (100µM; 24h), a PHD inhibitor, which strongly activates the HIF-pathway (404) and analysed by qPCR. As shown in Figure 4.8A, CoCl$_2$ treatment resulted in a significant increase in BNIP3 mRNA levels (HIF1α target gene; positive control; p<0.05) while neither SIRT5 nor 3 were affected.

These data indicated that the hypoxia-induced increase in SIRT5 and 3 is independent of the HIF pathway.

**Figure 4.8:** SIRT5 gene transcription is not regulated by the HIF1 pathway. PTECs were stimulated with CoCl$_2$ (100µM; 24h) and analysed by qPCR. Bar graphs showing mRNA levels of (A) B-cell lymphoma 2 19 kilodalton interacting protein 3 (BNIP3) (positive control for HIF1α activation), (B) SIRT5 and (C) SIRT3. mRNA data were normalised to hypoxanthine phosphoribosyltransferase 1 (HPRT1). Data are mean ± SD. To determine statistical significance a Mann-Whitey U test was carried out. *p<0.05.
OND-induced increase in SIRT5 mRNA levels may be a consequence of mTOR-pathway inhibition. Nutrient depletion (426) as well as hypoxia (427) reduces the activity of mammalian target of rapamycin complex 1 (mTORC1), which stimulates TFEB/3 shuttling from the cytosol to the nucleus and thereby, induces autophagy-related gene transcription (426, 428). Recently, Guedouari et al. showed that during starvation SIRT5 protects mitochondria from autophagic degradation and by doing so, is cytoprotective.(338) To test whether the OND-induced SIRT5 upregulation (Figure 4.6A and E) was mediated by mTOR inhibition, hPTECs were treated with TORIN-1 (100nM), a potent mTORC1/2 inhibitor (405) and analysed by qPCR (Figure 4.9).

As displayed in Figure 4.9A-C, TORIN-1 stimulation significantly increased mRNA levels of p62 (p<0.05; TFEB/3 target gene; positive control), SIRT5 (p<0.05) and SIRT1 (p<0.05; TFEB target gene; positive control). These data implied that SIRT5 mRNA upregulation during OND may be, at least in part, driven by mTOR inhibition.

The SIRT5 gene promoter contains putative TFEB/3 binding sites. It is well-accepted that mTORC1 inhibition results in autophagy induction (426) by promoting cytosol-to-nucleus shuttling of the master autophagy modulators TFEB and TFE3 (428, 429) while the transcriptional repressor of autophagy-related genes Zinc finger protein with KRAB and SCAN domains 3 (ZKSCAN3) translocates from the nucleus to the cytosol (430, 431). This ultimately results in the induction of autophagic and lysosomal genes.(430, 431) Shin et al. showed that the murine Sirt1 gene contains a TFEB binding site (CLEAR site), thereby, explaining the transcriptional mechanism through which Sirt1 is upregulated during starvation.(141) To test whether the human SIRT1 and SIRT5...
promoters also contain TF binding sites for MiTF/TFE3 as well as for their counter-regulator ZKSCAN3, promoters were screened using the Genomatrix software.

![Diagram](Image)

**Figure 4.10: Putative MiTF/TFEB and ZKSCAN3 binding sites in the human SIRT1 and SIRT5 gene promoters.** Genomatrix software was used to screen the human SIRT1 and SIRT5 gene promoters for transcription factor binding sites.

The software implements all coding transcripts and screens their distinct promoters (Appendix, Table 1). Promoter analyses of the primary transcripts indicated that both SIRT1 and SIRT5 gene promoters contain putative binding sites for MiTFE/TFEB family proteins (Figure 4.10; Appendix, Table 1). The SIRT1 promoter contains three full TFEB binding sites (CLEAR sites: TCACGTG) while the SIRT5 promoter used for the transcription of the primary transcript, contains one full CLEAR site (TCACGTG) and one site predicted to bind MiTF/TFE3 (CATGTG). Promoter screening for ZKSCAN3 binding sites (CCCC) revealed three binding sites in the SIRT5 promoter while none were found in the promoter of primary transcript of SIRT1 indicating that SIRT5 may be transcriptionally repressed during normal nutrient conditions.

**SIRT5 gene expression is not regulated by TFEB or TFE3.** Based on the results of the mTOR inhibition experiment (Figure 4.9) and the Genomatrix analysis (Figure 4.10), which both indicated that SIRT5 transcription may be regulated by TFEB/3, a siRNA-mediated knockdown approach was adopted using (Figure 4.11) siRNAs against TFEB and TFE3 followed by TORIN-1 treatment (100nM; 24h), 48h post
knockdown. Samples were analysed using qPCR. As this was a proof of principle experiment it was only performed once.

As shown in Figure 4.11A, TFEB RNAi reduced TFEB mRNA levels and furthermore, prevented the TORIN-1-induced increase in SIRT1 mRNA expression (positive control) indicating successful TFEB depletion. Subsequent screening for SIRT5 mRNA revealed that TFEB knockdown did not prevent the TORIN-1-induced increase in SIRT5 mRNA expression relative to the siRNA control which suggested that SIRT5 transcription was not regulated by TFEB. On the contrary, TFEB RNAi-treated cells showed significantly higher levels of SIRT5 mRNA compared to the siRNA control. Given the time/financial restraints of the project and the fact that the experiment had shown that SIRT5 was not regulated by TFEB this observation was not further investigated.

Figure 4.11: SIRT5 gene expression is not regulated by the transcription factors TFEB or TFE3. hPTECs were treated with siRNA (50nM; 48h) against the autophagy-associated transcription factors: (A) TFEB, or (B) TFE3 followed by treatment with the mTOR inhibitor TORIN-1 (100nM) or DMSO (0.05% v/v) for 24h. (A) Bar graphs showing mRNA levels of TFEB, SIRT1 (a known TFEB target) and SIRT5. (B) Bar graphs showing mRNA levels of TFE3, p62 (a known TFE3 target) and SIRT5. Data are from a single, proof of principle experiment with n=3 replicates/group. Data were normalised to hypoxanthine phosphoribosyltransferase 1 (HPRT1). Data are mean ± SD. To determine statistical significance a Two-Way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Next, it was tested whether SIRT5 gene expression is regulated by TFE3 (Figure 4.11B). TFE3 RNAi treatment reduced TFE3 mRNA levels and impaired TORIN-1 induced upregulation of the TFE3 target gene p62, indicating successful TFE3 depletion. Similar to the TFEB RNAi experiment, TFE3 RNAi did not prevent the TORIN-1-mediated increase in SIRT5 mRNA levels when compared to the siRNA control suggesting that SIRT5 gene expression in hPTECs is not regulated by TFE3.

4.4.3 SIRT5 depletion exacerbates OND-induced mitochondrial dysfunction

SIRT5 knockdown impairs cellular energy metabolism. SIRT5 has been identified as a central modulator of cellular energetics by targeting multiple enzymes involved in mitochondrial (OXPHOS, TCA cycle and FAO) as well as glycolytic metabolism.(288, 329, 330) To address whether SIRT5 depletion affected ATP metabolism and thus, may also be an important regulator of cellular bioenergetics in hPTECs, SIRT5 knockdown (by RNAi) followed by quantification of steady-state ATP levels as well as ATP production rate was carried out (Figure 4.12A-D).

Interestingly, SIRT5 RNAi reduced total ATP levels compared to the control RNAi group (Figure 4.12B; p<0.05). To differentiate between mitochondrial and glycolytic steady-state ATP levels, SIRT5 RNAi-treated hPTECs were pre-treated with either 2-deoxyglucose (2DG) or oligomycin A (O), respectively. SIRT5 RNAi significantly reduced steady-state ATP levels in both treatment groups (2DG and O; both: p<0.05). To assess whether SIRT5 depletion impaired ATP production rates (by glycolysis and OXPHOS) an ATP rate assay (using a Seahorse Analyzer) was performed. This assay measures oxygen consumption rate (OCR) at baseline and after sequential injections of O and rotenone and antimycin A (R+A). As shown in Figure 4.12C, SIRT5 RNAi slightly impaired total ATP generation as well as ATP production by glycolysis and OXPHOS (p<0.05), indicating that in hPTECs, SIRT5 knockdown impaired mitochondrial as well as glycolytic energy metabolism. Interestingly, the data also showed that hPTECs primarily depend on OXPHOS for ATP generation (OXPHOS/glycolysis = 60/40) and this bioenergetic profile was not affected by SIRT5 depletion (Figure 4.12D).

The ΔΨM is a fundamental parameter that regulates mitochondrial ATP synthesis.(432) To determine whether SIRT5 knockdown affected the ΔΨM and thus, may contribute to the reduction of mitochondrial ATP, control and SIRT5 RNAi-treated hPTECs were incubated with Tetramethylrhodamine methyl ester (TMRM) and analysed by flow cytometry (Figure 4.12E).
Figure 4.12: SIRT5 knockdown impairs cellular energy metabolism. SIRT5 in hPTECs was knocked down by RNAi. Non-targeting siRNA served as control. All experiments were carried out 72h after siRNA transfection. (A) WB showing SIRT5 protein levels. (B) Bar graph displaying steady-state ATP levels. hPTECs (control and SIRT5 RNAi) were analysed without inhibitor treatment to determine total ATP levels (glycolytic + mitochondrial ATP), after treatment with 2-deoxyglucose to inhibit glycolysis (mitochondrial ATP; OXPHOS) or after treatment with oligomycin A (O) to inhibit mitochondrial ATP synthase (glycolytic ATP; Glycolysis). ATP levels were normalised to cell number as measured by DNA quantification. Data are from four independent experiments with n=6 technical replicates/group. (C) Bar graph displaying ATP production rate and (D) bioenergetic profile. hPTECs (control and SIRT5 RNAi) were analysed on a Seahorse Analyzer. Oxygen consumption rate (OCR) was measured at baseline, after injection of O (inhibition of F1/F0-ATP synthase) and after injection of rotenone and antimycin A (inhibition of respiratory complexes I and III). ATP production rate and bioenergetic profile were determined with the Wave software tool. Data were normalised to cell number. Data are from four independent knockdown experiments with n=3 technical repeats/group. (E) Bar graph showing TMRM fluorescence intensity. hPTECs (control and SIRT5 RNAi) were incubated with TMRM (25nM), a fluorescent dye which accumulates in mitochondria based on the ΔΨM and fluorescence intensities determined by flow cytometry. Background fluorescence was assessed by FCCP treatment (10µM) prior to TMRM incubation and fluorescence intensities were subtracted from the TMRM fluorescence intensities. Data are from five independent experiments with n=2 technical replicates/group. Data are mean ± SD. To determine statistical significance a Mann-Whitney U test was applied. *p<0.05 and **p<0.01.
TMRM is a fluorescent dye, which accumulates in mitochondria based on the $\Delta \Psi_M$ and therefore, can be used to determine the $\Delta \Psi_M$. As shown in Figure 4.12E, SIRT5 RNAi-treated hPTECs displayed reduced TMRM fluorescence intensities compared to control RNAi-treated cells ($p<0.01$) suggesting that SIRT5 depletion impaired the $\Delta \Psi_M$.

Although the changes shown in Figure 4.12B-E appear to be rather small, it has to be stated that these data were generated from four (steady-state ATP levels and ATP rate assay) and five (FACS analysis) independent SIRT5 knockdown experiments which all showed the same effect. Therefore, these data suggested that SIRT5 depletion reduces both glycolytic and mitochondrial ATP levels; and, the decrease in the latter may at least be partly mediated by a reduction in $\Delta \Psi_M$.

**SIRT5 knockdown induces mitochondrial fragmentation.** Mitochondria are highly dynamic organelles that constantly undergo fusion and fission.(433) This process is central to bioenergetic adaptation (44) as well as mitoQC (42) and is dependent on the metabolic state of a cell and the $\Delta \Psi_M$, respectively. To assess whether SIRT5 knockdown impacts on mitochondrial morphology, control and SIRT5 RNAi-treated cells were incubated with Mitotracker Red CMXRos and analysed by confocal microscopy. Notably, SIRT5 RNAi resulted in mitochondrial fragmentation compared to control RNAi-treated cells (Figure 4.13A). As shown in Figure 4.13B, semi-quantitative image analysis revealed that SIRT5 RNAi induced an increase in individual mitochondria/mitochondrial footprint (0.87±0.28 versus 1.66±0.55; $p<0.0001$), increased the number of mitochondrial networks/mitochondrial footprint (0.11±0.046 versus 0.21±0.055; $p<0.0001$) and reduced the mean network size (21.1µm versus 10.1µm; $p<0.0001$) as well as the mean branch length (0.84±0.1µm versus 0.69±0.11µm; $p<0.0001$), compared to the RNAi control. Taken together these data suggested a role for SIRT5 in the regulation of mitochondrial structure.
Figure 4.13: *SIRT5* RNAi induces mitochondrial fragmentation. *SIRT5* knockdown by RNAi was carried out in hPTECs. Non-targeting siRNA served as control. All experiments were carried out 72h after siRNA transfection. **(A)** Cells were stained with Mitotracker Red CMXRos (100nM) and imaged by confocal microscopy. Scale bar: 20µm. **(B)** Mitochondrial morphology was quantitatively assessed using the Mitochondrial Network Analysis (MiNA) toolset (ImageJ). The number of individual mitochondria and mitochondrial networks per cell were quantified and normalised to the mitochondrial footprint; Mean network size (number of branches) and the mean branch length (µm) were determined. Forty-two control RNAi-treated and 51 *SIRT5* RNAi-treated cells from three independent experiments were analysed. Box and whisker plots display median interquartile range and minimum and maximum values. Mann-Whitney U test was applied. ****p<0.0001.

**SIRT5** knockdown disrupts the mitochondrial fission/fusion machinery. Mitochondria are highly dynamic organelles and their architecture is highly regulated by pro-fission and -fusion proteins.(44) While under physiological conditions fission and fusion dynamics are balanced, ischaemia provokes a shift towards fission resulting in excessive mitochondrial fragmentation.(39) Based on our previous findings
(Figure 4.13), the question was asked whether SIRT5 RNAi directly affected the expression of proteins involved in mitochondrial fission/fusion; and, whether this was exacerbated during OND. Although the WBs shown in Figure 4.14A and B are representative blots from a single SIRT5 knockdown experiment the data shown in the scatter plots are pooled from three to four independent SIRT5 RNAi experiments (biological replicates) which were all carried out in triplicate (technical replicates).

To assess whether SIRT5 RNAi impacts on the fission machinery, siRNA-treated hPTECs cultured under control conditions (21%O₂+CM) or OND (1%O₂+HBSS) were screened for DRP1, DRP1-S616 as well as the DRP1 receptors MiD51 and FIS1, by WB. As indicated in Figure 4.14A, DRP1 levels significantly increased (p<0.05) in the OND-treated SIRT5 RNAi-treated cells relative to the control RNAi group (21%O₂+CM). Furthermore, SIRT5 RNAi significantly increased (p<0.05) DPR1-S616 phosphorylation in control and OND-treated cells (both p<0.05). Of note, this increase was independent of OND treatment as DRP1-S616 levels did not change in control RNAi-treated cells exposed to OND suggesting that SIRT5 RNAi directly or indirectly affected DRP1 phosphorylation to promote fission. Interestingly, the levels of the DRP1 recruitment factors MiD51 and FIS1 were not affected by SIRT5 RNAi or OND.

To determine whether SIRT5 RNAi impacts on the fusion machinery, levels of the pro-fusion factors MFN1, MFN2 and OPA1 were investigated by WB (Figure 4.14B). First it was assessed whether SIRT5 knockdown affected their protein levels during normoxia. This showed that SIRT5 RNAi significantly reduced MFN1 (p<0.05), MFN2 (p<0.0001) and OPA1 (p<0.05) protein levels relative to the RNAi control suggesting that SIRT5 depletion may drive fragmentation by impairing the pro-fusion system. Next, it was assessed whether the protein levels of these three fusion factors were affected by OND, in the presence or absence of SIRT5. As shown in Figure 4.14B, OND treatment significantly decreased MFN1 (control and SIRT5 RNAi: p<0.01) and MFN2 (control and SIRT5 RNAi: p<0.0001) in both control and SIRT5 RNAi-treated cells relative to the normoxia siRNA control. Notably, SIRT5 knockdown triggered a significantly larger reduction in MFN2 protein levels (p<0.0001) relative to the RNAi control in OND hinting that SIRT5 depletion may exacerbate mitochondrial fragmentation during OND in a MFN2-dependent manner. OPA1 protein levels were also assessed. As shown in Figure 4.14B, OPA1 levels did not significantly decline in the RNAi control-treated cells exposed to OND, a finding which is in line with previous data (Figure 4.3C). However when SIRT5 was depleted, total OPA1 levels dropped to a similar level as found in normoxia (Figure 4.14B). While the drop in total OPA1 was significant larger after OND (p<0.01) compared to normoxia (p<0.05) relative to the normoxic siRNA control, there was no difference between the two SIRT5 RNAi-treated groups (normoxia versus OND).
This finding suggested that SIRT5 knockdown may induce OPA1 proteolysis independent of OND. As a result, total OPA1 levels were significantly lower in SIRT5 RNAi-treated cells compared to control RNAi-treated cells (p<0.05) exposed to OND.

Figure 4.14: SIRT5 knockdown disrupts the mitochondrial fission/fusion machinery. hPTECs were transfected using SIRT5 siRNA SIRT5 or non-targeting siRNA control. Seventy-two hours post-transfection cells were exposed to 6h OND (1%O₂+HBSS) or normoxia and complete medium (21%O₂+CM) as control. (A) WBs of pro-fission proteins showing protein levels of dynamin-related protein 1 (DRP1), DRP1-S616, the DRP1 receptors mitochondrial MiD51 and FIS1. Scatter plots showing densitometry results of DRP1, DRP1-S616, MiD51 and FIS1. DRP1 and DRP1-S616 were normalised to tubulin while MiD51 and FIS1 where normalised to actin. (B) WBs of pro-fusion proteins showing protein levels of MFN1/2 and OPA1. Scatter plots showing densitometric quantitation of MFN1, MFN2 and OPA1. Data are from three to four independent experiments with n=3 replicates/group. MFN1 and OPA1 were normalised to tubulin while MFN2 was normalised to actin. ImageJ was used for densitometric analysis. Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Taken together, the data indicated that SIRT5 depletion impairs mitochondrial dynamics by affecting the expression of central components of the mitochondrial fission and fusion machinery. By doing so, SIRT5 knockdown may induce mitochondrial fragmentation during normoxia, which may be exacerbated in OND.
*SIRT5* knockdown affects OPA1 processing by OMA1 and YME1L. As previously discussed (Section 4.1) the pro-fusion protein OPA1 exists as five isoforms. To examine whether *SIRT5* knockdown-induced reduction in ATP and the ΔΨ_M impacted on OPA1 processing, siRNA-treated hPTECs (control and *SIRT5*) exposed to normoxia (21%O_2+CM) or OND (1%O_2+HBSS) were screened for l/s-OPA1, YME1L and OMA1 by WB (Figure 4.15A).

![Figure 4.15: SIRT5 knockdown alters l-OPA1 processing by OMA1 and YME1L. hPTECs were transfected with SIRT5 siRNA or non-targeting siRNA control. Seventy-two hours post-transfection cells were exposed to 6h OND (1%O_2+HBSS) or normoxia as a control. (A) WBs showing protein levels of OPA1 (five isoforms: L1, L2, S1-3) and the OPA1 processing proteases, OMA1 (ATP-independent) and YME1L (ATP-dependent). (B) Scatter plots showing densitometric quantitation of long (l)-OPA1, short (s)-OPA1, OMA1 and YME1L. Data are from three to four independent experiments with n=3 replicates/group. Tubulin was used as a loading control. ImageJ was used for densitometric analyses. Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05, **p<0.01, and ***p<0.001.](image)

As shown in Figure 4.15B, *SIRT5* knockdown significantly reduced l-OPA1 protein levels (p<0.05) relative to the normoxic control RNAi cells suggesting that SIRT5 depletion may stimulate proteolytic cleavage of l-OPA1. It was then assessed whether l-OPA1 proteolysis in *SIRT5* RNAi and OND-treated control RNAi cells was mediated by OMA1. *SIRT5* RNAi provoked a significant decrease of YME1L (p<0.01) while OMA1 levels remained unchanged (Figure 4.15B) suggesting OMA1-mediated YME1L degradation leads to augmented l-OPA1 proteolysis. Of note, YME1L levels also declined as a consequence of OND treatment in control (p<0.001) and *SIRT5* RNAi (p<0.01) treated cells confirming the previous observation that YME1L proteolysis was induced by OND (Figure 4.6). Interestingly, OMA1 levels remained unchanged in control and *SIRT5* RNAi-treated cells exposed to OND.
Notably, proteolytic cleavage of l-OPA1 results in a shift towards s-OPA1 which is reflected in increased s-OPA1 levels. Unexpectedly, s-OPA1 levels dropped in SIRT5 RNAi cells under control conditions (p<0.05) as well as OND (p<0.01) and were not affected by OND in the control RNAi-treated cells (Figure 4.15B) suggesting that SIRT5 RNAi increased the proteolytic degradation of s-OPA1.

In conclusion, the data suggested that SIRT5 knockdown increased l-OPA1 proteolysis which may be a direct or indirect consequence of enhanced OMA1 activity.

**SIRT5 RNAi exacerbates OND-induced mitochondrial swelling.** The mitochondrial pro-fusion factor OPA1 is fundamental to mitochondrial ultrastructure as OPA1 depletion disrupts cristae formation (155, 159, 397) and induces mitochondrial swelling (434). This is particularly interesting in the context of renal ischaemia as mitochondrial swelling is a central pathophysiologic feature of PTEC injury.(435, 436) To determine the impact of SIRT5 knockdown on mitochondrial ultrastructure, siRNA-treated hPTECs exposed to normoxia or OND were analysed by transmission electron microscopy (TEM).

As shown in Figure 4.16A, in normoxic conditions SIRT5 RNAi induced mitochondrial swelling relative to the siRNA control which was amplified by OND, suggesting that SIRT5 depletion exacerbates OND-induced changes in mitochondrial ultrastructure. Quantification of the mitochondrial diameter confirmed this finding (Figure 4.16B). SIRT5 RNAi resulted in a significant increase in mitochondrial diameter compared to the RNAi treatment control (p<0.0001). After OND, mitochondrial diameter further increased in SIRT5-depleted cells only (p<0.01) compared to the normoxia control, while no significant increase could be detected in the control RNAi-treated cells (normoxia versus OND). In the OND treatment group, SIRT5 RNAi cells showed significantly increased mitochondrial diameter relative to the control RNAi cells (p<0.0001).

Taken together these data suggested that SIRT5 depletion caused mitochondrial swelling which was exacerbated by OND.
Figure 4.16: SIRT5 knockdown aggravates mitochondrial swelling in OND. hPTECs were transfected with SIRT5 siRNA or non-targeting siRNA control. Seventy-two hours post-transfection, cells were exposed to 6h OND (1%O₂+HBSS) or normoxia and complete medium (21%O₂+CM). (A) TEM images showing transfected (control or SIRT5 RNAi) hPTECs exposed to control conditions or OND. Red arrows indicate mitochondria. Data are from two independent experiments with n=2 technical replicates/group. Magnification: 1500x and 8000x. (B) Box and whisker plots display mitochondrial diameter (in µm) as median interquartile range and minimum and maximum values. Between 162-273 mitochondria from >10 cells/treatment group were analysed using ImageJ. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and ****p<0.0001.

SIRT5 RNAi exacerbates metabolic dysfunction after OND. Mitochondrial form and function are tightly-linked and morphological changes including ischaemia-induced mitochondrial swelling reduce energetic output, which promotes disease progression.(72, 194) To assess the effect of SIRT5 knockdown on mitochondrial function after OND, transfected hPTECs (control and SIRT5 RNAi) exposed to OND were analysed using a Seahorse respirometer. As shown in Figure 4.17, SIRT5 RNAi significantly impaired mitochondrial and glycolytic energy metabolism, evident as a reduction in basal respiration, respiration coupled to ATP production, maximal respiration and glycolytic capacity (all: p<0.05).
These data suggest that in hPTECs SIRT5 depletion worsens OND-induced metabolic dysfunction by impairing both glycolytic and mitochondrial energy metabolism.

**Figure 4.17: SIRT5 knockdown aggravates OND-induced mitochondrial dysfunction.** hPTECs were transfected with SIRT5 siRNA or non-targeting siRNA control. Seventy-two hours post-transfection, cells were exposed to 6h OND (1%O₂+HBSS) or normoxia and complete medium (21%O₂+CM). Representative profile of Mito Stress Test data showing oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) with arrows indicating injection of the specific stressors oligomycin (O), FCCP (F) and rotenone plus antimycin A (R+A). Relative values of the parameters were normalised to the non-targeting siRNA control. Wave software (Agilent) was used for data analyses. Data are from four independent experiments with n=3 technical replicates/group. Data are mean ± SD. Mann-Whitney U test was used to determine statistical significance. *p<0.05.

**SIRT5 depletion increases mitophagy during normoxia and OND.** Excessive mitochondrial fragmentation and mitochondrial dysfunction are prerequisites for autophagy-mediated degradation (mitophagy).(437) To assess whether SIRT5 RNAi increased mitophagy during normoxia and/or OND, hPTECs were loaded with a pH-dependent, fluorescent probe (Mtphagy dye; magenta), exposed to 6h of normoxia or OND and stained with a lysosomal dye (Lyso dye; cyan). The Lyso dye stains all lysosomal structures (lysosomes and autophagolysosomes) while the Mtphagy dye only stains lysosome-engulfed mitochondria and therefore, can be used to determine mitophagy. Co-localisation of both dyes (magenta and cyan) appears white and indicates lysosome-engulfed mitochondria. Figure 4.18 displays three ways of visualising co-localisation of the dyes using SIRT5 RNAi-treated hPTECs exposed to 6h OND as an
example: (A) Maximum projection laser-scanning confocal micrograph, (B) 3D reconstruction of Z-stack microscope images and (C) cross-sectional view of 3D images. To quantify mitophagy in hPTECs and assess the impact of SIRT5 depletion on mitophagy, 3D reconstructions were used followed by measurement of the integrated optical density (IOD) of the Mtophagy dye.

Figure 4.18: Mitochondrial and lysosomal co-labelling to assess mitophagy. hPTECs were transfected with SIRT5 siRNA. Seventy-two hours post-transfection cells were exposed to 6h OND (1%O₂+HBSS). Representative images showing lysosomes (cyan) and lysosome-engulfed mitochondria (magenta). Co-localisation of the dyes appears white. (A) Maximum projection laser-scanning confocal micrograph, (B) 3D image generated from laser-scanning confocal micrographs; and, (C) cross-section of 3D image. Z-stacks were analysed using Image-Pro Premier 10 (Media Cybernetics).

Subsequently, control and SIRT5-depleted hPTECs were analysed to determine whether SIRT5 RNAi affected mitophagy during normoxia and/or OND. As shown in Figure 4.19A, SIRT5 depletion increased mitophagy during normoxia, which was further increased by OND, compared to control RNAi-treated cells. This finding was confirmed by quantitative image analysis (p<0.05; Figure 4.19B). OND significantly increased mitophagy in both control and SIRT5 RNAi-treated cells (both: p<0.0001). Depletion of SIRT5 significantly increased OND-induced mitophagy compared to control RNAi-treated hPTECs (p<0.0001). These data suggested that SIRT5 knockdown increased mitochondrial susceptibility to degradation under both control conditions and OND.
Figure 4.19: SIRT5 depletion enhances OND-induced mitophagy. hPTECs were transfected using SIRT5 siRNA or non-targeting siRNA control. Seventy-two hours post-transfection cells were exposed to 6h OND (1%O₂+HBSS) or normoxia and complete medium (21%O₂+CM) as control. (A) Maximum projection laser-scanning confocal micrographs showing lysosomes (cyan; red arrow) and lysosome-engulfed mitochondria (magenta; yellow arrow). hPTECs were incubated with a fluorescent, pH-dependent probe (Mtphagy dye; magenta), exposed to 21%O₂+CM or 1%O₂+HBSS and subsequently, incubated with a lysosomal dye (Lyso dye; cyan). Scale bar: 25µm. (B) Integrated optical density (IOD) of Mtphagy dye was assessed using Image-Pro Premier 10 (Media Cybernetics). Data are from two independent experiments with >40 cells analysed/condition. Data are mean with 95% CI. To determine statistical significance a Kruskal-Wallis test was carried out followed by Dunn’s post hoc test to normalise for multiple comparisons. *p<0.05 and ****p<0.0001.

SIRT5 RNAi enhanced mitochondrial mass reduction during OND. As SIRT5 depletion increased OND-induced mitophagy in hPTECs (Figure 4.19), it was assessed whether this also provoked a reduction in mitochondrial mass. Three proxy markers of mitochondrial mass were assessed: (i) protein levels of key mitochondrial markers (by WB), (ii) a citrate synthase (CS) activity assay, which is commonly used to assess mitochondrial mass (384) and (iii) mtDNA copy number (Figure 4.20).
First, SIRT5 RNAi- and control RNAi-treated cells were screened by WB for the mitochondrial markers: succinate dehydrogenase A (SDHA), voltage-dependent anion channel 1 (VDAC1), translocase of inner membrane 23 (TIM23) and TOM20. As shown in Figure 4.20A, under normoxia SIRT5 RNAi significantly decreased VDAC1 (p<0.05) and TIM23 (p<0.01) protein levels compared to control RNAi-treated cells, suggesting that SIRT5 knockdown reduces mitochondrial mass. This was exacerbated by OND as TIM23 (p<0.001), TOM20 (p<0.01) and SDHA (p<0.05) protein levels further declined in
the SIRT5-depleted cells resulting in significant differences between control RNAi- and SIRT5 RNAi-treated cells (TIM23: p<0.01; SDHA: p<0.05).

Then, CS activity was measured in transfected hPTECs (control and SIRT5 RNAi) exposed to control conditions or OND for 6h. As shown in Figure 4.20B, SIRT5 knockdown reduced CS activity under both normoxia (p<0.05) and OND (p<0.05) compared to control RNAi-treated cells.

As final proxy measure of mitochondrial content, mtDNA levels were determined by qPCR in hPTECs (SIRT5 RNAi and control RNAi) exposed to OND or control conditions. Interestingly, as shown in Figure 4.20C, mtDNA levels increased upon SIRT5 RNAi-treatment compared to the control RNAi treated cells (p<0.01). Notably, mtDNA levels in control RNAi-treated hPTECs were not affected by OND relative to the respective normoxia control. However, in SIRT5 RNAi-treated hPTECs mtDNA levels declined upon OND relative to SIRT5-depleted hPTECs exposed to normoxia (p<0.001).

In summary, the data suggested that SIRT5 depletion may reduce mitochondrial mass in hPTECs exposed to normoxia and this effect is enhanced in OND.

4.4.4 Mitochondria/ ER and Ca^{2+} studies

Mitochondria and ER contact sites (MERCs) have emerged as important regulators of mitochondrial fission and Ca^{2+} homeostasis. Filadi et al. showed that ablation of MFN2, which is located on mitochondria and on the ER, increases MERCs, stimulates ER-to-mitochondrial Ca^{2+} transfer and causes mitochondrial swelling. Interestingly, SIRT5 RNAi reduced MFN2 protein levels (Figure 4.14B) and enhanced fragmentation (Figure 4.13) as well as mitochondrial swelling (Figure 4.21) indicating that these changes in mitochondrial architecture may be a consequence of increased MERCs. To test this hypothesis, SIRT5 RNAi and control RNAi-treated cells exposed to control conditions or OND were analysed by TEM and super-resolution confocal microscopy.
Figure 4.21: 

**SIRT5 knockdown may enhance mitochondria ER contact sites (MERCs).** hPTECs were transfected with SIRT5 siRNA or non-targeting siRNA control. Seventy-two hours post-transfection, cells were exposed to 6h OND (1%O₂+HBSS) or normoxia and complete medium (21%O₂+CM). TEM images showing close (red arrow) and distant (yellow arrow) MERCs. Data are from two independent experiments with n=2 technical replicates/group. Scale bar: 500nm.

First, TEM images were investigated to assess whether SIRT5 RNAi affected the distance between mitochondria and the ER. As shown in Figure 4.21, MERCs appeared closer in SIRT5 knockdown cells compared to control RNAi-treated cells. Notably, it appeared that OND increased MERCs in control siRNA-treated but not SIRT5 RNAi-treated cells compared to the control conditions. It should be emphasised that this is a qualitative observation and MERCs have not yet been quantified. TEM was carried out by Glenn Anderson and Monika Balys (Department of Histopathology, Great Ormond Street Hospital). Time constraints limited the number of images of the cells that could be taken in available time which precluded quantitative analyses.

For this reason, an alternative approach was tried using super-resolution microscopy (Leica SP8). For this, control and SIRT5 RNAi-treated hPTECs exposed to control conditions or OND were co-labelled with the ER marker calnexin (cyan), and the mitochondrial marker TOM20 (magenta), by IF. As shown in Figure 4.22, SIRT5 RNAi provoked mitochondrial fragmentation (magenta) compared to control RNAi-treated hPTECs. The tubular structure of the ER (cyan) appeared similar in control and SIRT5
knockdown cells suggesting that SIRT5 depletion had no impact on ER architecture. Of note, co-localisation of mitochondria and ER (TOM20 and calnexin; white) in control RNAi-treated cells did not appear different from SIRT5-depleted cells.

Figure 4.22: Super-resolution confocal image analysis of MERCs. hPTECs were transfected using SIRT5 siRNA or non-targeting siRNA control. Images were taken 78 h post-transfection. Mitochondria were visualised by TOM20 (magenta) and the ER by calnexin (cyan) immunolabelling. Images show (A-C) control RNAi- and (D-F) SIRT5 RNAi-treated cells. Images show (A,B) maximum projection laser-scanning confocal micrographs; (B,E) 3D render of confocal micrographs; and, (C,F) cross-sectional view. Data are from two independent experiments with n=2 technical replicates. Images were analysed with Image-Pro Premier 10 (Media Cybernetics).
It should be emphasised that the close proximity of ER and mitochondria (nm range; shown in the TEM images (Figure 4.21)) may mean that confocal microscopy lacks the resolution to assess whether SIRT5 RNAi increases MERCs in hPTECs. To address this in a different way, a functional approach was adopted (Figure 4.23).

Increased MERCs result in enhanced Ca\(^{2+}\) transfer from the ER to mitochondria.\(^{440}\) Under acute metabolic stress conditions, this mechanism is cytoprotective and helps cells to adapt.\(^{440}\) Chronic metabolic stress, however, leads to mitochondrial Ca\(^{2+}\) overload, a maladaptive process that has been shown to induce apoptosis.\(^{441, 442}\) To test whether SIRT5 RNAi affected mitochondrial Ca\(^{2+}\) homeostasis, control and SIRT5-depleted hPTECs were incubated with the Ca\(^{2+}\) dyes Fluo-4AM (binds cytosolic Ca\(^{2+}\)), Rhod-2AM (binds mitochondrial Ca\(^{2+}\)) as well as the mitochondrial dye Mitotracker Deep Red and analysed by confocal microscopy (Figure 4.23).

![Figure 4.23: Co-localisation of Ca\(^{2+}\) dyes Rhod2-AM and Fluo4-AM with Mitotracker dye in hPTECs.](image)

Cells were transfected with SIRT5 siRNA or non-targeting siRNA control. Seventy-two hours post-transfection, cells were exposed to 6h OND (1%O\(_2\)+HBSS), incubated with the Ca\(^{2+}\) dyes Rhod2-AM (1 µM, mitochondrial Ca\(^{2+}\), red), Fluo4-AM (5 µM, cytosolic Ca\(^{2+}\), green) and Mitotracker Deep Red (100nM, blue) for exactly 20mins at room temperature, followed by de-esterification at 37°C for 30min and analysed live using confocal microscopy. Images show maximum Z-projection confocal micrographs. Representative images are shown from three independent experiments with n=2 replicates/group. ImageJ was used for image processing. Scale bar: 10µm.

Of note, dye distribution indicating mitochondrial and cytosolic Ca\(^{2+}\) levels appeared very heterogeneous in both control and SIRT5 RNAi-treated cells independent of whether the cells were exposed to OND or control conditions (only control conditions
shown). Furthermore, both Ca$^{2+}$ dyes strongly co-localised and seemed to accumulate in vesicular structures (Figure 4.23).

To determine whether Ca$^{2+}$ dyes accumulated in fragmented mitochondria, potentially as a consequence of increased MERC formation, Z-stacks were reconstructed to 3D images and analysed using ImagePro 10. Figure 4.24 shows SIRT5 RNAi-treated hPTECs exposed to 6h OND selected as a representative sample. 3D analysis confirmed that both dyes co-localised, and also revealed that the vesicular structures primarily reside next to the mitochondria rather than within mitochondria. Mirmikjoo et al., has shown that Rhod2-AM also localises to lysosomes and pinocytotic vesicles (443) which might explain this observation.

Figure 4.24: Rhod2-AM and Fluo4-AM primarily accumulate in extra-mitochondrial vesicles. hPTECs were transfected with SIRT5 siRNA or non-targeting siRNA control. Seventy-two hours post-transfection, cells were exposed to 6h OND (1%O$_2$+HBSS). Cells were then incubated with the Ca$^{2+}$ dyes Rhod2-AM (1 µM, mitochondrial Ca$^{2+}$, red), Fluo4-AM (5 µM, cytosolic Ca$^{2+}$, green) and Mitotracker Deep Red (100nM, blue) for 20 min at room temperature, followed by de-esterification at 37°C for 30 min and analysed live using confocal microscopy. Images showing (A) maximum projection confocal micrographs, (B) 3D rendered Z-stack and (C) cross-sectional view of 3D image. Representative images are shown from three independent experiments n=2 replicates. ImagePro 10 was used for image analysis. Scale bar: 1µm.

Taken together the qualitative TEM data suggested that SIRT5 depletion may cause an increase in MERCs. To confirm this quantitatively and assess the functional consequences, additional analyses will be required to establish whether SIRT5 RNAi does affect MERC number and whether this has an impact on mitochondrial Ca$^{2+}$ levels and thus, alters mitochondrial architecture.
4.5 Discussion

Renal ischaemia manifesting as IRI is a major cause of AKI.\(^{(390)}\) The cell type primarily affected by IRI are PTECs due to their limited ability to switch from OXPHOS to an anaerobic metabolism under low-oxygen conditions.\(^{(45, 46)}\) These cells also have the highest mitochondrial turnover in the kidney \(^{(59)}\) which likely increases susceptibility to cellular injury. The NAD\(^+\)-dependent K de-suicinylase/-malonylase/-glutarylase SIRT5 has been shown to regulate both, mitochondrial \(^{(296, 337)}\) and glycolytic \(^{(288)}\) energy metabolism; and has been found to protect mitochondria from degradation \(^{(338)}\). However, its role in the kidney and in hPTECs remains unknown. To assess the function of SIRT5 in hPTECs, a combined OND model was developed to closely replicate the conditions found in renal ischaemia \emph{in vivo} \((i.e. \text{both hypoxia and nutrient-deprivation})\. The OND model had not been characterised with respect to autophagy/mitophagy induction, two aspects which have been shown to occur in IRI \emph{in vivo} \(^{(55)}\). It was also important to compare OND to hypoxia alone \((\text{an approach often used to model IRI \emph{in vitro})}\ . The first part of Chapter 4 is therefore dedicated to the characterisation of the model.

\textbf{OND stimulates mitophagy and provokes mitochondrial fragmentation.} To dissect the impact of the individual stimuli \((\text{hypoxia or starvation})\, \text{hPTECs were exposed to hypoxia (1\%O}_2+\text{CM}), nutrient deprivation (HBSS), or combined OND (HBSS+1\%O}_2) and compared to control conditions (21\%O}_2+\text{CM}). Induction of hypoxia and starvation was confirmed by qPCR via screening for markers of downstream-activated pathways \((\text{Figure 4.2): Hypoxia-induction was confirmed by screening for the HIF1\alpha-target genes LDHA, GLUT1 and BNIP3 (444). Nutrient starvation was confirmed by screening for p62, a marker of autophagy stimulation (421). Interestingly, while both hypoxia and OND increased HIF1\alpha target gene expression, p62 mRNA levels only increased during OND suggesting that OND induced autophagy. To assess autophagy induction as well as autophagic flux, protein levels of LC3-I/II and p62 were determined. When autophagy is induced, LC3-I is converted to LC3-II which acts as an autophagosomal linker protein connecting autophagosomes to cellular components \((\text{e.g. mitochondria})\) marked for degradation via autophagy/mitophagy receptors \((\text{e.g. p62})\) \((187, 421)\) Consequently, increased autophagic flux can be detected as a decrease in LC3-I and p62 protein levels as well as an increase in LC3-II protein levels.\(^{(421)}\) Interestingly, WB analysis revealed a decrease of LC3-I protein levels upon starvation, hypoxia and OND, however, only showed an increase in LC3-II protein levels upon HBSS and OND treatment \((\text{Figure 4.3B)}\ . Furthermore, p62 protein levels only declined in HBSS- and OND- but not hypoxia only-treated cells indicating that autophagy induction and autophagic flux were
only enhanced in OND but not hypoxia alone (Figure 4.3C+E). One explanation for the decline of LC3-I in the hypoxia-treated cells without a significant increase in LC3-II, may be that hypoxia reduced MAP1LC3B gene transcription (codes for pro-LC3) in hPTECs. However the goal was to find a model which induces autophagy rather than explain situations where it was not induced, hence, this was not further investigated. Taken together, given that autophagy induction is a key feature of renal ischaemia in vivo (55) the data suggest that combining hypoxia and nutrient deprivation is key to replicate in vivo ischaemia in an in vitro setting.

Based on the finding that autophagic flux appeared to be increased in OND compared to hypoxia only, the hypothesis was tested that OND-treated cells also showed higher levels of mitophagy. To address this, WB for TOM20, a marker of mitochondrial mass (445), and the pro-fusion factor OPA1 was carried out. Interestingly, although only OND-treated cells showed increased levels of autophagy, there was a significant reduction in TOM20 levels in OND- and hypoxia-treated cells (Figure 4.4B) suggesting that both stimuli equally induced mitophagy. This finding fits with the BNIP3 mRNA expression data which showed a significant increase of BNIP3 mRNA levels in hypoxia- and OND-treated hPTECs (Figure 4.2C). BNIP3 is a HIF1α-inducible mitophagy receptor (444) which strongly promotes clearance of damaged mitochondria in IRI (422) and therefore, would explain this finding. Notably, WB analysis of the mitochondrial marker protein OPA1, showed no change in total OPA1 levels which initially appeared to contradict the TOM20 data. However, WB clearly indicated a reduction of the two l-OPA1 isoforms in OND-treated cells while no visible change could be detected in the hypoxia-only treated group (Figure 4.4A). Subsequent densitometry confirmed this observation and revealed that the starvation component is required to stimulate l-OPA1 degradation.

As discussed in Section 4.1, OMA1 (ATP-independent) and YME1L (ATP-dependent) are two central l-OPA1 degrading enzymes. (158) OMA1 can degrade all l-OPA1 isoforms (S1 cleavage site) while YME1L degrades around 50% l-OPA1 isoforms (S2 cleavage site). (163, 446) Furthermore, OMA1 and YME1L undergo reciprocal degradation, adding another layer of complexity. (158) Cellular stress manifesting in a reduction of the ΔΨ_M activates OMA1, thereby, impairing the balance between OMA1 and YME1L and resulting in enhanced YME1L degradation, which subsequently drives l-OPA1 degradation. (158, 164) Treating hPTECs with CCCP, an uncoupling agent, which results in dissipation of the ΔΨ_M (158), leads to a decline in YME1L levels (Figure 4.6A), most likely because of impairment of the OMA1/YME1L equilibrium. Of note, at the time the experiment was carried out no suitable antibody for OMA1 detection was available so it was not possible to examine a shift from YME1L to OMA1. The dramatic reduction in l-OPA1 protein levels with CCCP treatment suggested this may be
due to increased OMA1 activity. To test whether this mechanism might explain I-OPA1 degradation in OND-treated but not hypoxia-treated cells, YME1L protein levels were determined by WB (Figure 4.6B). In line with the I-OPA1 data, WB analysis revealed a reduction in YME1L protein in OND- but not in hypoxia-treated cells which, again, implied that OMA1-mediated YME1L degradation may be the underlying mechanism driving I-OPA1 proteolysis. Nonetheless, in order to confirm the hypothesis that I-OPA1 degradation is driven by OMA1, protein levels would have to be quantified and ideally OMA1 activity would also have to be inhibited (i.e. via OMA1 knockdown) to show that the phenotype can be rescued. However, detailed dissection of the mechanism driving I-OPA1 degradation was beyond the scope of this project and was not analysed further.

The primary goal of the work in the first part of this Chapter was to determine whether OND is a better in vitro model than hypoxia alone to mimic in vivo renal ischaemia. The pro-fusion protein I-OPA1 is fundamental for normal mitochondrial from and function and its ablation results in excessive mitochondrial fragmentation and bioenergetic dysfunction (155, 159, 447), both of which are characteristics of mitochondrial dysfunction in IRI (3, 72). Given that hypoxia alone did not stimulate I-OPA1 proteolysis combined with the fact that IRI-induced reduction of I-OPA1 has emerged as important pathophysiological mechanism driving renal disease in mice in vivo (398), the data hinted that the OND model is the better in vitro model in which to mimic in vivo ischaemia.

Cellular stress including mild oxidative, metabolic or genotoxic stress has been shown to induce the expression of several SIRTs (SIRT1, 3 and 5) as a compensatory mechanism to protect cells from damage.(287, 331, 448) This together with our previous finding that IRI increased SIRT5 protein levels in murine PTECs in vivo (Figure 3.10B) suggested SIRT5 may also play a protective role in the kidney. The second part of this Chapter was therefore dedicated to testing the hypothesis that SIRT5 expression is also regulated by OND in hPTECs and if that was the case, to determine the underlying transcriptional network driving SIRT5 gene expression.

**OND increases SIRT5 mRNA and protein levels in hPTECs in vitro.** The development of the combined OND model facilitated the differential analysis of two components of ischaemia i.e. hypoxia and starvation, independently and in combination. mRNA and protein expression analyses showed that SIRT5 levels were increased by hypoxia and starvation (although this did not reach statistical significance), with the highest induction when the two were combined (Figure 4.7A). This suggested that multiple stress-induced transcriptional networks might drive SIRT5 expression. The
observation is also consistent with previous studies which found that cellular stress in vitro using cigarette smoke extract (331), a potent ROS inducer, as well as in vivo using hypoxia (332) increased SIRT5 gene expression. Interestingly, in this Chapter SIRT3 and SIRT1 mRNA expression were also found to be stimulated by OND in hPTECs (Figure 4.7). However, it emerged that upregulation of SIRT3 was a consequence of hypoxia while the increase in SIRT1 transcription is due to nutrient depletion. These observations were surprising as both SIRT1 and SIRT3 have previously been identified as being induced by both caloric restriction (449, 450) and hypoxia (316, 451) in multiple non-renal cells. A possible explanation for this might be that different cell types respond differently to cellular stress by activating distinct, cell-specific transcriptional networks, enhancers and epigenetic programs.(452-456)

While the findings showed that SIRT5, 3 and 1 mRNA expression was increased by OND, taken together the data underscore that in order to fully dissect the regulation of SIRT gene transcription, it is important to also (i) analyse the two stimuli driving metabolic disturbance in OND (hypoxia and starvation) individually and (ii) consider that within the kidney there may be a cell- and context-dependent regulation.

**SIRT5 upregulation in OND is likely to be independent of the HIF- and TFEB/3-pathways.** Based on the observation that in hPTECs SIRT5 and SIRT3 expression was upregulated by hypoxia, it was tested whether the increase was mediated by the HIF-pathway. To specifically activate the HIF-pathway, hPTECs were stimulated with CoCl₂, a PHD inhibitor (457), which suggested that the increase in SIRT5 and SIRT3 mRNA expression may not be driven by HIF (Figure 4.8). This was an interesting finding as it highlighted that hypoxia, which is quite often primarily associated with the HIF pathway, is complicated and results in the activation of a large number of hypoxia-responsive TFs including activating protein 1 (AP1), SP1 and C/EBPβ (458). Due to the complexity of the hypoxia-activated transcriptional network, however, it is difficult to dissect the exact underlying mechanism driving SIRT5 gene expression in oxygen-deprived hPTECs. Interestingly, Demetriades et al. showed that hypoxic stress and energetic stress (starvation) result in the inhibition of mTORC1, a protein complex, which controls protein synthesis.(459) Since SIRT5 was induced by hypoxia as well as starvation (although this did not reach statistical significance) it was tempting to speculate that mTORC1 inhibition plays a central role in the regulation of SIRT5 expression during OND. To test this hypothesis, hPTECs were treated with the mTORC1 inhibitor TORIN-1, and analysed by qPCR. Interestingly, TORIN-1 treatment increased mRNA levels of SIRT5 as well as SIRT1 and p62 in hPTECs (Figure 4.9) suggesting that
mTORC1 inhibition may indeed be a potential explanation for the enhanced transcription of these genes in OND (Figure 4.2D and 4.7C).

Settembre et al. were the first to show that starvation-induced mTORC1 inhibition results in cytosol-to-nucleus shuttling of a TF called TFEB, which induces transcription of genes involved in lysosomal biogenesis and autophagy. As discussed in 1.2.1.2 TFEB is a member of the MiTF/TFEB family of TFs which consists of 4 members: MiTF, TFEB, TFE3 and TFEC (129, 130), of which TFEB is regarded the master regulator of autophagy and also lysosomal biogenesis. (130, 131) Recently Mansueto et al. discovered that TFEB also drives mitochondrial biogenesis (138), thereby, acting as a key regulator of mitoQC. Notably, the MiTF/TFEB family of TFs has emerged as a central regulator of PGC1 gene transcription (126-128), which is one way in which this TF family can enhance mitochondrial biogenesis. PGC1α (via PPARα and ERRα) has also been shown to stimulate SIRT5 and 3 expression in murine hepatocytes (293) hinting that the TFEB-PGC1α-PPARα/ERRα axis may regulate SIRT5 gene expression in OND. Interestingly, TF binding analysis of the SIRT5 and 1 promoters using the Genomatrix software tool, indicated two and three putative MiTF/TFEB binding sites, respectively, (Figure 4.10) which suggested that MiTF/TFEB may directly regulate SIRT5 and 1 gene expression. Notably, Shin et al. reported that in mice Sirt1 gene expression is regulated by TFEB and confirmed this using siRNA against Tfeb. (141) mTOR inhibition (via i.e. TORIN-1 or HBSS) is known to trigger nucleus-to-cytosol shuttling of ZKSCAN3, a master transcriptional repressor of autophagy, and thereby, induce transcription of autophagic genes. (430) MiTF/TFEB and ZKSCAN3 are regarded as opposing players with respect to autophagy induction (127, 430, 431). The Genomatrix software tool, also showed that the SIRT5 promotor contains three ZKSCAN3 binding sites while none could be found in the SIRT1 promotor (Figure 4.10). Considering that the SIRT5 promotor contained both, MiTF/TFEB and ZKSCAN3 binding sites, the data implied that SIRT5 transcription may be inhibited under high nutrient conditions (by ZKSCAN3) and activated as part of the cellular autophagy program (by MiTF/TFEB). This appeared plausible, particularly, as SIRT5 has been shown to protect mitochondria from degradation during nutrient deprivation. (338) To test the hypothesis that SIRT5 is regulated by TFEB or its family member TFE3, siRNA-mediated knockdown was carried out. TFEB was chosen on the premise that it is regarded the master regulator of autophagy induction (460) and TFE3 due to its high renal expression (129) and its overlapping targets with TFEB (428, 461). As positive controls, SIRT1 and p62 were chosen, both of which are TFEB and TFE3 targets (138, 428, 461). Thus, it could be confirmed that SIRT1 and p62 expression are regulated by TFEB and TFE3, respectively (Figure 4.11). However, it emerged that neither TFEB nor TFE3 depletion reduced SIRT5
expression when stimulated with TORIN-1 (Figure 4.11). These data, therefore suggested that SIRT5 is not regulated by TFEB/TFE3. However it should be emphasised that this finding did not exclude the involvement of MiTF or TFEC, both of which are expressed in the kidney (129), or the transcriptional repressor, ZKSCAN3. While potentially of interest, the focus of the project was the consequences of SIRT5 depletion during OND on mitochondrial function/mitophagy. Therefore, the role of MiTF, TFEC or the transcriptional repressor ZKSCAN3 in the regulation of SIRT5 gene expression in hPTECs was not studied further.

In summary, the findings indicated that the OND-induced increase in SIRT5 expression is driven by hypoxia and to a lesser extent, starvation (possibly via mTOR inhibition). Furthermore, targeted activation of the HIF-pathway (with CoCl2) as well as knockdown of TFEB/3 followed by TFEB/3 activation (with the mTORC1 inhibitor TORIN-1) revealed that SIRT5 expression during OND is unlikely to be regulated by either of these two transcriptional networks.

Mitochondrial dysfunction is a major pathophysiologic feature of AKI and CKD, which manifests in structural and functional changes of the organelle.(3, 4) SIRT5 has been shown to boost mitochondrial metabolism and furthermore, protects mitochondria from fragmentation during nutrient-deprivation.(338) mTORC1 inhibition by rapamycin has been shown to protect mice from IRI.(462, 463) Taking into consideration that mTORC1 inhibition increased SIRT5 mRNA expression, it is tempting to speculate that SIRT5 may be a positive contributor to restoring renal function by promoting mitochondrial function in AKI. To date, it has not been established whether this also applies to hPTECs. Therefore, the third part of this Chapter tested the hypothesis that the OND-induced increase in SIRT5 expression in hPTECs averts mitochondrial dysfunction and protects renal cells from injury.

**SIRT5 RNAi exacerbates OND-induced mitochondrial dysfunction.** The work presented in this Chapter showed that SIRT5 depletion (by RNAi) reduced steady-state ATP levels and ATP production rate (OXPHOS and glycolysis) compared to control RNAi-treated hPTECs (Figure 4.12). SIRT5 RNAi caused a decline in steady-state ATP levels (total ATP: ~15%; glycolytic and mitochondrial ATP: ~21%) and ATP production rate (total: 5%; glycolytic: 5%; OXPHOS: ~10%). Although these changes are small, it is important to highlight that these experiments were repeated four times (independent knockdowns; biological replicates) with consistent results suggesting the changes are biologically relevant. These data are in line with a study in murine hearts and livers which reported that SIRT5 ablation caused a ~20% decrease in cardiac ATP levels (330) and
reduced hepatic F1/F0-ATP synthase activity (~15%) (336), respectively. Conversely, Buler et al. found that in a human hepatocyte cell line (HepG2), SIRT5 knockdown did not significantly affect steady-state ATP levels, while SIRT5 overexpression significantly increased ATP levels (~11%).(293) Their findings suggested that although SIRT5 is not critical for basal energy metabolism, it can increase ATP levels. Of note, in the present study knockdown efficiency was >85% while Buler et al. only achieved ~44% reduction in SIRT5 protein levels (293) hinting that a threshold of SIRT5 depletion has to be reached to see an effect on ATP metabolism. The fact that Buler et al. achieved only a partial knockdown could be one explanation for the different impact on steady-state ATP levels seen upon SIRT5 depletion, although cell type-specific differences cannot be excluded. In line with this hypothesis, Fisher-Wellman et al. recently showed that in cardiac mitochondria from Sirt5-/− mice there is a ~15% reduction in ATP generation and a reduction of oxygen consumption.(464) Taken together, the observations in the present study and others hint that on one hand a certain threshold of SIRT5 depletion may need to be reached to see an effect on the other, that even complete ablation of SIRT5 (464) may not dramatically impact on basal cellular energy metabolism.

Mitochondrial ATP production is regulated by the ΔΨₜ and reductions in the ΔΨₜ fundamentally impair ATP biosynthesis.(432) Although the changes in the ΔΨₜ were modest, a consistent and statistically significant effect of SIRT5 RNAi on the ΔΨₜ (22% decline) was detected (Figure 4.12C) (data from five independent knockdown experiments). These data are in keeping with a study on cardiac mitochondria from Sirt5−/− mice, which reported a small but consistent reduction in the ΔΨₜ compared to the ΔΨₜ measured in mitochondria from WT hearts.(464) The data therefore suggested that SIRT5 RNAi impaired basal cellular energy metabolism in hPTECs and reduced the ΔΨₜ. Of note, PTs express a variety of cell membrane transporters (465), which have the potential to extrude fluorescent dyes such as TMRM (466). In fact, Hall and colleagues have shown that the TMRM fluorescence intensity decays over time in PTs but not in DTs and this was not a result of photobleaching indicating that TMRM was extruded from PTs. With this in mind, it is important to state that it cannot be excluded that SIRT5 RNAi affected these extrusion mechanisms by either changing the expression levels or activity of membrane transporters in PTECs.

Mitochondria are highly dynamic organelles which constantly undergo structural changes (433), a process which is fundamental to bioenergetic adaptation (44) as well as mitoQC (42), both of which are dependent on the metabolic state of the cell and the ΔΨₜ. SIRT5 depletion induced mitochondrial fragmentation in hPTECs (Figure 4.13). In keeping with this finding, Guedouari et al. recently showed that Sirt5−/− MEFs display fragmented mitochondria and disturbed mitochondrial metabolism.(338) However, it is
worth noting that there are some mechanistic differences in MEFs compared to hPTECs. While here it was found that mitochondrial fragmentation in SIRT5-depleted hPTECs arises from increased fission (DRP1-S616) and decreased fusion (MFN1/2 and OPA1), in Sirt5<sup>−/−</sup> MEFs fragmentation is driven by excessive fission (MiD51 and FIS1) only (338). There are also differences in the underlying mechanisms driving fission in hPTECs and MEFs. In SIRT5-depleted hPTECs DRP1-translocation is phosphorylation-dependent (DRP1-S616) with no changes in MiD51 and FIS1 while in Sirt5<sup>−/−</sup> MEFs fission is DRP1 phosphorylation-independent due to increased levels of MiD51 and FIS1 (338). This is important because it highlights potential species-specific and/or cell type-/ differentiation dependent differences in the mechanisms regulating mitochondrial dynamics. However, these differences may also relate to levels of SIRT5 expression in that Sirt5<sup>−/−</sup> MEFs lack any SIRT5 whereas SIRT5 RNAi-treated hPTECs have some residual SIRT5; or be a consequence of compensatory mechanisms in Sirt5<sup>−/−</sup> MEFs versus SIRT5 RNAi hPTECs, which may have developed as a result of germline deletion. It is worth mentioning that Poletta et al. carried out a short hairpin (sh) RNA (shRNA)-mediated knockdown (~50% knockdown efficiency) as well as used a SIRT5 inhibitor (MC3482) in murine muscle cells (C2C12) as well as human breast cancer cells (MDA-MB-231) and reported reduced levels of total OPA1 and MFN2.(300). This finding is in line with our observations and furthermore, suggested mechanisms induced by transient SIRT5 depletion might cause a different response to the one seen in MEFs derived from a Sirt5<sup>−/−</sup> germline knockout. The present study also investigated the impact of SIRT5 RNAi on pro-fusion/-fission proteins in the context of OND. SIRT5-depleted, OND-treated hPTECs displayed significantly lower levels of the pro-fusion proteins MFN2 and OPA1 compared to the control RNAi, OND-treated cells (Figure 4.14). Furthermore, although not statistically significant, SIRT5 RNAi-treated cells showed a trend to increased DRP1-S616 protein levels compared to control RNAi-treated hPTECs. Therefore, the data suggested that SIRT5 depletion exacerbated mitochondrial fragmentation induced by OND.

Mitochondrial architecture is determined by two tightly-linked elements, the ΔΨ<sub>M</sub> as well as the ATP pool, and their depletion, as occurs during ischaemia, is a fundamental driver of mitochondrial fragmentation (45) and swelling (467). The fact that SIRT5 depletion reduced both the ΔΨ<sub>M</sub> and ATP levels, suggested that mitochondrial fragmentation in hPTECs may be a consequence of metabolic disturbance. Notably, Rainbolt et al. showed that the combination of ΔΨ<sub>M</sub> loss and ATP depletion caused mitochondrial fragmentation through excessive l-OPA1 degradation in human neuroblasts due to an imbalance between the two l-OPA1 processing enzymes, OMA1 and YME1L.(158) In hPTECs, SIRT5 depletion increased l-OPA1 processing suggesting
that fragmentation may potentially also be triggered by an imbalance between OMA1 and YME1L (Figure 4.15B).

While it is generally accepted that SIRT5 boosts enzymes involved in glycolytic and mitochondrial energy metabolism (288, 329) it remains to be tested whether the effect of SIRT5 RNAi on mitochondrial structure is a consequence of decreased bioenergetics or is more direct through regulation of fission/fusion proteins. Interestingly, using an immunoprecipitation (IP) approach, it has been shown that SIRT3 (but not SIRT5) binds, deacetylates and activate OPA1 in MEFs and thus, promotes mitochondrial fusion.(318) Lee et al. recently reported that deacetylation of MFN1 is fundamental for its function as pro-fusion factor during nutrient depletion in MEFs (468) and SIRT1 was found to deacetylate MFN2 and thereby, alleviate mitochondrial dysfunction induced by hepatic ischaemia in mice (469). It has yet to be determined whether SIRT5 binds and modifies K-acylations on MFN1, MFN2 or OPA1 in hPTECs, further analyses to address this are required. Although SIRT5 did not bind OPA1 in MEFs (468), this may not be the case hPTECs and this remains to be investigated. In addition, it would be interesting to test whether SIRT5 binds OMA1 or YME1L directly to regulate their activities. To date, no K-acylation sites have been identified on either of these proteins. However, in human mesenchymal stem cells OMA1 phosphorylation on tyrosine 216/276 by glycogen synthase kinase 3 (GSK-3) was shown to be important for OMA1 degradation (470) indicating that OMA1 levels are also regulated by phosphorylation. To determine whether OMA1 or YME1L are regulated through other PTMs including KAc, KSucc or KMal further studies including structural analyses to screen OMA1 and YME1L for K-residues are required; and, if that was the case, IP of OMA1 and YME1L followed by screening for the PTMs (by WB) would be essential to determine whether SIRT5 is directly involved in OMA1/YME1L regulation rather than just activating OMA1 as a consequence of SIRT5 RNAi-induced metabolic disturbance.

Intriguingly, the data showed that SIRT5 depletion not only caused a decline of l-OPA1 but also reduced s-OPA1 (Figure 4.15B). To date, very little is known about the function of s-OPA1 or how it is degraded. Recently, Lee et al. reported that s-OPA1 is required for cristae formation and the maintenance of mitochondrial energetics.(397) This suggests that the reduction of s-OPA1 induced by SIRT5 RNAi might provide another explanation for the changes in mitochondrial structure found in SIRT5-depleted hPTECs.

Taken together, the findings in the present study showed that SIRT5 RNAi impacted on mitochondrial dynamics, however further analyses are required to dissect whether the increased fragmentation in SIRT5-depleted hPTECs is a direct effect
through regulation of mitochondrial fusion/fission proteins, a consequence of impaired bioenergetics or a combination of both.

As mentioned earlier, mitochondrial swelling in PTECs is a hallmark of ischaemic AKI. Here, it was shown that SIRT5 depletion exacerbated OND-induced swelling in hPTECs (Figure 4.16). Interestingly, studies in the ischaemic canine heart found that mitochondrial swelling correlates with ATP depletion (472) and also revealed that increased mitochondrial matrix volume stimulated respiration rates (467), indicating that swelling might be a compensatory mechanism to adapt to energetic stress. The observation that SIRT5 depletion impaired cellular bioenergetics, suggested that reduced ATP levels may be the underlying cause, leading to exacerbated OND-induced swelling in hPTECs. While slight swelling may be beneficial, excessive swelling primes the opening of the mitochondrial permeability transition (MPT) pore (473), a mega-channel made up of proteins on the mitochondrial outer/inner membranes associated with the induction of cell death (83). Interestingly, mitochondrial respiration, ATP production-linked oxygen consumption and maximal respiration were significantly reduced in SIRT5-depleted hPTECs post-OND (Figure 4.17) which implied that SIRT5 RNAi aggravated OND-induced mitochondrial dysfunction in hPTECs. Notably, Chiba et al. recently analysed Sirt5−/− kidney homogenates by high-resolution respirometry (using an Oxygraph-2k system (Oroboros)) and reported no difference of basal respiration, however, they showed reduced complex II activity between WT and Sirt5−/− kidneys, which dissipated after IRI. Although these data appear to contradict the findings in hPTECs, it should be pointed out that in addition to species differences (human versus mouse), the group also analysed whole kidney homogenates rather than isolated PTECs, which may have diluted the effects of SIRT5 ablation on mitochondrial function in PTECs. Furthermore, in their study, an Oxygraph-2k respirometer was used and oxygen consumption was measured upon injection of various substrates (i.e. pyruvate/malate, ADP, glutamine and succinate) rather than mitochondrial toxins (i.e. oligomycin A, FCCP, antimycin A and rotenone). Thus, the group actually analysed substrate usage efficiency (by OXPHOS) rather than respiratory capacity (by ETC) which would be key to allowing a direct comparison to our Seahorse data and to determine whether Sirt5−/− kidneys/PTECs show a reduction in i.e. maximal respiratory capacity and/or ATP production-linked oxygen consumption. Therefore, further analyses including use of mitochondrial toxins in conjunction with the Oxygraph-2k respirometer (analysis of whole kidney homogenates) and more cell-type specific analysis of Sirt5−/− PTECs using a Seahorse respirometer are fundamental to elucidate the impact of Sirt5 knockout on mitochondrial function and put it in the context with the data presented in this Chapter.
ECAR which is why Chiba et al. could not assess the effect of $\text{Sirt}5^+$ on glycolytic capacity. (371) In the present study, however, it was shown that $\text{SIRT}5$ RNAi-treated hPTECs displayed reduced glycolytic capacity (Figure 4.17). Given that PTECs inherently exhibit very limited glycolytic capacity (45), together with the fact that glycolysis is vital to prevent mitochondrial fragmentation during ischaemia (45, 46), it is evident that impairing this metabolic pathway is likely to exacerbate PTEC injury. Intriguingly, immunofluorescence co-labelling of SIRT5 with markers of the different nephron segments in human kidneys revealed that SIRT5 levels peak in human distal tubular epithelial cells (DTECs), with little expression in PTECs (Figure 3.3). Of note, Chiba et al. found that SIRT5 levels peaked in murine DTECs. (371) This is particularly interesting, since the metabolically-flexible DTECs have been shown to be protected from ischaemic injury (45), which may suggest that SIRT5 contributes to DTEC injury resistance. This, together with our observation that renal ischaemia in vivo and in vitro increased SIRT5 levels in PTECs, suggests that inducing $\text{SIRT}5$ expression in hPTECs may be a compensatory mechanism to boost cellular bioenergetics and minimise mitochondrial dysfunction during ischaemia. To link injury resistance to SIRT5 levels in DTECs it would be central to carry out the same set of experiments in this cell type. However, as mentioned in the Chapter 3 (Section 3.5), to our knowledge, there is currently no well-characterised hDTEC cell line. Isolation of primary DTECs from human kidneys was attempted using an antibody against the sodium chloride cotransporter (NCC) (474) followed by magnetic-activated cell sorting (MACS). However, isolation of primary differentiated DTECs turned out to be challenging and isolated cells appeared NCC$^-$ one week post isolation.

Mitochondrial form and function are tightly-linked and excessive fragmentation is associated with reduced bioenergetic output (52) and increased susceptibility to degradation by mitophagy. (42) The data in this Chapter provided evidence that SIRT5 depletion exacerbated metabolic dysfunction after OND (by impairing both mitochondrial function and glycolytic capacity). In addition, the data also showed that $\text{SIRT}5$ RNAi increased mitophagy and thereby, reduced mitochondrial mass under control conditions (CM+21%$\text{O}_2$) and OND (Figure 4.19 and 20). This could be demonstrated by applying super-resolution confocal microscopy to detect engulfed mitochondria (Mtophagy/Lysosome dye) and WB, CS assay as well as mtDNA quantification to determine mitochondrial mass. Although the overall $\text{SIRT}5$ RNAi-induced increase in mitophagy was modest, to our knowledge, this is the first study to show directly that mitophagy rates increase when SIRT5 is depleted. Previous studies in MEFs only reported a reduction in mitochondrial mass as measured by WB (338) and did not analyse mitophagy. This is
an important point as a reduction of mitochondrial marker proteins is not necessarily a consequence of increased mitophagy but could arise from reduced mitochondrial biogenesis.\(^{(53)}\) Interestingly, mtDNA quantification, an additional proxy marker for mitochondrial mass, revealed a \textit{SIRT5} RNAi-induced increase in mass during normoxia (Figure 4.20C). At first this finding may appear illogical as a decline in mtDNA would have been expected. However, one plausible explanation may be the reduced MFN2 levels caused by \textit{SIRT5} depletion: Sitarz et al. found that patients with Charcot-Marie-Tooth type 2A (CMT2A) disease, a neuropathological disorder caused by MFN2 mutations, show increased mtDNA levels in blood leukocytes.\(^{(475)}\) Based on their finding, the group hypothesised that this might be a compensatory response to an underlying bioenergetic crisis (referred to as the “sick mitochondrion hypothesis”).\(^{(475)}\) To determine whether \textit{SIRT5} RNAi-induced MFN2 depletion caused the increase in mtDNA in hPTECs, MFN2 reduction could be rescued by using a MFN2 overexpression plasmid followed by quantification of mtDNA levels. Alternatively a knockdown of \textit{MFN2} (by e.g. siRNA or shRNA) could be performed in hPTECs. Nonetheless, independent of whether the \textit{SIRT5} RNAi-induced increase in mtDNA is mediated by MFN2, the data supported the sick mitochondrion hypothesis and suggested a compensatory response as a consequence of \textit{SIRT5} depletion in hPTECs.

As discussed in Section 4.1, mitochondria are highly dynamic organelles that constantly undergo fusion and fission, a process which is driven by pro-fusion/ -fission factors (e.g. MFN1/2 or DRP1).\(^{(44, 396)}\) Intriguingly, Friedman et al. discovered that the ER marks fission sites by forming constriction sites (referred to as “pre-DRP1 constriction step”).\(^{(197)}\) For this, the ER wraps around mitochondria and causes local constriction, which is central to the subsequent recruitment of DRP1.\(^{(197)}\) Interestingly, Lewis et al. found that newly synthesised mtDNA molecules localised to these constriction sites\(^{(476)}\) suggesting that ER signalling might be involved in the regulation of mtDNA replication. Qualitative observations of TEM images suggested that \textit{SIRT5} knockdown seemed to promote mitochondria/ER juxtaposition and thus, increase the number of mitochondria - ER contact sites (MERCs) (Figure 4.21). Due to time constraints of our collaborators on the TEM work, it was not possible to confirm these observations quantitatively. Efforts were made to determine MERCs by confocal microscopy (staining for the mitochondrial marker TOM20 and the ER marker calnexin), however, control RNAi-treated cells did not appear different from \textit{SIRT5}-depleted cells. No further in-depth image analysis was carried out as it was presumed that the resolution achieved by confocal microscopy may not be adequate (MERCs are defined as close contacts <20nm). Possible experimental approaches to assess whether \textit{SIRT5} RNAi increases MERCs, which could be applied in the future, include: (1) a dimerisation-dependent assay, which employs two monomers
(one localised to the ER and one to the mitochondrial outer membrane) (477); or (2) a fluorescence resonance energy transfer (FRET) assay as an indicator of mitochondria/ER proximity (153). In the present study, another, more functional readout indicating MERC formation was applied: examining changes in Ca$^{2+}$ signalling (using Fluo4-AM and Rhod2-AM). Given that elevated Ca$^{2+}$ levels in mitochondria promote swelling (473), this was felt to be a reasonable approach as this experiment was likely to also provide an explanation for the structural changes induced by SIRT5 RNAi. Worth noting, a slight elevation of mitochondrial Ca$^{2+}$ has been shown to increase OXPHOS (by activating mitochondrial dehydrogenases and F1/F0-ATPase), however, Ca$^{2+}$ overload causes excessive swelling (rat brain, kidney and liver mitochondria) and provokes opening of the MPT pore (473). Based on the fact that the ER is a major source of mitochondrial Ca$^{2+}$ (478) combined with the observation that SIRT5 RNAi potentially increased MERCs, it was tempting to speculate that SIRT5 knockdown increased MERCs, increased mitochondrial Ca$^{2+}$ levels and thus, sensitised the organelle to OND-induced swelling. Quantification of cytosolic (by Fluo4-AM) and mitochondrial (by Rhod2-AM) Ca$^{2+}$ as well as the movement of Ca$^{2+}$ proved difficult as the Ca$^{2+}$ dyes appeared to accumulate in vesicles, adjacent to mitochondria rather than within mitochondria (Figure 4.24). In addition, the two Ca$^{2+}$ dyes (Fuo4-AM and Rhod-2AM) strongly co-localised suggesting a non-specific accumulation and therefore, technical artefacts. Interestingly, Mirnikjoo et al. reported that in MEFs Rhod2-AM accumulates in mitochondria, pinocytotic vesicles, the ER and lysosomes indicating that the present observation may indeed be an artefact. It is worth mentioning that, during the experiments significant time-dependent dye toxicity was apparent with the formation of large, Fluo4-AM, Rhod-2A and Mitotracker Deep Red FM, triple-positive vesicles (images not shown). One reason for this may be that Fluo4-AM and Rhod2-AM have been shown to inhibit Na$^+$/K$^+$/ATPase and thereby, cause cytotoxicity (479). A more specific and less toxic would be to use genetically-encoded calcium indicators (GECIs) (480). These sensors, which can be FRET-based, ratiometric or intensiometric, can also be modified to specifically target organelles (e.g. mitochondria) and therefore, are a better tool to detect and evaluate Ca$^{2+}$ movements (480).

Intriguingly, the pro-fusion protein MFN2, which also localises to ER membranes, has been shown to be involved in mitochondria/ER tethering by engaging in homo- or heterotypic interactions with MFN2 or MFN1 on the OMM (152). Filadi et al. reported that Mfn2$^{-/-}$ MEFs as well as MFN2-depleted SH-SY5Y cells (human neuroblastoma cells) showed an increase in MERC formation (439). Although further studies will be required to determine the impact of SIRT5 depletion on MERC formation, the findings reported by Filadi et al. (439) together with the present observation (Figure 4.14) propose a
mechanistic model in which SIRT5 RNAi-induced decline in MFN2 (Figure 4.14) may stimulate an increase in MERCs in hPTECs.

4.6 Conclusion

Taken together, the data presented in this Chapter revealed that combined OND provides a better *in vitro* model of the mitochondrial dysfunction in hPTECs caused by renal ischaemia *in vivo* and highlights the significance of nutrient deprivation in this model. Furthermore, it was shown that metabolic stress induced by OND increased *SIRT5* mRNA and protein levels in hPTECs and established that this increase was independent of HIF1/2 and TFEB/TFE3, which are TFs activated by hypoxia and nutrient deprivation, respectively. Finally, the data showed that in hPTECs SIRT5 is an important enzyme that contributes to metabolic homeostasis, protects mitochondria from fragmentation/degradation, and decreases susceptibility to OND-induced mitochondrial dysfunction (Figure 4.25).

*Figure 4.25: Proposed model of how SIRT5 depletion affects mitochondrial function and exacerbates OND-induced mitochondrial dysfunction in hPTECs. Under normoxic conditions (21%O2+CM), SIRT5 depletion (by RNAi) causes mitochondrial fragmentation, induces mitophagy and reduces mitochondrial mass. OND (1%O2+HBSS) induces mitochondrial dysfunction in control RNAi-treated hPTECs, which is exacerbated when by SIRT5 depletion (by RNAi). Thus, SIRT5-depleted cells show increased OND-induced mitochondrial dysfunction apparent as enhanced metabolic dysfunction and mitochondrial fragmentation and swelling.*

These findings give rise to a model in which OND-induced increase in SIRT5 alleviates the mitochondrial dysfunction caused by OND. Further studies will be needed to provide
a comprehensive understanding of the function of SIRT5 in the ischaemic kidney and moreover, assess its function in a more chronic setting which may potentiate the effects seen in acute ischaemic injury
Chapter 5

Loss of SIRT5 protects against ischaemic AKI but exacerbates nephrotoxic CKD.
5.1 Introduction

Mitochondrial dysfunction is a major pathophysiologic feature of AKI as well as CKD (3, 4) and improving mitochondrial function has been shown to alleviate renal damage in multiple models of acute and chronic injury in mice. (356, 481) The nephron segment most susceptible to injury is the PT. (30) The reason for this is on one hand the high ATP demand (due to Na\(^+\)/K\(^+\)-ATPase-driven reabsorption processes) as well as the dependence on mitochondrial FAO for ATP generation (metabolic inflexibility) (32, 34, 45); and, on the other hand, the fact that PTECs display the highest mitochondrial turnover in the kidneys, as indicated by increased mitophagy rates (59). Therefore, to facilitate normal mitochondrial function, PTECs are highly reliant on oxygen supply as well as mitochondrial biogenesis to meet the energetic demand and maintain mitochondrial mass (see PGC1\(\alpha\) expression data; Figure 3.2A), respectively. Acute and chronic injury, however, impair mitochondrial function as well as biogenesis (indicated by decline in metabolic output and PGC1\(\alpha\) levels) which causes PTEC dysfunction and thereby, drives kidney disease. (63, 356, 482, 483)

The SIRT family of NAD\(^+\)-dependent KDACs are fundamental stress sensors which have been found to promote tubular cell function in murine kidneys. (6) In particular, SIRT1 (393, 484-486), 3 (322, 323, 370, 487) and 6 (488-491) have emerged as key family members which protect murine kidneys from acute and chronic injury. The underlying mechanisms of how the SIRTs protect kidneys from injury are numerous, however, preservation of mitochondrial function appears to be one common denominator. (6, 320, 491) Notably, SIRT1 has been shown to deacetylate and activate PGC1\(\alpha\) (492-494), which in turn stimulates mitochondrial biogenesis (61) and also increases the expression of the mitochondrial SIRTs, SIRT3 and 5 (293), suggesting an important role for the SIRT1/PGC1\(\alpha\)/SIRT3,5 axis. SIRT3 (294, 308) and SIRT5 (330, 333) deacylate K residues of central metabolic enzymes involved in mitochondrial FAO and by doing so, increase mitochondrial ATP production. In addition to their ability to enhance mitochondrial bioenergetics, both SIRT3 (318, 320) and 5 (338) have been shown to prevent excessive mitochondrial fission, a pathophysiologic feature which drives disease progression in AKI (60, 481) and CKD (4). Given the importance of mitochondrial function to facilitate ATP generation in PTECs, it is not surprising that ablation of SIRT3 increased susceptibility to tubular injury (320, 370) while overexpression was protective. (322, 370, 487) So far it is not known whether this also applies to SIRT5, although, multiple studies have shown that SIRT5 ablation exacerbates injury in the heart (330, 339) and brain (395) suggesting that loss of SIRT5 may also increase renal injury.
5.2 Specific aims

The aims of the studies described in this Chapter were to evaluate the function of SIRT5 in the kidney in vivo, (i) at baseline, (ii) during acute injury (IRI) and (iii) during chronic injury (FAN).

5.3 Methods

5.3.1 Crude mitochondrial isolation

WT and Sirt5⁻/⁻ mice were sacrificed by cervical dislocation and left kidneys were harvested. Kidneys were decapsulated, transferred to a glass petri dish on ice, ice-cold, iso-osmotic homogenisation buffer (0.32M sucrose, 1mM EDTA, 10mM Tris-HCl; pH 7.4) was added and kidneys were chopped into small (~2mm) pieces with scalpels. Tissue pieces were transferred to a pre-cooled glass tube, homogenisation buffer was added (total volume of 2ml) and tissues were homogenised by 15 up and down strokes of the rotating pestle (1000rpm) of a Potter Elvehejm tissue homogeniser (Eurostar). The homogenate was transferred to a 50ml Falcon tube (Corning; Cat# CLS430828) and centrifuged at 1500xg for 10min at 4°C. The mitochondria-containing supernatant was transferred to a new 50ml Falcon tube and centrifuged at 11,500xg for 10min at 4°C. The supernatant was carefully removed and the pellets re-suspended in 500µl homogenisation buffer. Crude mitochondrial extracts were flash-frozen in liquid N₂ and stored at -80°C until analysed.

5.3.2 Complex II activity assay

Succinate ubiquinone oxidoreductase (complex II) activity was measured in crude mitochondrial extracts from WT and Sirt5⁻/⁻ kidneys. A modified version based on the method of Hatefi and Stiggall (495) was used. This assay measures the succinate-ubiquinone reductase activity indirectly through the CoQ₂ (ubiquinone-2)-dependent reduction of 2,6-dichloroindophenol (DCIP), a blue dye which shows maximal absorption at 600nm. The time-dependent decrease in DCIP absorption allows the determination of complex II activity. However, before complex II activity could be assessed, mitochondrial extracts were subjected to three freeze-thaw cycles to release mitochondrial enzymes: Samples were flash-frozen in liquid nitrogen and subsequently thawed in a water bath at 30°C for 2min.

To determine complex II activity, absorption was determined in a test cuvette and compared to the absorption of a reference cuvette. An assay solution was prepared: potassium phosphate buffer (50mM; K₂HPO₄; VWR™, Cat# 103494G; KH₂PO₄; VWR™,
141

Cat# 29608), EDTA (100µM; VWR®; Cat# 443885J), DCIP (74µM; Sigma®, Cat# D1878-5G), sodium-succinate (20mM; Sigma®, Cat# 14160), sodium-cyanide (1mM; Sigma®, Cat# S3296; complex IV inhibitor which prevents electron flow to O₂), rotenone (10µM; Sigma®, Cat# R8875; complex I inhibitor which prevents electron flow along complex I). The reference cuvette contained the assay solution and in addition, the complex II inhibitor 2-thenoyltrifluoroacetone (TTFA; 1mM; Sigma®, Cat# T27006) and the sample (shows succinate-dependent, CoQ2-independent reduction of DCIP). The test cuvette also contained the assay buffer. Sequential addition of sample, CoQ2 and TTFA allowed determination of complex II activity. The detailed process was carried out as follows: (1) Assay solution was added to the cuvette. (2) At t=50s sample was added to the assay solution, mixed carefully and absorption was measured at t=120s and t=180s to determine succinate-dependent, but CoQ2-independent reduction of DCIP. (3) At t=200s, CoQ2 (50µM; Sigma®, Cat# C8081) was added to the assay solution, mixed thoroughly and absorption measured at t=240s and t=360s, which facilitated quantification of complex II activity. (3) Finally, TTFA was added which inhibits complex II activity. Based on the molar extinction coefficient of DCIP (21,000 M⁻¹·cm⁻¹) the rate of the complex II activity can be determined:

\[
\text{Rate (µmol/min/mg)} = \frac{\text{Absorption}}{\text{time}} \times \frac{1,000,000}{21000} \times \text{Protein conc.}
\]

A Hitachi U-3310 UV-Vis spectrophotometer was used to measure absorption of DCIP. All measurements were carried out in quadruplicate at 30°C. Complex II enzyme activities were normalised to the total protein content of the sample as determined by the RC DC protein assay (Bio-Rad).

5.3.3 Immunohistochemistry

The detailed protocol for IHC staining is given in Chapter 2, Section 2.3.1.1. The antibodies used are listed below:
Table 5.1: Antibodies for immunohistochemical analyses

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Clonality (clone)</th>
<th>Provider</th>
<th>Cat#</th>
<th>Dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>Rabbit</td>
<td>pc</td>
<td>DAKO</td>
<td>A0452</td>
<td>1:1000</td>
</tr>
<tr>
<td>SIRT5</td>
<td>Rabbit</td>
<td>mc (D8C3)</td>
<td>Cell Signalling</td>
<td>8782</td>
<td>1:200</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Rabbit</td>
<td>mc (EPR3776)</td>
<td>Abcam</td>
<td>ab92547</td>
<td>1:2000</td>
</tr>
<tr>
<td>Control IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>Rabbit</td>
<td>pc</td>
<td>R&amp;D Systems</td>
<td>AB-105-C</td>
<td>*</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EnVision+/HRP reagent Anti-Rabbit</td>
<td>Goat</td>
<td>pc</td>
<td>DAKO</td>
<td>K400211-2</td>
<td>IHC (ready-to-use)</td>
</tr>
</tbody>
</table>

**Key:** HRP: Horseradish peroxidase, IHC: Immunohistochemistry, mc: monoclonal, pc: polyclonal, * equivalent concentrations of IgG to the relevant primary antibody were used.

### 5.3.4 Histochemistry

For all histochemical stains, paraffin-embedded kidney sections (3µm) were used.

**Periodic Acid Schiff (PAS) stain.** PAS specifically stains polysaccharides (e.g. glycogen), mucus substances (e.g. glycoproteins and glycolipids) and basement membranes. In the kidney, PAS visualises the brush border of proximal tubules as well as the basement membranes of renal tubules and glomerular capillary loops. PAS staining was done by UCL IQ Path, UCL Queen Square Institute of Neurology.

**Masson’s Trichrome (MT) stain.** The MT stain visualises nuclei, cytoplasm and collagen fibres and therefore, can be used to assess the degree of fibrosis in the kidney. The MT stain was also carried out by UCL IQ Path.

**Picro Sirius Red (PSR) stain.** PSR stains collagen type I and III fibrils and can be used to quantitate interstitial fibrosis. In brief, after deparaffinisation and rehydration, sections were incubated with PSR solution (0.1% Direct Red 80 (w/v); Fluka, Cat# 43665) in saturated aqueous picric acid solution (1.3% (v/v); Sigma; Cat# P6744-1GA) for 1h. Sections were rinsed twice with 1% acidified water (1% acetic acid in water (v/v)), dehydrated in three ethanol washes (3min each) followed by two washes in xylene (3min each). Finally sections were mounted with toluene-free mounting medium (DAKO,
Cat# CS705) and stored at RT for analysis. Images were collected on a Nikon ECLIPSE Ci-L microscope (Nikon Digital Sight D5-Fi2 camera) and analysed using ImageJ (NIH).

5.3.5 Renal injury score

Murine kidneys were analysed in a blinded manner to minimise observer bias. At least 10 images per kidney (magnification: 20x) were collected on a Nikon ECLIPSE Ci-L microscope (Nikon Digital Sight D5-Fi2 camera).

Acute kidney injury. To determine the degree of tubular damage following renal ischaemia (30min or 40min ischaemia, 24h reperfusion), PAS-stained kidney sections were analysed for changes in staining of the brush border membrane of PTs, changes in tubular structure (atrophy, dilation, tubular casts) and immune infiltration as a measure of tissue injury. Tubular injury in the renal cortex was evaluated according to the following scoring system: 0=no tubular injury; 1≤10% tubules injured; 2=11%–25% tubules injured; 3=26%–50% tubules injured; 4=51%–74% tubules injured; and 5≥75% tubules injured.

Chronic kidney injury. To evaluate the degree of tubulointerstitial damage in the FAN model (14d), MT-stained kidney sections were analysed for changes in collagen, tubular structure (atrophy, dilation, tubular casts) and immune infiltration as an indicator of tissue injury. Tubulointerstitial damage was evaluated according to the following scoring system: 0=no tubulointerstitial injury; 1≤10% tubules injured/interstitial fibrosis; 2=11%–25 tubules injured/interstitial fibrosis; 3=26%–50% tubules injured/interstitial fibrosis; 4=51%–74% tubules injured/interstitial fibrosis; and 5≥75% tubules injured/interstitial fibrosis.

5.3.6 Gene expression analyses by RT-qPCR

The detailed protocol for RNA extraction, cDNA synthesis and RT-qPCR are described in Chapter 2, Section 2.3.2. β-actin was used as an internal control (housekeeping gene) for all RT-qPCR analyses. The primers used are listed in Table 5.2:
**Table 5.2: PCR primers for quantitative gene expression analyses**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction (5'→3')</th>
<th>Primer sequences</th>
<th>Amplicon size (bp)</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Sense</td>
<td>GATTCCATACCAAGAAGGAAGGC</td>
<td>128</td>
<td>Kipp et al., 2013 (496)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CACTGGCCGACTCTTTCCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αSma</td>
<td>Sense</td>
<td>GCTGCTCCAGCTATGTGTGA</td>
<td>834</td>
<td>Markella Ponticos⁺</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CGCTGACTCCATCCAAATGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col1α1</td>
<td>Sense</td>
<td>TGGAAAGACGGAGAGTAC</td>
<td>264</td>
<td>Mason et al., 2002 (497)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GCGCAGGAAGGTCAGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fn-EDA</td>
<td>Sense</td>
<td>AGTAACCAACATTGATCGGCCCTA</td>
<td>76</td>
<td>Haruta Mogami et al., 2012 (498)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTCCCAAGCAATTGGATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Sense</td>
<td>GAAATGCACAACCTTTTGACAGTG</td>
<td>116</td>
<td>Kong et al., 2019 (499)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TGGATGCTCTCTCATGACAGAC</td>
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<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Sense</td>
<td>ATCCTCTGGAAACCCCAACAC</td>
<td>58</td>
<td>Kong et al., 2019 (499)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GAACTTTTCTGACTCTCTGT</td>
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</tr>
<tr>
<td>Kim1</td>
<td>Sense</td>
<td>TCAGCTCGGAATGCACAA</td>
<td>67</td>
<td>Honarpisheh et al., 2018 (500)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TGGTTGCCTCTCGTCTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mcp1</td>
<td>Sense</td>
<td>TTTAAAACACTTGGATCGGAAACAA</td>
<td>121</td>
<td>Kong et al., 2019 (499)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GCATTAGCTCTCATGATACAGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ngal</td>
<td>Sense</td>
<td>AATGTCACTCCATCTGGTG</td>
<td>102</td>
<td>Honarpisheh et al., 2018 (500)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ATTTCCAGAGTGAACTGGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pgc1α</td>
<td>Sense</td>
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<td>75</td>
<td>Yu et al., 2013 (329)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
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</tr>
<tr>
<td>Sirt1</td>
<td>Sense</td>
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<td>183</td>
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<td>ACACAGAGACGGCTGGAACCT</td>
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</tr>
<tr>
<td>Sirt3</td>
<td>Sense</td>
<td>TACAGGCCCAATGTCACCTCA</td>
<td>168</td>
<td>Tian et al., 2014 (501)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ACAGACGGCTGATGTAGCTG</td>
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</tr>
<tr>
<td>Sirt4</td>
<td>Sense</td>
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<td>179</td>
<td>Kawamura et al., 2010 (502)</td>
</tr>
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<td></td>
<td>Antisense</td>
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<tr>
<td>Sirt5</td>
<td>Sense</td>
<td>CCACCGACAGATTCAGGTTT</td>
<td>164</td>
<td>Yu et al., 2013 (329)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTTCCGTTAGTGCCCGTCTT</td>
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<tr>
<td>Tfam</td>
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<td>GAGCGAGCTAATCCAGTCTG</td>
<td>108</td>
<td>Li et al., 2017 (503)</td>
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<td></td>
<td>Antisense</td>
<td>GAGCGGAATCATCCTTTGCCCT</td>
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<tr>
<td>Vcam1</td>
<td>Sense</td>
<td>CTGCGGAAGCTGGAACCAAG</td>
<td>115</td>
<td>Kong et al., 2019 (499)</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>GCCAAACACTTGACGCTG</td>
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<td></td>
</tr>
</tbody>
</table>

*personal communication*
5.3.7 Quantification of mitochondrial DNA (mtDNA)

The Qiagen DNeasy® Blood & Tissue kit (Qiagen, Cat# 69504) was used to isolate total DNA from kidney lysates. Flash-frozen murine kidney slices stored at -80°C were lysed with 200µl buffer ATL supplemented with proteinase K (Qiagen) on ice. Lysates were transferred to 1.5ml Eppendorf tubes, incubated at 56°C for 5min and homogenised by vortexing to ensure complete lysis. DNA was extracted as described in the manufacturer’s protocol and quantified with a NanoDrop 8000 spectrophotometer (Labtech). Samples were diluted to a final concentration of 5ng/µl and stored at -20°C. All qPCRs (LightCycler® 96 System, Roche) were performed in triplicate with an input of 1µl DNA (5ng/µl) in a total reaction volume of 20µl. The 2x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, Cat# PB20.15-20) reaction mix was used, with SYBR Green as a fluorescent reporter. To quantify mtDNA, primers located in the 16S rRNA gene were used and were normalised to the amount of nuclear DNA (nDNA) as measured by relative amount of the single-copy nuclear gene hexokinase 2 (Hk2). The primers (Table 5.3) were as described by Quiros et al., 2018 (504) and were obtained from Sigma®. Specificity of the primers was confirmed by using the NCBI Primer BLAST tool.

Table 5.3: PCR primers for mtDNA quantification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction (5’→3’)</th>
<th>Primer sequences</th>
<th>Amplicon size (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hk2 (nDNA)</td>
<td>Sense</td>
<td>GCCAGCCTCTCCTGATTTTAGTTG</td>
<td>116</td>
<td>Quiros et al., 2018 (504)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGGAAACACAAAGACCTCTTCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>Sense</td>
<td>CCGCAAGGGAAGATGAAAGAC</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>(mtDNA)</td>
<td>Antisense</td>
<td>TCGTGTGTTCCGGGTTTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The qPCR was run under the same conditions as described in Chapter 2, Section 2.3.2 and the ΔΔCt method was used to determine mtDNA levels.

5.3.8 Western blot analyses

The detailed protocol for murine kidney lysate preparation and WB analysis is described in Chapter 2, Section 2.3.3. β-actin (referred to as actin) and α-tubulin (referred to as tubulin) were used as loading controls. Antibodies used are listed below (Table 5.4):
## Table 5.4: Antibodies for Western blot analyses

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Clonality (clone)</th>
<th>Provider</th>
<th>Cat#</th>
<th>Dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Loading controls</strong></td>
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<td></td>
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</tr>
<tr>
<td>Actin</td>
<td>Rabbit</td>
<td>pc</td>
<td>Sigma</td>
<td>A2066</td>
<td>1:5000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Rabbit</td>
<td>mc (EP1332Y)</td>
<td>Abcam</td>
<td>ab52866</td>
<td>1:500,000</td>
</tr>
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<td><strong>SIRT5</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SIRT5</td>
<td>Rabbit</td>
<td>mc (D8C3)</td>
<td>Cell Signalling</td>
<td>8782</td>
<td>1:1000</td>
</tr>
<tr>
<td><strong>Autophagy-associated proteins</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3-I/II</td>
<td>Rabbit</td>
<td>pc</td>
<td>Cell Signalling</td>
<td>4108</td>
<td>1:1000</td>
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<tr>
<td><strong>Mitochondrial proteins</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SDHA</td>
<td>Mouse</td>
<td>mc (F-2)</td>
<td>Santa Cruz</td>
<td>sc-390381</td>
<td>1:1000</td>
</tr>
<tr>
<td>NDUFS2</td>
<td>Mouse</td>
<td>mc (B-3)</td>
<td>Santa Cruz</td>
<td>sc-390596</td>
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<tr>
<td>TOM20</td>
<td>Rabbit</td>
<td>pc</td>
<td>Proteintech</td>
<td>11802-1-AP</td>
<td>1:3000</td>
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<tr>
<td><strong>Mitochondrial fusion-associated proteins</strong></td>
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<tr>
<td>MFN1</td>
<td>Mouse</td>
<td>mc (3C9)</td>
<td>Abcam</td>
<td>ab57602</td>
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<td>MFN2</td>
<td>Mouse</td>
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<td>OPA1</td>
<td>Mouse</td>
<td>mc (18/OPA1)</td>
<td>BD Biosciences</td>
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<tr>
<td><strong>Mitochondrial fission-associated proteins</strong></td>
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<tr>
<td>DRP1</td>
<td>Mouse</td>
<td>mc (C-5)</td>
<td>Santa Cruz</td>
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<td>YME1L</td>
<td>Rabbit</td>
<td>pc</td>
<td>Proteintech</td>
<td>11510-1-AP</td>
<td>1:1000</td>
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<tr>
<td><strong>Post-translational modifications</strong></td>
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<tr>
<td>KSucc</td>
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<td>pc</td>
<td>PTM Bio</td>
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<tr>
<td>KMal</td>
<td>Rabbit</td>
<td>mc mix</td>
<td>Cell Signalling</td>
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<tr>
<td><strong>Tubular injury marker</strong></td>
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<tr>
<td>KIM1</td>
<td>Goat</td>
<td>pc</td>
<td>R&amp;D systems</td>
<td>AF1817</td>
<td>1:1000</td>
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<td><strong>Pro-fibrotic proteins</strong></td>
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<tr>
<td>αSMA</td>
<td>Rabbit</td>
<td>pc</td>
<td>Abcam</td>
<td>ab5694</td>
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<td>COL1</td>
<td>Goat</td>
<td>pc</td>
<td>Merck</td>
<td>AB758</td>
<td>1:2000</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Rabbit</td>
<td>mc (EPR3776)</td>
<td>Abcam</td>
<td>ab92547</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

**Key:** mc: monoclonal, pc: polyclonal

### 5.3.9 Serum and urine analyses

Serum and urine were analysed by the Clinical Pathology Service, Mary Lyons Centre, Medical Research Council Harwell. Sera were screened for urea as well as creatinine and urine samples were screened for protein, all of which are a measure of renal function.
5.4 Results

There were some issues with breeding the Sirt5\(^{-/-}\) mice which affected the number of animals available and hence, the experimental design of this study: (1) Sirt5\(^{+/-}\) x Sirt5\(^{+/-}\) breeding pairs did not produce a Mendelian distribution of the three genotypes (50% Sirt5\(^{+/-}\), 25% Sirt5\(^{-/-}\) and 25% Sirt5\(^{+/-}\) (WT)) with a majority of Sirt5\(^{+/-}\) pups (>75%); and, (2) most (>70%) of litters were female dominated. To address issue (1) WT x WT and Sirt5\(^{-/-}\) x Sirt5\(^{-/-}\) breeding pairs were set up which did produce the correct genotypes. However, issue (2) still remained which meant it was not possible to reach an adequate number of male Sirt5\(^{-/-}\) and WT animals (6-10 per group) for both IRI (AKI model) and FAN (CKD model) study. The FAN model was prioritised and the majority of male Sirt5\(^{-/-}\) and WT mice were used for this study.

5.4.1 Loss of SIRT5 does not impair renal function

Sirt5\(^{-/-}\) mice have normal renal function. The Sirt5\(^{-/-}\) mice used in this study were kindly provided by Prof. Johan Auwerx, Ecole Polytechnique Fédérale in Lausanne (Switzerland). His group generated the mice and published the first report of their metabolic phenotype.\(^{(329)}\) The group carried out glucose tolerance tests and investigated the expression of multiple metabolic enzymes in liver and gastrocnemius muscle in mice fed normal chow and a high fat diet, however the impact of SIRT5 ablation on the kidney was not investigated. The first step was therefore, to characterise the renal phenotype in Sirt5\(^{-/-}\) mice by WB, IHC and serum analyses.

Successful ablation of the K de-succinylase/ -malonylase SIRT5, was confirmed by WB (Figure 5.1A) and IHC (Figure 5.1C). Kidneys from Sirt5\(^{-/-}\) mice showed hypersuccinylation/ -malonylation compared to WT kidneys demonstrating functional consequences of Sirt5 deletion (Figure 5.1B).
Based on the observations in Chapter 4 (Figure 4.13) and the findings of others (338) indicating that depletion of SIRT5 induces mitochondrial fragmentation, mitochondrial structure in WT and Sirt5\(^{-/-}\) kidneys was analysed by TEM, with a particular focus on PTs and DTs.

Sirt5\(^{-/-}\) in PTs did not cause dramatic mitochondrial fragmentation when compared to WT PTs (Figure 5.2). However, mitochondria in WT PTs appeared longer and more interconnected (red arrowhead) hinting that loss of SIRT5 induced a mild structural change in this cell type. No differences in cristae structure were detected between WT and Sirt5\(^{-/-}\) PTs.

**Figure 5.1:** Loss of SIRT5 results in increased renal lysine succinylation and malonylation. Male WT and Sirt5\(^{-/-}\) mice (8-10 weeks) were analysed (n=3). WBs showing (A) SIRT5 protein levels and (B) levels of lysine succinylation and malonylation in total kidney lysates from WT and Sirt5\(^{-/-}\) mice. Actin was used as a loading control for SIRT5 and tubulin was used as a loading control for KMal and KSucc. (C) IHC stain for SIRT5 in FFPE kidney sections. IgG served as a control. Scale bar: 100µm.

**Figure 5.2:** Effect of SIRT5 ablation on mitochondrial structure in PTs. Kidneys from male WT and Sirt5\(^{-/-}\) mice (12 weeks) were analysed by transmission electron microscopy (TEM) (n=2). Micrographs showing transverse sections of PTs. Scale bar: 3µm. Red arrow indicating interconnected mitochondria.
Mitochondrial structure in DTECs did not appear different between WT and Sirt5<sup>−/−</sup> DTs (Figure 5.3).

![Figure 5.3: Effect of SIRT5 ablation on mitochondrial structure in DTs.](image)

To investigate whether Sirt5 deletion affected the glomerular filtration barrier, podocyte foot processes were investigated. As shown in Figure 5.4, WT and Sirt5<sup>−/−</sup> glomeruli appeared identical with no evidence of foot process effacement (red arrowhead), an indicator of an impaired glomerular filtration barrier (505).

![Figure 5.4: Effect of SIRT5 ablation on podocyte foot processes.](image)

Next, to assess whether loss of SIRT5 affected renal function, urea and creatinine levels were measured in sera from WT and Sirt5<sup>−/−</sup> mice. As shown in Figure 5.5A and B, no differences between Sirt5<sup>−/−</sup> and WT mice were detected indicating that SIRT5 ablation did not impair renal function (i.e. glomerular function). This finding is consistent with the observation that Sirt5<sup>−/−</sup> podocytes did not show foot process effacement.
Loss of SIRT5 protects from ischaemic AKI

Multiple SIRT family members including SIRT1, 3 and 6 have been shown to play protective roles in murine kidneys during acute injury conditions. However, it is not known whether this also applies to SIRT5. To address this question, IRI was induced in WT and Sirt5-/- mice by clamping of the renal artery for 30 or 40 min. After 24h tissues were harvested, processed and tissue damage was assessed. Unlike in Chapter 3 (Figure 3.10) where bilateral ischaemia was used as a model of acute renal injury, here mice were subjected to unilateral ischaemia. The reasons for this were the previously mentioned breeding issues (Section 5.4), which limited the number of animals available for the study and meant that a protocol, which allowed the use of the contralateral (CL) kidney, was required.

Loss of SIRT5 alleviates tubular injury. Initially, WT and Sirt5-/- mice were subjected to 30 min renal ischaemia and the kidneys harvested after 24h. As shown in Figure 5.6A, 30 min ischaemia induced only very mild injury in both WT and Sirt5-/- kidneys. For this reason, a longer ischaemic period (40 min) was applied which provoked clear morphological changes in the tubules (i.e. tubular dilation, flattened tubular epithelium, loss of brush border).

To determine the degree of injury and assess whether there were differences between WT and Sirt5-/- kidneys, tubular injury in PAS-stained kidney sections was scored using a semi-quantitative system (Section 5.3.5). In line with the observation described in the previous paragraph, 30 min ischaemia caused a mild (<10%) but statistically significant tubular injury in Sirt5-/- kidneys compared to the CL Sirt5-/- control (p<0.05; Figure 5.6B). However, there was no significant difference between WT and Sirt5-/- kidneys exposed to 30 min ischaemia. Scoring of PAS-stained kidney sections from WT and Sirt5-/- mice that underwent 40 min renal ischaemia showed that 40 min...
ischaemia caused a significant increase in tubular injury in WT (p<0.01; ~60% damage) and Sirt5\(^{-/-}\) (p<0.0001; ~40% damage) kidneys relative to respective CL kidneys (Figure 5.6C). Interestingly, Sirt5\(^{-/-}\) IRI kidneys showed significantly reduced injury compared to WT IRI kidneys (p<0.05).

Taken together, the data indicated that an ischaemic period of 40min was required to induce pronounced renal injury (>40%) in kidneys harvested 24h post-ischaemia. Furthermore, the data suggested that Sirt5 deletion protected from ischaemic injury (40min ischaemia). For subsequent analyses only 40min IRI kidneys were investigated.

---

**Figure 5.6:** Histological analyses of WT and Sirt5\(^{-/-}\) kidneys exposed to renal ischaemia. Male WT and Sirt5\(^{-/-}\) mice (10-12 weeks) underwent clamping of the renal artery for 30min or 40min followed by 24h reperfusion (IRI) (n=3-5). Contralateral (CL) kidneys served as a control. (A) PAS staining of FFPE kidney sections. Scale bar: 100µm. Tubular injury score in WT and Sirt5\(^{-/-}\) mice exposed to (B) 30 min ischaemia and (C) 40min ischaemia. Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and ****p<0.0001.

Sirt5\(^{-/-}\) IRI kidneys display reduced expression of injury markers. Kidney injury molecule 1 (KIM1) and neutrophil gelatinase-associated lipocalin (NGAL) are two widely used early markers of tubular injury.(506) To test whether Kim1 and Ngal mRNA
expression were affected by 40min ischaemia in WT and Sirt5⁻/⁻ mice, a qPCR analysis using whole kidney RNA extracts was conducted. As shown in Figure 5.7A, IRI increased mRNA expression of Kim1 in both WT (p<0.0001) and Sirt5⁻/⁻ (p<0.01) kidneys relative to the respective CL kidneys. Interestingly, Kim1 mRNA levels were significantly higher in WT IRI kidneys compared to Sirt5⁻/⁻ IRI kidneys (p<0.001) again suggesting that SIRT5 ablation protected from tubular injury. Quantification of Ngal mRNA levels revealed a significant increase in WT IRI kidneys (p<0.01) relative to the CL WT kidneys while Ngal levels in Sirt5⁻/⁻ mice did not change significantly (Figure 5.7B). Ngal mRNA levels were significantly higher in WT IRI compared to Sirt5⁻/⁻ IRI kidneys (p<0.05).

**Figure 5.7:** Sirt5 deletion alleviates IRI and the decline in renal function. Male WT and Sirt5⁻/⁻ mice (10-12 weeks) underwent clamping of the renal artery for 40min followed by 24h reperfusion (IRI) (n=3-5). Contralateral (CL) kidneys served as a control. (A,B) qPCR analyses. Bar graphs showing mRNA levels of the tubular injury markers (A) kidney injury molecule 1 (Kim1) and (B) neutrophil gelatinase-associated lipocalin (Ngal). Data were normalised to actin. Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey's post hoc test to normalise for multiple comparisons. **p<0.01, ***p<0.001 and ****p<0.0001. (C,D) Sera analyses. Bar graphs showing (C) serum urea levels and (D) serum creatinine levels in WT and Sirt5⁻/⁻ mice as a measure of renal function. Data are mean ± SD. Statistical significance was determine by Mann-Whitney U test. *p<0.05.

Renal function was assessed in WT and Sirt5⁻/⁻ mice by measuring serum creatinine and urea levels as clearance of the two solutes is widely-used as an indication of renal function.(507) As shown in Figure 5.7C, sera from WT IRI mice showed a trend to increased creatinine levels compared to sera from Sirt5⁻/⁻ IRI mice. Notably, sera from Sirt5⁻/⁻ IRI mice showed significantly lower levels of urea (p<0.05) compared to WT IRI sera (Figure 5.7D).
Taken together, the mRNA expression and renal function data indicated that SIRT5 ablation protects renal tubules from ischaemic injury.

**Loss of SIRT5 prevents the IRI-induced decline in Pgc1α and Tfam.** Renal ischaemia causes a decline in mitochondrial mass, due to an imbalance between mitochondrial degradation and biogenesis which strongly affects tubular cell recovery post-ischaemia. (54, 401, 422, 508) Conversely, stimulating mitochondrial biogenesis and thereby, preserving mitochondrial mass has been shown to alleviate tubular damage in AKI. (64, 67, 494) As an indication of whether mass/biogenesis was affected by SIRT5 ablation in CL or IRI kidneys, mtDNA levels (504) as well as the mRNA expression of Tfam, a key transcription factor involved in mtDNA replication (509), and Pgc1α, the master regulator of mitochondrial biogenesis (61), were measured using qPCR.

![Figure 5.8: Effect of IRI on mitochondrial mass-/ biogenesis-associated markers. Male WT and Sirt5−/− mice (10-12 weeks) underwent clamping of the renal artery for 40min followed by 24h reperfusion (n=3-5). Contralateral (CL) kidneys served as a control. DNA and RNA were extracted from total kidney lysates. Bar graphs showing (A) mitochondrial DNA (mtDNA) and mRNA of (B) Pgc1α, the master regulator of mitochondrial biogenesis and (C) mitochondrial transcription factor A (Tfam), a key regulator of mtDNA replication. mtDNA levels were normalised to nuclear DNA (nDNA) and mRNA was normalised to actin. Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

As shown in Figure 5.8A, IRI did not affect mtDNA abundance in WT and Sirt5−/− IRI kidneys relative to the corresponding CL kidneys. No difference in mtDNA levels could be detected between WT kidneys and Sirt5−/− kidneys (CL and IRI groups).

Sirt5−/− CL kidneys showed a trend to increased Tfam levels compared to WT CL kidneys (Figure 5.8B). Notably, IRI significantly reduced Tfam mRNA levels in WT kidneys (p<0.05) with no change in Sirt5−/− kidneys relative to the CL controls. As a result, Tfam mRNA levels were significantly higher in Sirt5−/− IRI kidneys compared to WT IRI kidneys (p<0.01).

As shown in Figure 5.8C, Sirt5−/− CL kidneys showed a trend to increased Pgc1α levels compared to WT CL kidneys. Pgc1α mRNA levels dramatically declined in WT IRI kidneys (p<0.001) with no change in Sirt5−/− IRI kidneys compared to the respective CL
control kidneys. Consequently, Pgc1α mRNA expression was significantly higher in Sirt5−/− IRI kidneys (p<0.01) relative to WT IRI kidneys.

In summary, the data suggested that mitochondrial mass (measured by mtDNA as proxy marker) was neither affected by Sirt5 deletion nor IRI. Notably, compared to WT IRI kidneys, Sirt5−/− IRI kidneys had significantly higher levels of Tfam and Pgc1α, two key factors known to promote mitochondrial biogenesis, indicating that loss of SIRT5 might alleviate mitochondrial dysfunction post IRI.

**Sirt5−/− kidneys show increased levels of Sirt3 and 4 after IRI.** Expression of the SIRT family members SIRT1 (393, 485) and 3 (323, 487) has been linked to renal recovery after acute injury. To determine whether SIRT5 ablation affected the expression of mitochondrial function-associated SIRTs, CL and IRI kidneys were screened for Sirt3, 4, 1 and 5 (Figure 5.9).

![Figure 5.9: Sirt mRNA expression in WT and Sirt5−/− kidneys.](image)

*A* was confirmed by qPCR (Figure 5.9A). IRI resulted in a significant reduction of Sirt5 mRNA levels in WT IRI kidneys (p<0.01) compared to the CL WT kidneys.
Kidneys from WT and Sirt5\textsuperscript{−/−} mice were screened for Sirt3, Sirt4 and Sirt1 mRNA expression. As shown in Figure 5.9B-D, Sirt5\textsuperscript{−/−} CL kidneys showed a trend to increased mRNA levels of Sirt3, 4 and 1 in CL kidneys relative to the WT CL controls. When IRI was superimposed, mRNA expression of Sirt3 (p<0.001), Sirt4 (p<0.0001) and Sirt1 (p<0.01) significantly decreased in WT kidneys compared to CL WT kidneys. In Sirt5\textsuperscript{−/−} IRI kidneys, only Sirt4 levels decreased significantly (p<0.05) compared to Sirt5\textsuperscript{−/−} CL kidneys (Figure 5.9C). Sirt5\textsuperscript{−/−} IRI kidneys displayed significantly higher mRNA levels of Sirt3 (p<0.05) and Sirt4 (p<0.05) and showed a trend to increased Sirt1 levels compared to WT IRI kidneys.

In summary, the data indicated that SIRT5 ablation caused a minor increase in Sirt3, 4 and 1 mRNA levels at “baseline” in CL kidneys (WT versus Sirt5\textsuperscript{−/−}), which did not achieve statistical significance. IRI Sirt5\textsuperscript{−/−} kidneys showed higher levels of Sirt3, 4 and Sirt1 compared to WT IRI kidneys suggesting that this may contribute to the renoprotection.

\textbf{Sirt5\textsuperscript{−/−} kidneys show decreased complex II activity.} Chouchani et. al showed that complex II is a major contributor to reperfusion injury in the heart and kidney.\textsuperscript{(510)} In the liver and heart, SIRT5 has been shown to target and regulate a variety of proteins involved in the TCA cycle as well as the ETC, including complex II.\textsuperscript{(296, 326, 333, 335, 336)} To test whether SIRT5 ablation impaired complex II activity in the murine kidney and therefore, may be one putative explanation for the protection seen in Sirt5\textsuperscript{−/−} kidneys exposed to IRI, complex II activity was measured in crude mitochondrial extracts from WT and Sirt5\textsuperscript{−/−} kidneys.

As displayed in Figure 5.10, mitochondria from Sirt5\textsuperscript{−/−} kidneys showed significantly reduced complex II activities (p<0.01) compared to mitochondria from WT kidneys.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{complex_ii_activity.png}
\caption{SIRT5 ablation impairs complex II activity. Mitochondria were isolated from whole kidneys (WT and Sirt5\textsuperscript{−/−}) and screened for complex II activity (n=5). Enzyme activity was determined indirectly by measuring the time-dependent reduction in absorbance (at 600nm) of 2,6-dichloroindophenol (DCIP), a blue dye which is reduced by complex II. Bar graph showing complex II activity. Samples were measured in quadruplicate. Enzyme activities were normalised to the total protein. Data are mean ± SD A Mann-Whitney U test was carried out to determine statistical significance. **p<0.01.}
\end{figure}
These data suggested that Sirt5<sup>−/−</sup>-induced reduction in complex II activity may be one mechanism by which SIRT5 ablation reduced tissue damage in IRI.

### 5.4.3 Loss of SIRT5 enhances nephrotoxic CKD

Reduced renal expression of SIRT1 and 3 have been associated with an increased susceptibility to CKD in mice. However it is not known whether this is also the case for SIRT5 and whether the desuccinylase plays a role in the pathogenesis of CKD. To investigate the function of SIRT5 in a chronic renal injury model, male WT and Sirt5<sup>−/−</sup> mice were challenged with one dose of folic acid (FA; 240µg/g body weight (BW)) in 0.3M NaHCO<sub>3</sub> buffer (vehicle). Thirteen days post-injection, animals were placed in metabolic cages for 18h and then culled.

**Sirt5<sup>−/−</sup>** kidneys do not show increased Sirt3, 4 or 1 expression after FA treatment. Based on the changes in Sirt3, 4 and 1 expression seen in the IRI model, mRNA levels of these mitochondrial metabolism-associated SIRTs were also assessed in WT and Sirt5<sup>−/−</sup> kidneys in response to FA. Sirt5 deletion was confirmed by WB and qPCR (Figure 5.11A-C). FA treatment had no effect on SIRT5 protein expression in WT kidneys compared to the vehicle-treated WT control. Notably, qPCR analysis showed no difference in Sirt3, 4 or 1 expression between WT and Sirt5<sup>−/−</sup> vehicle control kidneys (Figure 5.11D-F). FA treatment only causes a minor, non-statistically significant decline in Sirt3, 4 or 1 mRNA expression (WT and Sirt5<sup>−/−</sup>) relative to the vehicle-treated control kidneys.
Sirt5⁻/⁻ enhances FA-induced tubular injury. To evaluate FA-induced tissue damage, FFPE kidneys were stained with MT to assess tubulointerstitial damage (fibrosis, immune infiltration and tubular atrophy).

As shown in Figure 5.12A, compared to vehicle controls FA treatment induced tissue injury in both WT and Sirt5⁻/⁻ kidneys, as indicated by increased immune cell infiltration and tubular dilation, although there was only limited fibrosis (blue stained areas indicated by black arrowheads). Injury appeared slightly more in Sirt5⁻/⁻ FA-treated kidneys (increased tubular atrophy and dilation) than WT FA kidneys (in both the cortex and medulla).

Semi-quantitative scoring showed that FA treatment provoked significant damage in Sirt5⁻/⁻ kidneys in both the cortex (p<0.01) and medulla (p<0.01) compared to the vehicle control (Figure 5.12B and C). Although damage in both tissue compartments
appeared higher in FA-treated Sirt5−/− mice compared to FA-treated WT mice this did not achieve statistical significance due, at least in part, to the very heterogeneous response to FA treatment ranging 0-75% damage.

To further characterise damage induced by FA, mRNA and protein expression analyses were carried out screening for the tubular injury markers NGAL and KIM1 (513). As shown in Figure 5.13A and B, Ngal and Kim1 expression increased in both WT and Sirt5−/− after FA treatment compared to the respective vehicle controls. However, only Sirt5−/− FAN kidneys showed a statistically significant increase (Ngal: p<0.05; Kim1:
p<0.01) compared to vehicle-treated kidneys. There was no significant difference between WT FA and Sirt5<sup>Δ/Δ</sup> FA-treated kidneys.

Figure 5.13: Effect of FA injection on renal expression of *Ngal* and *Kim1* in WT and Sirt5<sup>Δ/Δ</sup> kidneys. Male WT and Sirt5<sup>Δ/Δ</sup> mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO<sub>3</sub> buffer (vehicle control). Renal tissue was harvested 14d post-injection (n=6-11) and analysed by qPCR and WB. Bar graphs showing mRNA levels of the renal injury markers (A) neutrophil gelatinase-associated lipocalin (*Ngal*) and (B) kidney injury molecule 1 (*Kim1*) as assessed by qPCR. Data were normalised to actin. (n=6-11 kidneys/group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and **p<0.01. (C) WB showing protein levels of KIM1. Actin served as loading control. (D) Bar graph showing protein levels of KIM1 normalised to actin. (n=5-6 kidneys/group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and ***p<0.001. ImageJ was used for densitometry.

KIM1 protein levels were determined by WB (Figure 5.13C and D). FA treatment increased KIM1 protein levels in both WT and Sirt5<sup>Δ/Δ</sup> kidneys compared to the vehicle control kidneys. As mentioned earlier and shown in the WB, the response of both WT and Sirt5<sup>Δ/Δ</sup> mice to FA was heterogeneous and reflected in the variation in KIM1 protein levels (Figure 5.13C). However, densitometry revealed a significant increase of KIM1 in FA-treated WT (p<0.05) and Sirt5<sup>Δ/Δ</sup> (p<0.001) kidneys relative to the respective vehicle control kidneys (Figure 5.13D). Even though KIM1 levels appeared higher in Sirt5<sup>Δ/Δ</sup> FA kidneys compared to WT FA kidneys, this difference did not reach statistical significance.
Taken together, the qPCR and WB data suggested that FA injection induced mild tubular injury in WT and Sirt5−/− kidneys, which appeared enhanced in Sirt5−/− FA kidneys.

Renal fibrosis is a hallmark of CKD and arises from an imbalance between deposition and degradation of extracellular matrix (ECM).(514) Tubulointerstitial fibrosis significantly impairs renal function and therefore, is a key indicator for disease progression. Infiltrating immune cells which generate a pro-inflammatory environment are important contributors to renal fibrosis and drive renal disease progression.(515)

*Sirt5−/− enhances immune infiltration and pro-inflammatory gene expression in FAN kidneys.* To evaluate whether FA treatment stimulated immune infiltration in WT and Sirt5−/− kidneys, IHC staining for the T-cell marker cluster of differentiation 3 (CD3) (516) was carried out.

As shown in Figure 5.14A, FA treatment stimulated CD3+ cell infiltration into the cortex and to a lesser extent into the outer medulla of both WT and Sirt5−/− kidneys (Figure 5.14A). An observation which was confirmed by semi-automated quantification of the CD3+ area (Figure 5.14B and C). However, quantification of cortical immune cell infiltration showed no significant increase of CD3+ cells when compared to the vehicle control kidneys (Figure 5.14B). Interestingly, analysis of immune cell infiltration into the renal medulla indicated a significant increase in Sirt5−/− FA kidneys compared to the Sirt5−/− vehicle control kidneys while there was no significant increase in WT FA kidneys compared to WT vehicle control kidneys (Figure 5.14C). No statistically significant differences between Sirt5−/− and WT FA-injected kidneys could be detected.
Figure 5.14: Effect of FA-injection on immune cell infiltration in WT and Sirt5⁻/⁻ kidneys. Male WT and Sirt5⁻/⁻ mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO₃ buffer (vehicle control). Renal tissue was harvested 14d post-injection (n=6-11). (A) Images showing renal cortex and medulla of FFPE kidney sections stained for the T-cell marker CD3 by immunohistochemistry (IHC). Scale bar: 100µm. (B, C) Bar graphs showing protein CD3 protein expression in kidney sections: (B) cortex and (C) medulla. (n=4-8 kidneys/group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05.

Expression of the pro-inflammatory markers interleukin-(IL)-1β (IL-1β), IL-6 and vascular cell adhesion molecule 1 (Vcam1) was determined by qPCR (Figure 5.15). As shown in Figure 5.15A, IL-1β mRNA expression mildly increased in WT and Sirt5⁻/⁻ kidneys compared to the vehicle control kidneys, although this was not statistically significant (p>0.05). Similar to IL-1β, IL-6 mRNA expression was only slightly increased in FA-injected WT (p>0.05) and Sirt5⁻/⁻ (p<0.01) kidneys compared to the respective control kidneys with no difference between the WT and Sirt5⁻/⁻ FA kidneys (Figure 5.15B). Measurement of Vcam1 mRNA levels showed that FA treatment only caused a significant increase in Vcam1 in Sirt5⁻/⁻ FA kidneys compared to Sirt5⁻/⁻ vehicle control kidneys (p<0.05; Figure 5.15C), however, there was no significant difference between WT and Sirt5⁻/⁻ FA-treated kidneys.
Figure 5.15: Effect of FA injection on mRNA expression of pro-inflammatory markers in WT and Sirt5−/− kidneys. Male wild-type (WT) and Sirt5−/− mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO₃ buffer (vehicle control). Renal tissue was harvested 14d post-injection (n=6-11). RNA was extracted from whole kidney lysates. Bar graphs showing mRNA levels of the inflammatory markers (A) interleukin (IL)-1β (IL-1β), (B) IL-6 and (C) vascular adhesion molecule 1 (Vcam1) (n=6-11 kidneys/group were analysed). Data were normalised to actin. Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and **p<0.01.

Taken together, the qPCR and WB data suggested that FA injection induced a mild inflammatory response in WT and Sirt5−/− kidneys, which appeared enhanced in Sirt5−/− FA kidneys.

Sirt5−/− enhances fibrosis and pro-fibrotic gene expression in FAN kidneys.
As previously mentioned, renal fibrosis is a major contributor to CKD progression.(514) Common pro-fibrotic markers are vimentin (mesenchymal marker), α-smooth muscle actin (αSMA) (a marker of myofibroblast differentiation) and collagen I (COL1).(517, 518) Increased levels of vimentin have been shown to promote disease progression in unilateral ureter obstruction (UUO) kidneys, which could be alleviated by vimentin deletion.(519) To test whether FA increased the level of vimentin+ cells, WT and Sirt5−/− kidneys were screened by IHC.

As shown in Figure 5.16A, FA treatment increased the vimentin+ area in the renal cortex in both WT and Sirt5−/− kidneys with only very little increase in the medulla. Vimentin+ area was quantified using ImageJ (Figure 5.16B and C). FA increased the vimentin+ area in the renal cortex of WT and Sirt5−/− kidneys compared to the vehicle control kidneys, although this did not reach statistical significance (Figure 5.16B). In the renal cortex, no significant difference in vimentin+ area could be detected between WT and Sirt5−/− FA-treated kidneys. Vimentin levels in the outer medulla of WT and Sirt5−/− kidneys did not significantly change following FA injection (Figure 5.16C).
Next, the effect of FA injection on renal fibrosis in WT and Sirt5⁻/⁻ kidneys was assessed by: (1) PSR staining to determine the degree of fibrosis in FFPE kidney sections; (2) qPCR and (3) WB for mRNA and protein levels of collagen 1 (COL1), αSMA, extra domain A (EDA)-containing fibronectin (Fn) (Fn-EDA; drives fibroblast-to-myofibroblast differentiation (520)) and vimentin.

As shown in Figure 5.17, following FA injection there was only a small increase in the PSR⁺ area in WT and Sirt5⁻/⁻ kidneys relative to the respective vehicle control kidneys indicating very mild fibrosis. Given this mild response no quantification was carried out.
Collagen deposition in WT and Sirt5-/- kidneys in response to FA.

Male WT and Sirt5-/- mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO3 buffer (vehicle control). Kidneys were harvested 14d post-injection (n=6-11). Images showing renal cortex and medulla of FFPE kidney sections stained with PSR to visualise collagen I and III fibres. (n=6-11 kidneys/ group were analysed). Scale bar: 100µm.

Analysis of pro-fibrotic gene expression (Figure 5.18A-C) showed that FA injection increased Col1a1 mRNA levels in both WT and Sirt5-/- kidneys compared to the respective vehicle control kidneys (both: p<0.05; Figure 5.18A). While it appeared that Col1a1 expression was slightly higher in Sirt5-/- FA kidneys, no statistically significant difference in Col1a1 expression between WT FA and Sirt5-/- FA kidneys was detected. Analysis of αSma expression showed a non-statistically significant increase in Sirt5-/- FA kidneys and no change in WT FA kidneys compared to the respective vehicle control kidneys (Figure 5.18B). Interestingly, compared to WT FA kidneys, Sirt5-/- FA kidneys displayed significantly higher αSma mRNA levels (p<0.05) potentially indicating a higher number of myofibroblasts in Sirt5-deficient FA kidneys. When Fn-EDA mRNA levels were determined it emerged that FA injection only slightly, yet, not significantly increased Fn-EDA mRNA expression in WT and Sirt5-/- kidneys compared to the respective vehicle control kidneys (Figure 5.18C). No difference in Fn-EDA mRNA levels could be detected between WT FA and Sirt5-/- FA kidneys.

Protein levels of COL1, vimentin and αSMA were determined by WB (Figure 5.18D-G). As shown in Figure 5.18D, FA injection caused an increase of all three markers in WT and Sirt5-/- kidneys compared to the vehicle control kidneys. Densitometric analysis of the blots showed that COL1 protein levels (COL1A1/2) were significantly increased in Sirt5-/- FA kidneys relative to the vehicle control kidneys (p<0.05) with only a minor, non-significant increase in WT FA kidneys compared to the relevant controls (Figure 5.18E). Vimentin significantly increased in both WT FA (p<0.01) and Sirt5-/- FA kidneys (p<0.0001) when compared to the vehicle control kidneys.
αSMA protein levels showed a non-significant increase in WT and Sirt5⁻/⁻ FA kidneys compared to the respective vehicle control kidneys (Figure 5.18G). αSMA protein expression appeared enhanced in Sirt5⁻/⁻ FA kidneys compared to WT FA kidneys.

Figure 5.18: Expression of pro-fibrotic markers in FA-treated WT and Sirt5⁻/⁻ kidneys. Male wild-type (WT) and Sirt5⁻/⁻ mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO₃ buffer (vehicle control). Renal tissue was harvested 14d post-injection (n=6-11). Whole kidney lysates were used for mRNA and protein analyses. Bar graphs showing mRNA levels of the pro-fibrotic markers (A) collagen 1a1 (Col1a1), (B) α-smooth muscle actin (αSma) and (C) fibronectin (Fn)-extra domain a (EDA) (Fn-EDA). (n=5-6 kidneys/group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and **p<0.01. (D) WB showing protein levels of the fibrosis markers collagen 1 (COL1), vimentin and αSMA. Actin and tubulin were used as loading control. Bar graphs showing protein levels of (E) COL1, (F) VIMENTIN and (G) αSMA. Data was normalised to actin or tubulin. (n=5-6 kidneys/group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and **p<0.01. ImageJ was used for densitometry.
In summary, the data from PSR staining, qPCR and WB suggested that FA induced only a mild fibrotic response in WT and Sirt5⁻/⁻ kidneys, which appeared more pronounced in Sirt5⁻/⁻ FA kidneys.

Mitochondrial dysfunction caused by excessive fragmentation, reduced biogenesis and increased degradation results in impaired tubular energy metabolism and is a major contributor to CKD.(4) Data in Chapter 4 showed that knockdown of SIRT5 in hPTECs provoked mitochondrial fragmentation possibly by reducing the protein levels of MFN1/2 and OPA1 (Figure 4.14 and 4.15).

**Loss of SIRT5 does not impair mitochondrial fusion/fission dynamics.** Next the effect of Sirt5 deletion on renal expression of the three pro-fusion proteins MFN1/2 and OPA1 was explored and whether levels were affected by FA (Figure 5.19A). As shown in Figure 5.19B-D, densitometry revealed that protein levels of MFN1/2 and OPA1 were similar in Sirt5⁻/⁻ vehicle kidneys and WT vehicle kidneys. Although it appeared that MFN1 and OPA1 showed a trend to increase and MFN2 a trend to decrease in protein levels in Sirt5⁻/⁻ vehicle kidneys compared to WT vehicle kidneys. In WT kidneys, FA injection reduced expression of MFN2 (p<0.01) and OPA1 (p>0.05) compared to the WT vehicle control while MFN1 expression was unaffected (Figure 5.19B-D). In Sirt5⁻/⁻ kidneys, FA treatment resulted in a non-significant reduction in MFN1 and 2 as well as a significant decline in OPA1 (p<0.01) compared to the Sirt5⁻/⁻ vehicle controls (Figure 5.19B-D). MFN1 and MFN2 protein expression was similar in WT and Sirt5⁻/⁻ FA kidneys. OPA1 levels appeared lower in Sirt5⁻/⁻ FA kidneys compared to WT FA kidneys.

The pro-fusion protein OPA1 exists as five isoforms (2 isoforms of l-OPA1 and 3 isoforms of s-OPA1), which are key for mitochondrial form and function.(159, 397) In Chapter 4 it was shown that SIRT5 depletion in hPTECs reduced l-OPA1 levels (Figure 4.15), providing a potential explanation for SIRT5 RNAi-induced mitochondrial fragmentation. To test whether this also applied to Sirt5⁻/⁻ kidneys, l-OPA1 and s-OPA1 protein levels were quantified (Figure 5.19E and F). Vehicle-treated WT and Sirt5⁻/⁻ kidneys showed no difference in s- or l-OPA1 levels. FA injection reduced s- and l-OPA1 levels in both WT (both: p>0.05) and Sirt5⁻/⁻ (s-OPA1: p>0.05 and l-OPA1: p<0.05) kidneys compared to the respective vehicle control kidneys. Both s- and l-OPA1 levels appeared lower in Sirt5⁻/⁻ FA kidneys compared to WT FA kidneys, however, there was no statistically significant difference.
Figure 5.19: Expression of mitochondrial pro-fusion proteins in WT and Sirt5−/− kidneys in response to FA. Male WT and Sirt5−/− mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO3 buffer (vehicle control). Renal tissue was harvested 14d post-injection (n=6-11) and proteins prepared from whole kidney lysates. (A) WB showing expression of the pro-fusion proteins mitofusin (MFN) 1 (MFN1), MFN2 and optic atrophy 1 (OPA1). Actin and tubulin served as loading controls. (B-E) Bar graphs showing densitometric analysis of expression of (B) MFN1, (C) MFN2, (D) total OPA1, (E) short- (s) OPA1 (s-OPA1) and (F) long- (l)-OPA1 (l-OPA1). (n=5-6 kidneys/ group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05 and **p<0.01. ImageJ was used for densitometry.

Based on the observation that l-OPA1 levels were slightly reduced in Sirt5−/− FA kidneys compared to WT FA kidneys, protein levels of the l-OPA1 processing enzyme YME1L were determined (Figure 5.20A). Although YME1L can degrade l-OPA1 (~50% of all l-OPA1 isoforms), it equally acts as an l-OPA “protector” by degrading the peptidase OMA1 (which can degrade all l-OPA1 isoforms). Only YME1L levels were determined as
the anti-OMA1 antibody used in Chapter 4 (Figure 4.15) did not detect any specific bands in murine kidney lysates.

As shown in Figure 5.20B, YME1L levels appeared to be lower in Sirt5<sup>−/−</sup> vehicle kidneys compared to the WT vehicle kidneys although this was not statistically significant. FA treatment significantly reduced YME1L expression in both WT (p<0.001) and Sirt5<sup>−/−</sup> (p<0.0001) kidneys compared to the respective vehicle controls, although there was no difference between WT and Sirt5<sup>−/−</sup> FA kidneys.

![Figure 5.20: Expression of the l-OPA1 degrading enzyme YME1L in WT and Sirt5<sup>−/−</sup> kidneys in response to FA.](image)

Male WT and Sirt5<sup>−/−</sup> mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO<sub>3</sub> buffer (vehicle control). Renal tissue was harvested 14d post-injection (n=6-11). Whole kidney lysates were used for protein analyses. (A) WB showing protein levels of the protease YME1L. Tubulin served as a loading control. (B) Bar graphs showing densitometric analysis of YME1L expression. (n=5-6 kidneys/group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. ImageJ was used for densitometry.

Taken together, the data suggested that loss of SIRT5 did not significantly disrupt fusion dynamics in murine kidneys at “baseline”, but may enhance l-OPA1 proteolysis in FA kidneys.

**Loss of SIRT5 increases autophagy in FA-treated kidneys but does not exacerbate mitochondrial mass decline.** The effect of SIRT5 ablation on mitochondrial mass was also assessed. For this, specific markers driving mitochondrial biogenesis (i.e. mtDNA, Tfam and Pgc1α), mitochondrial marker proteins (i.e. TOM20, SDHA and NDUFS2) and markers of autophagy induction (LC3-II) were measured (Figure 5.21).

As depicted in Figure 5.21A, mtDNA levels did not differ between WT and Sirt5<sup>−/−</sup> vehicle kidneys. FA treatment only caused a minor, non-significant decline in mtDNA levels in both WT and Sirt5<sup>−/−</sup> kidneys compared to the vehicle controls with no statistically significant difference between WT and Sirt5<sup>−/−</sup> FA kidneys.
Figure 5.21: Expression of autophagy and mitochondrial marker proteins in WT and Sirt5−/− kidneys in response to FA. Male WT and Sirt5−/− mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO₃ buffer (vehicle control). Kidneys were harvested 14d post-injection (n=6-11) and screened for mtDNA, RNA and protein. Bar graphs showing (A) mitochondrial DNA (mtDNA); and, mRNA levels of (B) Pgc1α, the master regulator of mitochondrial biogenesis and (C) Tfam, a key regulator of mtDNA replication. mtDNA levels were normalised to nuclear DNA (nDNA) and mRNA was normalised to actin. (D) WB showing expression of mitochondrial proteins succinate dehydrogenase A (SDHA; Complex II), NADH:ubiquinone oxidoreductase core subunit S2 (NDUFS2; Complex I), transition outer membrane 20 (TOM20) and the autophagy marker Microtubule-associated proteins 1A/1B light chain 3 (LC3)-I and LC3-II. Actin was used as a loading control. Bar graphs showing densitometric analysis of protein expression of (E) LC3-I, (F) LC3-II, (G) LC3-II/I ratio, (H) TOM20, (I) SDHA and (J) NDUFS2. Data were normalised to actin or tubulin. (n=5-6 kidneys/ group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons. *p<0.05, **p<0.01 and ***p<0.001. ImageJ was used for densitometry.
Subsequently, mRNA levels of *Pgc1α* and *Tfam* were determined (Figure 5.21B and C). *Pgc1α* and *Tfam* mRNA levels were not significantly different between WT and *Sirt5*−/− vehicle kidneys. However, it appeared that, similar to what has been described earlier (Figure 5.8), there was a slight increase in *Tfam* expression in *Sirt5*−/− kidneys compared to the WT kidneys. FA caused a minor decline in *Pgc1α* and *Tfam* expression in both WT and *Sirt5*−/− kidneys compared to the respective vehicle controls. Intriguingly, *Tfam* mRNA levels appeared to be higher in *Sirt5*−/− FA kidneys compared to WT FA kidneys.

Next, it was assessed whether *Sirt5* deletion or FA treatment increased autophagy and/or affected mitochondrial mass (Figure 5.21D). As shown in Figure 5.21E, *Sirt5*−/− kidneys (vehicle and FA) showed a trend to decreased LC3-I levels compared to the respective WT kidneys. FA treatment did not significantly affect LC3-I levels in WT and *Sirt5*−/− kidneys compared to vehicle control kidneys. Notably, FA treatment caused a significant increase in LC3-II levels in *Sirt5*−/− kidneys (p<0.05) compared to vehicle controls while LC3-II levels in WT FA kidneys remained unchanged (Figure 5.21F). Although LC3-II levels appeared higher in *Sirt5*−/− FA kidneys, no significant differences between WT FA and *Sirt5*−/− FA kidneys could be detected. Of interest, when the LC3-II/I ratio was determined, *Sirt5*−/− vehicle kidneys showed a trend to an increased LC3-II/I ratio compared to WT vehicle kidneys (Figure 5.21G). FA treatment did not affect the LC3-II/I ratio in WT kidneys but slightly increased the ratio in *Sirt5*−/− kidneys (p>0.05). *Sirt5*−/− FA kidneys showed a significantly higher LC3-II/I ratio compared to WT FA kidneys (p<0.05) suggesting *Sirt5* deletion increased autophagy.

Subsequently, it was tested whether mitochondrial mass was decreased in *Sirt5*−/− kidneys and whether this was exacerbated by FA treatment (i.e. due to enhanced autophagy). For this, protein levels of the mitochondrial marker proteins TOM20, SDHA and NADH:ubiquinone oxidoreductase core subunit S2 (NDUFS2) were measured by WB. As shown in Figure 5.21H–J, *SIRT5* ablation slightly increased TOM20 expression and reduced expression of SDHA and NDUFS2 (all: p>0.05). As shown in Figure 5.21H, FA treatment significant reduced TOM20 protein levels in *Sirt5*−/− kidneys compared to *Sirt5*−/− vehicle kidneys (p<0.05) while no significant reduction could be detected in WT FA kidneys compared to WT vehicle kidneys. Although TOM20 levels appeared lower in *Sirt5*−/− FA kidneys compared to WT FA kidneys this did not reach statistical significance.

SDHA protein levels declined in WT (p<0.01) and *Sirt5*−/− FA kidneys (p>0.05) compared to the vehicle control kidneys (Figure 5.21I). There was no significant difference between WT and *Sirt5*−/− FA kidneys. Quantification of NDUFS2 protein levels revealed a FA-induced reduction in both WT (p<0.001) and *Sirt5*−/− (p<0.01) kidneys compared to the
respective vehicle controls (Figure 5.21J). There was no difference between WT and Sirt5\(^{-/-}\) FA kidneys.

Taken together the data suggested that SIRT5 ablation per se had no significant impact on the induction of autophagy or on mitochondrial mass. Notably, FA treatment of Sirt5\(^{-/-}\) kidneys increased autophagy induction, although, this did not significantly enhance mitochondrial mass decline.

**FA treatment does not significantly impair renal function.** The effect of FA on renal function 14d post-injection was assessed by analysis of serum and urine samples for urea/creatinine or protein, respectively. As shown in Figure 5.22, 14d post-injection there was no significant increase in serum urea, serum creatinine or proteinuria suggesting FA treatment did not impair renal function.

![Figure 5.22: Renal function in FA-treated WT and Sirt5\(^{-/-}\) kidneys.](image)

Male WT and Sirt5\(^{-/-}\) mice (8-12 weeks) received a single intraperitoneal injection of FA (240µg/g body weight) or 0.3M NaHCO\(_3\) buffer (vehicle control). After 13d, mice were transferred to metabolic cages for 18h and urine and blood were collected 14d post-injection (n=6-11). Bar graphs showing levels of (A) serum urea, (B) serum creatinine and (C) proteinuria. (n=5-11 kidneys/ group were analysed). Data are mean ± SD. To determine statistical significance a two-way ANOVA was carried out followed by Tukey’s post hoc test to normalise for multiple comparisons.

### 5.5 Discussion

Accumulating evidence has identified SIRT5 as an important regulator of mitochondrial metabolism by targeting key proteins involved in FAO, the TCA cycle as well as the ETC.(296, 326, 333, 335, 336) Renal PTs are highly dependent on mitochondrial metabolism (34, 45) suggesting that Sirt5\(^{-/-}\) might impair this bioenergetic pathway and result in PT cell dysfunction. To date, there is only limited knowledge as to whether Sirt5 deletion causes mitochondrial dysfunction in renal tubules in vivo and whether this affects kidney function (371). Therefore, the first part of this Chapter was dedicated to assessing the impact of Sirt5\(^{-/-}\) on mitochondrial architecture in tubular cells in vivo as well as renal function.
Loss of SIRT5 does not impair renal function. Absence of SIRT5 was confirmed by WB and IHC (Figure 5.1). Sirt5 deletion caused an increase in renal KSucc and to a lesser extent, KMal levels. Both hypersuccinylation/-malonylation have been shown to impair cellular energy metabolism (266, 296, 326) hinting that this may result in functional consequences, in particular for highly metabolically-active tubular cells such as PTs (32).

Mitochondrial bioenergetic output is largely determined by mitochondrial structure and excessive fragmentation can result in functional impairment (42, 43). In Chapter 4 it was shown that SIRT5 RNAi in hPTECs provoked mitochondrial fragmentation and impaired mitochondrial energy production (Figure 4.12 and 4.13). Interestingly, TEM analysis of PTs in Sirt5−/− kidneys did not show a dramatic phenotypic change in mitochondrial morphology, though, Sirt5−/− PT mitochondria appeared shorter and less interconnected compared to WT PT mitochondria (Figure 5.2). This finding suggested that SIRT5 ablation had only a minor impact on mitochondrial structure in PTs in vivo. Notably, when mitochondria in DTs were analysed, no differences between WT and Sirt5−/− mitochondria were detected. These findings seem to contradict the in vitro findings described in Chapter 4 (Figure 4.13) and the observations of others in Sirt5−/− MEFs (338) and SIRT5 RNAi-treated HK-2 cells (521) that showed that SIRT5 depletion caused excessive mitochondrial fragmentation. However, it is important to note that there are multiple factors which may have caused this discrepancy: (i) whole kidney analysis instead of analysis of a single cell type (i.e. PTECs) in the absence of modifying influences from other cell types; or, (ii) analysis of non-immortalised cells in vivo versus the use of immortalised cell lines (MEFs, HK-2 and HKC-8 cells) in vitro. Furthermore, it may be that (iii) SIRT5 is not important in the kidney or that (iv) loss of SIRT5 may have been compensated for (e.g. by other SIRTs). To exclude the effect of in vitro cell culture, murine PTECs would have to be isolated from WT and Sirt5−/− kidneys followed by analysis of mitochondrial structure using confocal microscopy (e.g. by Mitotracker stain or TMRM) or TEM. It is also possible that the highly metabolically active PTs and DTs have developed a compensatory mechanism in vivo to mitigate the effects of germline deletion of Sirt5 and therefore, did not show any dramatic alterations in mitochondrial structure. To determine whether germline knockout may have caused a compensatory response, it would be of interest to repeat this experiment in tubular/ PT-specific, inducible Sirt5−/− mice (522) and assess mitochondrial structure by TEM or super-resolution microscopy (IF staining for e.g. TOM20).

Podocyte foot process effacement is a pathophysiological feature of glomerular injury which impairs the glomerular filtration barrier and results in decreased renal function (505). Although glomerular expression of SIRT5 (as measured by IHC) was low in WT kidneys (data not shown), the ultrastructural impact of Sirt5 deletion on podocyte
function was assessed. For this, podocyte foot processes, which are key determinants of the glomerular filtration barrier, were analysed by TEM (Figure 5.4). The data revealed no differences in foot process ultrastructure between WT and Sirt5⁻/⁻ podocytes indicating that SIRT5 is either dispensable for normal foot process architecture and most likely function or not relevant for it given that SIRT5 expression was low in WT podocytes. This hypothesis was confirmed functionally by measuring serum creatinine and urea levels (Figure 5.5), which are both proxy markers of the glomerular filtration rate (i.e. podocyte function) and therefore, an indicator of renal function. This observation is consistent with a very recent study which assessed blood urea nitrogen and serum creatinine in Sirt5⁻/⁻ mice and showed that SIRT5 ablation per se does not impair renal function.

Taken together, the data suggested that SIRT5 is not required for normal renal function under physiological conditions in adult mice (age 10-12 weeks). It is worth mentioning that Sirt5⁻/⁻ mice develop cardiac hypertrophy with age due to a defect in mitochondrial FAO. Given that renal tubular cells (PTs and DTs) primarily depend on FAO for ATP production, it would be of interest to investigate whether an age-induced decline in renal function is enhanced in Sirt5⁻/⁻ mice and more importantly, whether this increases susceptibility to acute and/or chronic injury.

Loss of SIRT5 protects murine kidneys from acute ischaemic injury. Based on the findings in Chapter 4, that SIRT5 RNAi reduced mitochondrial function in hPTECs (Figure 4.12), which is a pathophysiologic feature in AKI (52, 72), the hypothesis was tested that SIRT5 ablation exacerbates IRI. WT and Sirt5⁻/⁻ mice were challenged with 30min renal ischaemia (Figure 5.6). However, only very mild injury was observed 24h post-insult and no differences between WT and Sirt5⁻/⁻ IRI kidneys could be detected. To increase the severity of the model, the ischaemic insult was extended to 40min. As expected, renal injury increased with the duration of the ischaemia: While 30min ischaemia induced only mild damage in both WT and Sirt5⁻/⁻ kidneys, 40min ischaemia induced severe tubular injury (dilation, atrophy and casts; Figure 5.6). Surprisingly, when kidneys exposed to severe 40min ischaemia were scored for tubulointerstitial injury, it emerged that loss of SIRT5 alleviated rather than exacerbated damage.

To assess tubular injury on a molecular level, WT and Sirt5⁻/⁻ IRI kidneys (40min ischaemia) were screened for expression of two tubular injury markers, Kim1 and Ngal. In keeping with the tubulointerstitial injury data, these data also suggested that SIRT5 ablation conveyed protection from IRI as Sirt5⁻/⁻ IRI kidneys expressed significantly lower levels of Kim1 and Ngal compared to WT IRI kidneys (Figure 5.7). This idea was confirmed on a functional level (serum creatinine/urea levels as a proxy marker for renal function) as values for serum creatine from Sirt5⁻/⁻ IRI mice showed a trend to lower
creatine (~14% lower) and significantly lower levels of urea (~27% lower) relative to sera from WT IRI mice (Figure 5.7). Notably, although creatinine levels in sera from WT and Sirt5−/− IRI kidneys did not reach a statistically significant difference, these data support the notion that loss of SIRT5 improves renal function in IRI; especially as mice were subjected to unilateral ischaemia and therefore, contained one fully functioning kidney, which makes it more difficult to detect changes in renal function. The fact that a difference in renal function could be seen between the two genotypes in the IRI treatment group therefore suggested that this finding is biologically relevant. Of interest, in line with the present data, a very recent study by Chiba et al. found that Sirt5−/− mice subjected to unilateral IRI (22min) showed enhanced creatinine clearance and thus, improved renal function compared to WT mice.(371) Notably, the group chose 7d post-ischaemia as the time-point to evaluate rather than 24h, and carried out a CL nephrectomy on day 6, which removed the potentially confounding influence of having one healthy kidney. Thus, the group was able to show a significant difference in serum creatinine levels between WT and Sirt5−/− mice.(371)

In Chapter 4 it was shown that SIRT5 RNAi reduced mitochondrial mass in hPTECs (Figure 4.20). As an indication as to whether Sirt5−/− kidneys may equally display a reduction in mitochondrial mass and whether this is affected by IRI, changes in mtDNA (proxy marker of mitochondrial content) as well as mRNA levels of genes involved in mitochondrial biogenesis and function were determined (by qPCR): TFAM is an important TF driving mtDNA replication (524) and PGC1α is regarded as a master regulator of mitochondrial biogenesis (61).(64, 525) Quantification of mtDNA levels showed no difference between the genotypes (WT versus Sirt5−/−; CL and IRI) or treatments (CL versus IRI) suggesting that mitochondrial mass was not affected by SIRT5 ablation or ischaemic insult (Figure 5.8). This was surprising as an IRI-induced reduction in mtDNA might have been expected given that the ischaemic insult was quite severe. Nonetheless, it is possible that 24h post-ischaemia might have been too early to see the full repercussion of the IRI. Injury-induced reduction in renal Tfam or Pgc1α expression (by IRI, FA or UUO) has been positively correlated with amplified damage.(64, 525) Conversely, increasing their expression alleviated renal injury (64, 525) suggesting that TFAM and PGC1α are key determinants of renal protection in response to injury. When CL kidneys (both WT and Sirt5−/−) were screened for Tfam and Pgc1α mRNA, it appeared that SIRT5 ablation stimulated a slight though not statistically significant increase in both gene transcripts (Figure 5.8). When WT and Sirt5−/− kidneys exposed to IRI were analysed, ischaemic injury only reduced Tfam and Pgc1α mRNA levels in WT kidneys but not in Sirt5−/− kidneys. Consequently, Sirt5−/− IRI kidneys displayed significantly higher mRNA levels of Tfam and Pgc1α compared to WT IRI
kidneys (Figure 5.8) suggesting that Sirt5<sup>−/−</sup> IRI kidneys show enhanced mitochondrial biogenesis post-ischaemia, which may promote overall mitochondrial function. To test this hypothesis, additional markers of mitochondrial mass (such as CS assay or WB analysis to screen for mitochondrial marker proteins) as well as metabolic characterisation of WT and Sirt5<sup>−/−</sup> IRI kidneys (by e.g. Oxygraph-2k (Oroboros) or ETC enzymatic assays) would need to be performed.

Nasrin et al. showed that SIRT4 ablation in vivo provoked a compensatory increase in hepatic expression of Pgc1α, Sirt1 and 3 (295), all of which are important regulators of mitochondrial metabolism (61, 294, 296, 526). To address whether Sirt5 deletion may have elicited a similar response, Sirt1, 3, and 4 mRNAs were assessed in 40min CL and IRI kidneys (Figure 5.9). Transcript analyses showed no significant difference between the two genotypes, although there was trend to increased expression of Sirt1, 3 and 4 in Sirt5<sup>−/−</sup> CL kidneys compared to WT CL kidneys, similar to what was observed for Tfam and Pgc1α (Figure 5.8). Considering that Sirt5 deletion has been shown to impair mitochondrial metabolism in murine kidneys (371), it is tempting to speculate that the elevation in Tfam, Pgc1α, Sirt1, 3 and 4 mRNA levels may be a compensatory response to boost mitochondrial biogenesis and function to alleviate the bioenergetic dysfunction. Surprisingly when mRNA levels of Sirt5 were determined in IRI kidneys, it emerged that in WT kidneys IRI caused a decrease instead of an increase in Sirt5 levels (Figure 5.9). This was unexpected as data presented in Chapter 3 showed that IRI resulted in increased levels of SIRT5 protein (measured by IHC; Figure 3.10). While these findings seem to be contradictory, it is important to note that the surgical model, the duration of ischaemia and therefore, the severity of the models differed between the two IRI studies (30min bilateral ischaemia (archived tissue from Prof. Alan Salama) versus 40min unilateral ischaemia). For this reason, the data cannot be compared directly. Also, although mRNA levels are an indicator of protein expression, SIRT5 protein levels would have to be measured to allow a direct comparison.(360) When mRNA levels of Sirt1, 3 and 4 were measured, it emerged that ischaemia reduced expression of all three transcripts in WT kidneys only, while in Sirt5<sup>−/−</sup> kidneys just Sirt4 declined relative to the respective CL control kidneys. Sirt5<sup>−/−</sup> IRI kidneys, therefore, showed significantly higher levels of Sirt3 as well as 4 and showed a trend to increased Sirt1 mRNA levels. This is an important finding as preserving SIRT1 and 3 expression in AKI has been shown to protect against injury.(77) However, it is difficult to determine whether the increased levels of Sirt1, 3 and 4 are a result of or contribute to reduced tubular injury in Sirt5<sup>−/−</sup> IRI kidneys (Figure 5.6). The observation that Sirt5<sup>−/−</sup> IRI kidneys displayed significantly higher levels of Sirt3 mRNA expression is important given that SIRT5 and SIRT3 have a
considerable target overlap. One example of a mutual target is FoxO3A which has been shown to be renoprotective.

As mentioned above, Chiba et al. recently reported that loss of SIRT5 protected mice from IRI, which is in keeping with the data presented in this Chapter. Interestingly, the group found that SIRT5 ablation stimulated a switch from mitochondrial to peroxisomal FAO and proposed that this may be the underlying mechanism protecting Sirt5−/− mice from IRI, possibly by reducing oxidative stress. Although ROS levels were not quantified, this explanation seems plausible given that ROS are a central driver of tubular injury. However, this mechanism presupposes that the cell type protected contains peroxisomes. Studies in mice, rats and humans have shown that only the PT segments S2 (in cortex) and even more so the S3 segments (in outer medulla) have peroxisomes, while cortical PT S1 segments appear to lack these organelles. Given that the present study found tubular protection in cortical PTs, which are very likely predominantly S1 PTs, it seems unlikely that such a FAO switch is the only reason for the reduced ischaemic injury seen in Sirt5-depleted PTs. An alternative explanation could be that impairment of mitochondrial function per se alleviated IRI. This idea has been put forward in several studies which showed that inhibition of ETC complexes I (530-532) and II (510) or the F1/F0-ATP synthase (533), reduced ischaemic injury in the kidney. Two mechanisms have been implicated which entail (i) mitochondrial ROS production and (ii) ATP depletion:

(i) Mitochondria are a major source of ROS that cause PTEC death in ischaemic injury. One key mechanism generating mitochondrial ROS is reverse electron transfer from complex II to complex I in the reperfusion phase. The detailed mechanism causing ROS production is as follows: During ischaemia (low O2 levels), SDH (complex II subunit) reverses functionally and converts fumarate to succinate leading to succinate accumulation, whereas during reperfusion (restoration of physiological O2 levels) SDH converts the accumulated succinate to fumarate. Succinate oxidation leads to QH2 production (electron donor), which moves electrons from complex II to complex I (reverse electron transfer). At complex I, electrons escape the ETC leading to the reduction of NAD+ as well as molecular oxygen (O2) to form NADH and superoxide (O2−), respectively. NAD+ reduction (to NADH) as well as O2− production are enhanced by an increased Δp. ROS production is, therefore, highly regulated by the activities of the two ETC complexes (complex I and II) and the Δp as driving force. Pharmacological inhibition of SDH has been shown to decrease succinate accumulation/oxidation and to alleviate ischaemic tubular injury. This, together with the present finding and the observations of others that Sirt5 deletion reduced complex II activity in murine kidneys suggested that impairment of complex II may be
one mechanism of alleviating IRI. Although this has not been shown in the kidney, this idea may be supported by an interesting observation by Chiba et al. who found that Sirt5-/- IRI kidneys contained reduced KSucc levels compared to WT IRI kidneys. Given that Sirt5 deletion impaired complex II activity, this observation could be regarded as a functional readout for reduced succinate accumulation during ischaemia, which resulted in lower succinyl-CoA levels and thereby, may explain the decrease in KSucc in Sirt5-/- IRI kidneys. However, this is only a hypothesis and would need to be confirmed by quantification of succinate as well as succinyl-CoA levels in IRI kidneys (WT and Sirt5-/-) by mass spectrometry. As mentioned earlier, there are two additional elements involved in ROS production during reperfusion, namely complex I and the Δp. SIRT5 ablation has been shown to impair complex I activity in murine liver (336) and reduce the ΔΨM, a central component of Δp, in cardiac mitochondria (464) and SIRT5 RNAi hPTECs (Figure 4.12) hinting that this may also contribute to renoprotection in Sirt5-/- IRI kidneys.

To test these hypotheses, it would be fundamental to determine complex I activity (by enzymatic assay) and measure the ΔΨM in WT and Sirt5-/- kidney slices (by confocal microscopy; TMRM stain). Given that the key factor leading to tubular damage is ROS production rather than complex I or II activities, the most essential functional readout would be to measure mitochondrial ROS levels in IRI tissues. Since it is difficult to measure specifically mitochondrial ROS in tissue sections, a more general approach would have to be used e.g. IHC stain for 4-hydroxynonenal (535).

(ii) An important element, which has been implicated in ischaemic injury, in particular PTEC death, is the F1/F0-ATP synthase (complex V).(45, 50, 536) Multiple lines of evidence have shown that under low oxygen conditions the F1/F0-ATP synthase reverses functionally and consumes (glycolytic) ATP in order to maintain the ΔΨM.(48, 536) This mechanism requires a metabolic switch from OXPHOS to glycolysis to supply sufficient ATP.(45, 48, 536) PTECs, however, display very limited glycolytic capacity causing rapid ATP depletion and cell death (acute tubular necrosis) during ischaemia.(45, 50) Various studies have identified the F1/F0-ATP synthase as a target of SIRT5 and found that Sirt5 deletion reduced ATP production rates in murine liver and heart (336, 464). This would fit with the present findings in SIRT5-depleted hPTECs (Figure 4.12). Although the impact of SIRT5 ablation on F1/F0-ATP synthase reversal has not been investigated, these data hint that SIRT5-mediated reduction in F1/F0-ATP synthase activity may reduce ATP hydrolysis during ischaemia and thereby, alleviate IRI. To assess whether this would be an additional explanation for the reduced injury found in Sirt5-/- kidneys, biochemical assays would have to be carried out to measure F1/F0-ATP synthase activity in WT and Sirt5-depleted kidneys.(537) Furthermore, given that ATP levels are a critical factor that determine PT cell death (45, 49, 50), it would be
important to measure steady-state ATP levels in renal tissues post-ischaemia. Of note, maintaining ATP levels will most likely be at the expense of the $\Delta \Psi_M$. On one hand, this may be beneficial, as a reduction in the $\Delta \Psi_M$ is associated with reduced ROS production, as discussed in (i) above. On the other hand, a reduction in the $\Delta \Psi_M$ may exacerbate mitochondrial dysfunction during acute ischaemia and drive apoptosis. The latter, however, might not be an issue provided that dysfunctional mitochondria are removed by mitophagy (54, 55). Considering that SIRT5-depleted hPTECs showed increased mitophagy (Figure 4.19), it appears likely that reduced F1/F0-ATP synthase activity coupled with enhanced mitophagy might be another mechanism protecting Sirt5$^{-/-}$ IRI kidneys. As previously mentioned, to investigate whether SIRT5 ablation impacts on the $\Delta \Psi_M$, it would be essential to measure the $\Delta \Psi_M$ in kidney slices using confocal microscopy (TMRM stain). Further, to assess whether Sirt5 deletion enhanced mitophagy in vivo, Sirt5$^{-/-}$ mice could be crossed with mitophagy reporter mice (mitoQC mice) (59) and renal tissue analysed by confocal microscopy.

Worth mentioning, F1/F0-ATP synthase is inhibited by the inhibitory factor 1 (IF1) and IF1 overexpression has been shown to protect from ischaemic injury.(47) IF1 activity was found to be reduced by PKA-mediated phosphorylation on S39 (538) suggesting that IF1 activity is regulated post-translationally. Intriguingly, Park et al. found that murine Sirt5$^{-/-}$ livers showed a dramatic increase in IF1 K90 succinylation (Sirt5$^{-/-}$/WT ratio: 68/1) (335) indicating that SIRT5 may regulate IF1 activity in mouse liver. To test whether this also applied to the kidney, murine WT and Sirt5$^{-/-}$ kidneys would have to be screened for K$\text{SUcc}$ sites by mass spectrometry. Furthermore, to determine whether SIRT5 increases or reduces IF1 activity, it would be of interest to use site-directed mutagenesis to generate IF1 proteins which either lack the succinylation site (e.g. K90) or generate a constitutively active succinylation mimetic (539) (e.g. on K90). The potential impact of IF1 K$\text{SUcc}$ on F1/F0-ATP synthase activity could subsequently be measured in blue native gels (using a complex V activity stain (540)) or using a photometric assay (537).

Taken together, the data showed that SIRT5 ablation protected mice from IRI and suggested that this may, at least in part, be mediated by a reduction in mitochondrial function. While SIRT5 deficiency may be beneficial in an acute setting it will be important to investigate later time-points (e.g. 14d, 1 month and 6 months post-ischaemia) in order to assess the repercussion of Sirt5$^{-/-}$, and impaired mitochondrial function in a more chronic setting. Furthermore, to evaluate the therapeutic potential of SIRT5 inhibition in IRI, a fundamental next step would be to repeat the experiment using specific SIRT5 inhibitors (541, 542).
To date, nothing is known about the role of SIRT5 in chronic renal injury. Given that mitochondrial dysfunction has emerged as a major pathophysiological feature of CKD (4), the data in the third part of this Chapter tested the hypothesis that Sirt5⁻/⁻ exacerbates FA-induced CKD. At the outset it is important to mention that the response to FA was highly variable, which was reflected in large error bars in the data. This is not an uncommon phenomenon as mice seem to show a heterogeneous response to FA and injury has been shown to regress in longer-term models (28-42d).(543, 544) Despite this FAN is a widely-used model of CKD as it causes tubulointerstitial fibrosis and also allows urine analyses, which is not possible after, for instance, UUO, a CKD model which is less variable and causes more severe injury (543, 545), however, is also less clinically relevant.

**Loss of Sirt5 enhances FA-induced renal damage.** Absence of SIRT5 mRNA and protein expression in the kidneys of Sirt5⁻/⁻ mice was confirmed by qPCR and WB, respectively (Figure 5.11). When WT FA kidneys were investigated it emerged that in contrast to the findings in Chapter 3 (Figure 3.11), FA treatment did not reduce SIRT5 mRNA/protein expression suggesting that the single dose FAN model only caused mild damage. Based on the previous observation which showed that Sirt5⁻/⁻ IRI kidneys displayed elevated Sirt1, 3, and 4 mRNA levels (Figure 5.9) and to determine whether this also applied to the FAN model, Sirt transcripts were measured by qPCR (Figure 5.11). Interestingly, FA treatment did not significantly reduce mRNA levels of any of the analysed SIRTs again suggesting that the model only caused very mild damage.

Analysis of MT-stained vehicle and FA kidneys (WT and Sirt5⁻/⁻) confirmed this conclusion (Figure 5.12) as FA kidneys showed only minor tubulointerstitial injury (tubular atrophy, fibrosis, immune cell infiltration). Interestingly, although FA injection only induced mild damage, qualitative assessment suggested tubulointerstitial injury was enhanced when SIRT5 was removed. Quantitation of tubulointerstitial damage showed a trend to increased damage Sirt5⁻/⁻ FA kidneys compared to WT kidneys, although this did not reach statistical significance. The observation that FA only caused very mild injury in WT kidneys was surprising as the dose injected (240µg/g BW) had previously induced severe damage (14d post-injection) (365). Of note, the present study used exactly the same concentration of FA in NaHCO₃ buffer (20mg/ml). This is an important factor as FA is highly soluble in the alkaline bicarbonate buffer meaning that reducing FA concentration results in decreased tubular crystal formation and thereby, less damage.(543) One difference between the present study and that of Tomlinson et al. (365) is in the mouse strains (C56BL/6J versus mixed background (347)). This is particularly relevant given that C57BL/6J mice have been shown to be resistant to multiple forms of chronic renal injury (546-548).
To assess tubular injury on a molecular level, WT and Sirt5⁻/⁻ kidneys (vehicle and FA) were screened for Ngal and Kim1 (Figure 5.13). Only Sirt5⁻/⁻ kidneys showed a significant increase in Ngal and Kim1 as well as KIM1 protein levels indicating more severe damage in the absence of SIRT5. However, there was no difference between WT and Sirt5⁻/⁻ FA kidneys suggesting that SIRT5 ablation only mildly exacerbated FA-induced injury.

Renal fibrosis is a hallmark of CKD and a key indicator for disease progression.(514) Immune cell infiltration is an important factor driving renal fibrosis through the generation of a pro-inflammatory environment.(515) IHC staining revealed that FA treatment slightly increased CD3⁺ T-cell infiltration in the renal cortex and medulla of both WT and Sirt5⁻/⁻ kidneys compared to the respective vehicle controls (Figure 5.14). Again, there was no significant difference between WT and Sirt5⁻/⁻ FA kidneys, although Sirt5⁻/⁻ FA kidneys showed a trend to increased T-cell infiltrate. In line with this, mRNA expression analysis of pro-inflammatory markers (IL-1β, IL-6 and Vcam1) showed that FA-injection caused only a mild pro-inflammatory response with no difference between WT and Sirt5⁻/⁻ FA kidneys (Figure 5.15). To assess whether Sirt5⁻/⁻ exacerbated FA-induced renal fibrosis, expression of the pro-fibrotic markers vimentin (mesenchymal cell marker), αSma (myofibroblast marker), Col1 and Fn-EDA (both ECM components) were determined and tissue sections were stained with PSR to visualise collagen deposition (Figure 5.16-18). Consistent with the results discussed in the previous paragraph, the data indicated that FA caused only mild injury as evidenced by a small increase in pro-fibrotic marker expression. To enhance the injury severity in this model, the FA dose/concentration (i.e. to 250µg/g BW; >20mg/ml) could be increased; multiple FA doses administered; and, the duration of the model extended to 28d (545). An on-going experiment used two FA injections (Day 0: 240µg/g; D14: 120µg/g) and animals will be sacrificed at 28d.

Mitochondrial dysfunction in renal tubules is a major pathophysiological feature of CKD and is characterised by (i) excessive fragmentation and an imbalance between (ii) biogenesis and (iii) mitophagy which together causes a reduction in mitochondrial mass and impairs bioenergetic output.(4)

(i) To address whether SIRT5 ablation exacerbated FA-induced mitochondrial fragmentation, WB analysis was carried out to screen for the pro-fusion proteins MFN1/2 and OPA1 (Figure 5.19). In contrast to the in vitro findings in hPTECs which showed that MFN1/2 and OPA1 levels were reduced by SIRT5 RNAi (Figure 4.14), SIRT5 ablation in vivo had no significant effect on MFN1/2 or OPA1 protein expression. Analysis of WT and Sirt5⁻/⁻ FA kidneys showed that MFN2 and OPA1 levels declined in both WT and Sirt5⁻/⁻ kidneys while MFN1 levels were not affected. Although there was no statistically
significant difference in MFN2 or OPA1 levels between WT and Sirt5\(^{-/-}\) FA kidneys, total OPA1 levels were lower in Sirt5\(^{-/-}\) FA compared to WT FA kidneys. This observation is in line with our in vitro data in hPTECs, which suggested that metabolic dysfunction induced by SIRT5 depletion enhanced l-OPA1 proteolysis (Figure 4.14). Analysis of l-OPA1 and s-OPA1 showed a similar pattern indicating that Sirt5 deletion may enhance l-OPA1 proteolysis in FA kidneys. As discussed earlier (Chapter 4, Section 4.1), l-OPA1 degradation is regulated by OMA1 and YME1L. The OMA1 antibody available could not be used to detect murine OMA1 so only YME1L levels were analysed (Figure 5.20). FA caused a significant decline in renal YME1L levels in both WT and Sirt5\(^{-/-}\) kidneys which may be one explanation for FA-induced reduction in l-OPA1. Although there was a trend to decreased YME1L levels in Sirt5\(^{-/-}\) FA kidneys compared to WT FA kidneys, this did not achieve statistical significance. Of note, while the data indicated that there was no difference in YME1L levels in WT and Sirt5\(^{-/-}\) FA kidneys it is possible that activity of the ATPase YME1L is reduced in Sirt5-deleted FA kidneys. Measuring YME1L activity directly is difficult. However, given that YME1L can degrade OMA1 (158), screening for OMA1 protein levels (by WB) may provide an indication as to whether YME1L activity is reduced in Sirt5\(^{-/-}\) kidneys. The present in vitro (Figure 4.15) and in vivo (Figure 5.19) data, although not statistically significant, did suggest a role for SIRT5 in the regulation of l-OPA1 proteolysis in FAN. Currently there is only very limited knowledge of the role/importance of l-OPA1 in kidney disease. Only one study has shown that decreasing l-OPA1 proteolysis using an Oma1 knockout mouse, reduced acute ischaemic injury and thereby, improved renal function.(398) However, the authors did not investigate whether YME1L levels were affected by OMA1 ablation. This study only used a model of acute injury (398) and it would be of interest to investigate the role of l-OPA1 degradation in a chronic setting (e.g. FAN). While these analyses give an indication as to whether mitochondrial dynamics are perturbed in FA-treated WT and Sirt5\(^{-/-}\) kidneys, it would also important to examine mitochondrial ultrastructure by TEM or super-resolution confocal microscopy. FAN kidneys (WT and Sirt5\(^{-/-}\); n=2/group) have been fixed and embedded for TEM, however, due to time limitations on the part of our collaborators at the UCL Institute of Child Health, analyses are pending.

(ii) To address whether FA treatment reduced mitochondrial biogenesis and test whether this was exacerbated by SIRT5 ablation WT and Sirt5\(^{-/-}\) kidneys were screened for mtDNA as well as Pgc1a and Tfam mRNA levels (Figure 5.21). Surprisingly none of these markers was significantly reduced by FA treatment in either WT or Sirt5\(^{-/-}\) kidneys indicating that FA did not markedly impair mitochondrial biogenesis. Interestingly, both Pgc1a and Tfam mRNA levels have been shown to be reduced by FA treatment in CD-1 mice (90) and FvB mice (63). A slightly lower dose of FA was used in the present study
(240µg/g BW versus 250µg/g BW) however a more important difference is in the strain of mice. Compared to i.e. CD-1 mice, C57BL6/J mice have been shown to be resistant chronic renal injury.\(^{(547, 549)}\) Therefore, as discussed above, it would be important to examine the effect of an increased FA dose and/or concentration on renal injury and mitochondrial biogenesis.

(iii) To determine whether \textit{Sirt5} deletion enhanced autophagy/mitophagy, protein levels of the autophagy marker proteins LC3-I and LC3-II were assessed. SIRT5 ablation did not significantly affect the levels of LC3-I or LC3-II in the vehicle-treated group (Figure 5.21). Interestingly, when challenged with FA, \textit{Sirt5}\(^{-/-}\) kidneys displayed a significant increase in LC3-II while WT kidneys were not affected (Figure 5.21) suggesting that \textit{Sirt5} deletion in FAN kidneys enhanced autophagy. While this has not previously been shown in an \textit{in vivo} setting, Poletta et al. reported that SIRT5 depletion increased autophagy in human breast cancer cells (MDA-MB-231) as well as murine myoblasts (C2C12) \(^{(300)}\). Notably, it remains to be elucidated whether autophagy in CKD is beneficial or detrimental: On one hand, reduced autophagy has been shown to drive tubular/podocyte death and enhance fibrosis in multiple models of CKD including diabetic nephropathy \(^{(550)}\), obesity-induced nephropathy \(^{(551, 552)}\) and obstructive nephropathy \(^{(553)}\). While, on the other hand, excessive autophagy has been shown to aggravate renal damage (fibrosis and tubular atrophy), particularly, in obstructive nephropathy.\(^{(554, 555)}\) Proposed underlying mechanisms for this phenomenon have been autophagy-induced apoptosis and lipotoxicity due to increased lipid accumulation.\(^{(63, 554-556)}\) Bearing in mind that CKD is not one disease and can be induced by multiple aetiologies (e.g. obstruction/diabetes/obesity), it is quite likely that autophagy has different functions depending on the type of insult/form of CKD and furthermore, the outcome may depend on the duration/degree of autophagy: In UUO nephropathy, a severe, surgical model which causes extensive structural changes in the ligated kidney, it appears that initially induction of autophagy is protective \(^{(553)}\) while chronically induced autophagy drives disease progression \(^{(555)}\). However, in diabetes- and obesity-induced nephropathies, which are metabolic forms of CKD, reduced autophagy seems to be the factor promoting injury (i.e. fibrosis and tubular cell death).\(^{(550-552)}\) In a ground-breaking study, Kang et al. showed that impaired mitochondrial FAO and the resulting accumulation of lipids in renal tubules are major drivers of FAN in mice and CKD in humans.\(^{(63)}\) Given that \textit{Sirt5}\(^{-/-}\) FA kidneys showed increased autophagy (Figure 5.20) and SIRT5 ablation has been shown to reduce mitochondrial FAO in murine kidneys \(^{(371)}\) it is tempting to speculate that this may be one mechanism by which \textit{Sirt5} deletion enhanced renal injury. To test this hypothesis and determine whether SIRT5 ablation increased lipid accumulation, frozen renal tissue sections from WT and \textit{Sirt5}\(^{-/-}\) FA kidneys could be
stained with Oil Red O or BODIPY (557). Importantly, Chiba et al. showed that although SIRT5 ablation reduced mitochondrial FAO in murine kidneys, it also increased peroxisomal FAO, which protected mice from ischaemic AKI.(371) The group hypothesised that this metabolic switch lowered the amount of free-fatty acids and thereby, alleviated IRI, possibly due to reducing mitochondrial lipotoxicity. Nonetheless, as discussed in the previous section on AKI, it is likely that a combination of enhanced peroxisomal FAO and decreased mitochondrial ETC/TCA cycle enzyme activities (i.e. complex II) are the underlying renoprotective mechanisms in AKI. While this mechanism may protect PTECs from acute injury, it is unlikely that this switch also protects in chronic injury conditions given the reliance of PTECs on mitochondrial FAO for ATP production (34, 35). This idea is in line with the present findings in FAN (Section 5.4.3) which suggested that SIRT5 ablation enhanced FA-induced injury. Mitophagy is a highly specialised form of autophagy, which contributes to a reduction in mitochondrial mass.(176) Given that Sirt5−/− FA kidneys showed enhanced autophagy induction, it was tempting to speculate that this may have exacerbated the decline in mitochondrial mass. Sirt5−/− vehicle kidneys showed a trend to increased TOM20 and decreased SDHA and NDUFS2 levels, three proxy markers of mitochondrial mass suggesting that SIRT5 ablation may have resulted in a minor decrease in mitochondrial content (Figure 5.21). FA injection caused a decline in TOM20, SDHA and NDUFS2 in both WT and Sirt5−/− FA kidneys. Although there was a trend to lower expression of the three markers in Sirt5−/− FA kidneys, there was no significant difference in expression of these markers between WT and Sirt5−/− FA kidneys. This suggested that the Sirt5−/−-mediated increase in autophagy did not significantly enhance the decline in mitochondrial mass, however, needs to be confirmed (e.g. by CS activity assay). Mitophagy can be counterbalanced by the generation of new mitochondria, which may reduce mitochondrial mass decline when mitophagy is enhanced.(558) Sirt5−/− vehicle kidneys showed a trend to increased Tfam mRNA levels hinting at a potential increase in mitochondrial biogenesis. Notably, Pgc1α mRNA levels were not significantly affected by Sirt5 deletion. FA treatment did not significantly reduce these two markers of mitochondrial biogenesis indicating minor injury in FAN kidneys. Interestingly, Tfam mRNA levels showed a trend to increased expression in Sirt5−/− FA kidneys suggesting increased mitochondrial biogenesis. It would be important to confirm this at the protein level by screening vehicle and FA kidneys (WT and Sirt5−/−) for PGC1α and TFAM by WB. It would also be of interest to increase the FA dose/concentration to determine whether under more severe injury conditions Sirt5 deletion has a more pronounced effect on mitochondrial content.

Notably, for WB analyses whole kidney lysates were analysed. Although this approach may give an overview of the overall processes that occur in the kidneys and
illuminate large effects in WT and Sirt5−/− kidneys induced by FA treatment, it is highly likely that this approach may miss small and nephron segment-specific changes in protein levels. To address this, individual tubular segments could be isolated by dissection and analysed by WB. Alternatively, protein expression could be determined in kidney sections using IF, which would allow co-localisation of proteins of interest with segment-specific markers.

Finally, to evaluate whether Sirt5−/− aggravated FA-induced impairment of renal function, serum urea/creatinine and proteinuria were determined (Figure 5.22). Renal function data confirmed mild injury in FAN kidneys (WT and Sirt5−/−).
5.6 Conclusion

In summary, the data presented in this Chapter showed that loss of SIRT5 does not increase mitochondrial fragmentation in murine PTECs and DTECs in vivo. In addition, loss of SIRT5 does not impair renal function indicating that SIRT5 is not required for normal renal organ function. Furthermore, when injury is superimposed, Sirt5 deletion may have differential effects depending on the type of injury and the duration of the model (ischaemic AKI versus nephrotoxic CKD; Figure 5.23): In IRI, a model of AKI, loss of SIRT5 protected from injury, while in FAN, a model of CKD, SIRT5 ablation enhanced damage. This is most likely due to the impact of SIRT5 on mitochondrial function. Impairment of mitochondrial function may be beneficial during acute ischaemia but detrimental in the setting of chronic injury.

Figure 5.23: Proposed model of the role of SIRT5 in AKI and CKD. Loss of SIRT5 impairs mitochondrial function and thereby, protects from ischaemic renal injury (IRI). In folic acid nephropathy (FAN), a nephrotoxic model of CKD, loss of SIRT5 enhances injury. Kidney injury marker (Kim1) is a marker of tubular injury.

The data, therefore, suggested that SIRT5 inhibition or activation may be two new potential therapeutic approaches in the treatment of ischaemic AKI or nephrotoxic CKD, respectively. Further investigations including the use of specific SIRT5 inhibitors in experimental AKI as well as increasing the severity of the CKD model, will be required to fully assess the therapeutic potential of SIRT5 manipulation in kidney disease.
Chapter 6

Discussion
6.1 General discussion

Acute and chronic kidney diseases are a major global health issue and CKD is predicted to be fifth most common cause of death world-wide by 2040 (559, 560). To date, there are no curative treatments for kidney diseases, and current therapeutic strategies are aimed at managing disease-associated complications (e.g. hypertension) to slow progression rather than treat the underlying cause.(25, 26) In recent years, mitochondrial dysfunction has emerged as a central pathophysiologic feature of AKI and CKD (3, 4) and promoting mitochondrial function has been proposed as a promising new treatment option (64, 67, 72, 363). Various strategies including boosting mitochondrial biogenesis (64, 67), inhibiting mitochondrial fragmentation (52) and reducing mitochondrial ROS levels (72, 399) are currently under investigation. Another, more pleiotropic approach, is to target metabolic dysfunction by activating the sirtuins, a family of NAD⁺-dependent KDACs, which have been shown to be involved in the regulation of mitochondrial form, function and biogenesis.(146, 320, 324, 494) One family member, SIRT5, a K-demalonylase/ -desuccinylase/ -deglutarylase, primarily localises to mitochondria and boosts mitochondrial function.(308, 326, 335) While SIRT5 has been shown to be highly expressed in healthy murine kidneys (330), its expression and function in normal human kidneys as well as during acute and chronic renal injury, remains unknown. The three main aims of this PhD project were therefore to: (1) determine the expression pattern of SIRT5 in human kidneys and assess whether renal SIRT5 levels are affected by ageing and/or acute/ chronic injury; (2) investigate the role of SIRT5 in hPTECs in vitro in the cellular response to ischaemia; and, (3) evaluate the function of SIRT5 in AKI and CKD in vivo in mice.

(1) SIRT5 localises to mitochondrial-rich tubular segments and is affected by acute and chronic injury. Prior to this work, the expression pattern of SIRT5 along the human nephron was not known. This is, therefore, the first study to show that in humans, renal SIRT5 expression peaks in mitochondria-rich nephron segments including DTs, TALs and PTs (Figure 3.3). Intriguingly, IHC staining revealed that SIRT5 also localised to nuclei in renal tubules (Figure 3.5) suggesting that in human kidneys, SIRT5 function may not be restricted to mitochondria/cytosol, but may also modulate nuclear proteins. Of note, KSucc of nucleosomes has been shown to regulate gene expression (374, 561) hinting that SIRT5 may act as an epigenetic modulator. Although SIRT5 has not been shown experimentally to target nucleosome KSucc, the nuclear localisation of SIRT5 is an important finding to keep in mind as the manipulation of SIRT5 activity in humans may affect cellular processes beyond mitochondrial metabolism (561). Notably, a similar role has been suggested for SIRT7, which like SIRT5, is a K de-
succinylase, however, primarily localises to the nuclei.(561) To better understand the function of SIRT5 in the human kidney, it would be of interest to perform studies to investigate the effects of SIRT5 ablation/inhibition on gene expression (e.g. by RNAseq), which would provide insights into the wider effects of manipulation of this gene/protein.

Given that ageing and the associated reduction in mitochondrial function are major risk factors for AKI and CKD development (18, 19, 25, 78, 363), the impact of ageing on mitochondrial mass and SIRT5 levels was assessed. IHC analysis of healthy human kidneys (0-78 years) revealed that mitochondrial mass (measured by TOM20 expression) significantly declined with age (Figure 3.8), however, SIRT5 levels were not affected (Figure 3.9). At first, this finding was rather surprising as a decline of both mitochondrial mass and SIRT5 might have been expected, especially as an age-related reduction in the kidney has been demonstrated for other members of the sirtuin family including SIRT1.(78, 562, 563) It is worth noting that total SIRT5 protein levels per se may not be the decisive element but rather overall SIRT5 activity; and this may decrease with age. An age-related reduction in activity is particularly likely as SIRT5 has a high $K_M$ value for NAD$^+$ (980μM) and is thought to be highly affected by fluctuations in NAD$^+$ (291), which, in addition, has been shown to decline with age in mice (78, 562) and in man (564). Notably, age-related NAD$^+$ depletion was found to impair SIRT1 activity ($K_M$ [NAD$^+$]: 95μM (291)), and caused mitochondrial dysfunction in rats (562). Moreover, the reduction in NAD$^+$ with age has been linked to increased susceptibility to kidney disease (78). In keeping with this, replenishing NAD$^+$ by NMN supplementation, restored sirtuin activity and improved mitochondrial function and reduced AKI in aged rats.(78) Although Guan et al. only investigated the effect of boosting NAD$^+$ levels on SIRT1 activity (78), it is highly likely that NMN treatment activated multiple SIRTs including SIRT5 and 3 (236) and thereby, alleviated AKI. Notably, Ebrahimkhani et al. showed that AKI causes a significant decrease in renal NAD$^+$ levels in mice.(565) Given that SIRT activities depend on NAD$^+$ availability, it is highly likely that such AKI-induced NAD$^+$ decline substantially impairs the functionality of various SIRTs including SIRT5 and thus, drives injury. Recently, the Auwerx group reported that NAD$^+$ can be boosted in human proximal tubular cells (human kidney clone 2 (HK-2) cells) in vitro and in mice in vivo using a specific ACMSD inhibitor (TES-1025).(79) ACMSD is an enzyme, which is primarily expressed in the kidneys and the liver where it degrades an important intermediate of the de novo NAD$^+$ biosynthesis pathway and by doing so impairs NAD$^+$ generation (Figure 1.6).(79) Conversely, ACMSD inhibition enhanced de novo NAD$^+$ synthesis, increased NAD$^+$ levels and alleviated ischaemic as well as nephrotoxic AKI in mice.(79) In a recent ground-breaking study Samir Parikh’s group showed that boosting NAD$^+$ levels (by NAM supplementation) reduced the incidence of AKI in patients undergoing
cardiac surgery. These findings in rodents and humans suggest that increasing NAD⁺ levels may be a novel treatment strategy to prevent and potentially treat AKI. However, to fully understand the effects of boosting NAD⁺ in kidney disease, further in vivo experiments are required, especially in the setting of chronic injury. Interestingly, NAM supplementation in patients with ESRD (8 weeks), was shown to be safe and reduced alkaline phosphatase levels, a marker of tubular injury (566), suggesting that CKD patients may also benefit from enhancing NAD⁺ levels. An additional NAD⁺ boosting agent, which has been reviewed and regarded safe by the FDA, is NR (Niagen™), a vitamin B3 derivative. NR is turned over by NRK1 and NRK2, which primarily localise to the kidney and muscle, respectively, therefore may strongly increase NAD⁺ levels in both tissues. (249) No adverse effects were apparent in subjects given NR for 8 weeks compared to the placebo control. (567) However in CKD patients, 8 weeks would be considered short-term, as the disease takes years to evolve. Thus, further investigations (e.g. administration >1 year) need to be carried out to show that it is safe for long-term use. Given that NR is likely to have a similar safety profile to NAM (566), an initial experiment could be to treat patients with e.g. CKD stage 2-3, for a period of up to 8 weeks, assess whether it increases NAD⁺ levels and determine its effects on renal function. Based on the incidence of adverse effects as well as impact on renal function, treatment could be continued for a longer time period.

Analysis of archived murine kidneys from an ischaemic AKI study (Prof. Alan Salama; unpublished) and a nephrotoxic CKD study (FAN; Prof. Jill Norman (365)) revealed that acute and chronic injury had a differential effect on SIRT5 expression. On one hand, bilateral IRI (30min) increased SIRT5 levels indicating that SIRT5 may be a stress-inducible enzyme in the setting of acute injury. While, on the other hand, FA treatment reduced SIRT5 levels suggesting that chronic injury negatively affected SIRT5 expression. Given that SIRT5 is regarded a major regulator of mitochondrial function (296, 326, 335, 337), the data hinted that enhanced expression in acute IRI may be aimed at promoting mitochondrial metabolism while in a chronic setting (FAN) the reduction in SIRT5 expression may aggravate mitochondrial dysfunction.

(2) SIRT5 depletion impairs mitochondrial function in vitro. The findings in murine IRI and FAN tissues gave rise to the second major goal of this study, the question as to whether the data in mice can be translated to hPTECs and if that was the case, determine the function of SIRT5 in hPTECs. Using the OND model, an in vitro model developed to more closely mimic the ischaemic environment in vivo (nutrient starvation and oxygen deprivation), it was shown that the findings in murine PTs also applied to hPTECs (Figure 4.7). Although the details of the transcriptional network regulating SIRT5
expression in OND were not determined the data indicated that it was driven by hypoxia (HIF1-independent; Figure 4.7 and 4.8) and to a lesser extent by nutrient starvation (most likely by mTOR inhibition; Figure 4.9). To date, there is only very limited knowledge on how SIRT5 is regulated, however, Zhang et al. showed that intermittent hypoxia increased SIRT5 levels in rat hearts (332). As this treatment regime is known to be cardioprotective (332) and SIRT5 is a central enzyme involved in the regulation of mitochondrial energy metabolism (296, 313, 326, 335), it was hypothesised that the increase in SIRT5 expression may be aimed at enhancing mitochondrial function. In support of this hypothesis, increasing SIRT5 expression in hepatocytes has been shown to boost ATP levels.(293) Given a number of in vivo studies have shown that SIRT5 alleviated injury induced by cardiac (330, 337) and cerebral (395) ischaemia, the data suggested that increasing SIRT5 expression may be a protective mechanism in IRI.

The SIRT5 knockdown studies in hPTECs showed that SIRT5 depletion impaired cellular energy metabolism by means of reduced cellular ATP production (glycolysis and OXPHOS), reduced steady-state ATP levels (glycolysis and OXPHOS) as well as a decline in the ΔΨM (Figure 4.12). The findings in hPTECs are consistent with studies in murine livers (336), hearts (330), cardiac mitochondria (464) and HEK293T cells (568), which indicated that loss of SIRT5 impaired ATP production and reduced the ΔΨM. Given the significance of mitochondrial function to tubular cell viability (356), the data suggested that SIRT5 depletion may increase PTEC susceptibility to injury. Notably, the ΔΨM is a key determinant of mitochondrial structure and depletion has been shown to cause mitochondrial fragmentation (42, 46) inter alia by promoting proteolysis of the pro-fusion factor I-OPA1 (161). The present study showed that SIRT5 depletion causes significant mitochondrial fragmentation in hPTECs (Figure 4.13). Furthermore, in addition to decreasing the levels of the pro-fusion proteins MFN1/2 and increasing the levels of the pro-fission marker DRP1-S616, which perturbed mitochondrial fission/fusion dynamics to cause fragmentation, SIRT5 depletion also decreased I-OPA1 levels (Figure 4.14). Given that the activities of the I-OPA1-degrading enzymes OMA1 and YME1L, are dependent on the ΔΨM and cellular ATP levels (158, 164), respectively (both of which were reduced by SIRT5 knockdown; Figure 4.12), it is tempting to speculate that mitochondrial fission in SIRT5-depleted hPTECs was driven, or at least enhanced, by disrupting the OMA1/YME1L equilibrium (by increasing OMA1 activity and decreasing YME1L activity). To prove this hypothesis and link SIRT5 depletion to increased OMA1 activity, a rescue experiment using OMA1 RNAi could be carried out. Of note, a very recent study in HK-2 cells investigated the effect of SIRT5 depletion on mitochondrial structure and showed that SIRT5 knockdown reduced mitochondrial length (assessed by confocal microscopy) as well as increased DRP1-S616 levels (521), which is...
consistent with our findings. However, these investigators did not test whether SIRT5 depletion also affected pro-fusion proteins (e.g. MFN1/2 and I-OPA1) or the ΔΨM which would have allowed direct comparison with our data.\footnote{521} The group also reported that SIRT5 depletion enhanced cisplatin-induced mitochondrial dysfunction (20µM, 24h) in HK-2 cells while overexpression was protective.\footnote{521} Notably, we found that SIRT5 overexpression increased mitochondrial dehydrogenase activity (an indication of enhanced mitochondrial function and viability) in hPTECs exposed to cisplatin (20µM, 24h; Appendix Figure 1). Our finding, together with the study by Li et al. \cite{521} suggested that the OND- and IRI-induced increase in SIRT5 expression may be aimed at boosting mitochondrial bioenergetic output. In keeping with this idea, metabolic characterisation (by respirometry) of hPTECs showed that \textit{SIRT5} knockdown decreased mitochondrial bioenergetic output (Figure 4.17) post-OND proposing that SIRT5 depletion may exacerbate IRI. This hypothesis was also supported by the observation that \textit{SIRT5} knockdown enhanced OND-induced mitochondrial swelling (Figure 4.16), a central pathological feature of mitochondrial dysfunction in IRI \cite{194}. Up until now, \textit{SIRT5} knockdown had not been linked to enhanced mitochondrial swelling (in ischaemia). Interestingly, ablation of SIRT3, which targets similar metabolic pathways \cite{5}, has been reported to cause mitochondrial swelling in murine hearts \cite{569}. Further studies will be required to dissect the underlying mechanisms causing mitochondrial swelling in SIRT5-depleted hPTECs (for detailed discussion see Chapter 4, Section 4.5). Notably, SIRT5 has been reported to protect mitochondria from degradation in MEFs, MDA-MB-231 (human breast cancer cells) and C2C12 cells (mouse myoblasts)\cite{300,338}. In line with these reports, the present study showed that \textit{SIRT5} knockdown in hPTECs increased mitophagy rates (Figure 4.19) and reduced mitochondrial mass (Figure 4.20). Enhanced mitophagy in AKI can be protective by clearing damaged mitochondria\cite{55,422}. However, mitochondrial degradation has to be balanced with biogenesis as an imbalance leads to a reduction in mitochondrial mass and thereby, aggravates bioenergetic perturbation \cite{64,481}. Given that mitochondrial mass declined in SIRT5-depleted hPTECs, the data suggested that increased mitophagy may exacerbate metabolic dysfunction post-OND and thus, exacerbate ischaemic injury.

Taken together, the data up to this point indicated that SIRT5 depletion exacerbates four major aspects of mitochondrial dysfunction reported in AKI as well as CKD \cite{4,356,481,508}: (i) decreased bioenergetic output (pre- and post-OND), (ii) excessive fragmentation, (iii) mitochondrial swelling and (iv) decline in mitochondrial mass possibly due to increased mitophagy. Based on the presumption that mitochondrial dysfunction is a major driver of acute and chronic renal disease, the data (mitochondrial
structure/function analyses) suggested that SIRT5 depletion/ablation may aggravate tubular injury in vivo.

(3) Loss of SIRT5 protects from ischaemic AKI and enhances nephrotoxic CKD. As discussed previously, mitochondrial dysfunction, characterised by (i) structural and (ii) functional changes of the organelle, is regarded a major pathophysiologic feature of AKI and CKD. (3, 4)

(i) To test whether Sirt5 deletion caused ultrastructural changes in mitochondria similar to what was shown in SIRT5-depleted hPTECs and dissect the underlying mechanism, WT and Sirt5−/− kidneys were analysed by TEM and WB. Of note, the WB data discussed in the subsequent section is derived from vehicle control kidneys (FAN study) rather than untreated mice (due to the lack of WT and Sirt5−/− mice) as both were regarded as equivalent. Qualitative TEM analysis showed that SIRT5 ablation in vivo caused mild mitochondrial fragmentation in PTs while mitochondrial structure in DTs was unaffected (Figure 5.2 and 5.3) suggesting that compared to our observations in SIRT5-depleted hPTECs (Figure 4.13), Sirt5 deletion in kidneys had only a minor impact on mitochondrial structure. Furthermore, in contrast to the in vitro findings in hPTECs, complete ablation of SIRT5 did not reduce MFN1/2 or total OPA1 levels (Figure 5.19) in murine kidneys suggesting potential cell (hPTECs versus whole kidney lysate) or species differences (human versus mouse). Notably, to fully define the consequences of Sirt5−/− on proteins involved in mitochondrial dynamics, it would be of value to screen murine kidneys for the pro-fission marks DRP1-S616 and DRP1 as well as the receptors FIS1, MiF49, MiD51 and MFF. Furthermore, to overcome the potential limitations of 2D imaging of mitochondrial networks by TEM, it would be of interest to also analyse the 3D mitochondrial network in PTs in WT and Sirt5−/− kidneys by e.g. super-resolution confocal microscopy (tissue section stained for TOM20 by IF).

(ii) As an indication as to whether Sirt5 deletion affected mitochondrial function, complex II activity was measured in crude mitochondrial extracts (Figure 5.10). The data showed that renal Sirt5−/− mitochondria exhibited reduced complex II enzyme activity, a finding which is in line with a recent study (371). Interestingly, Chiba et al. also reported that Sirt5−/− kidneys showed reduced mitochondrial FAO (371), which is central to PTEC ATP metabolism (33). To complement these findings and get a more complete picture of the enzymes regulated by SIRT5 in PTECs, it would be of interest to isolate PTECs from Sirt5−/− kidneys and perform metabolic characterisation by e.g. measuring ETC/TCA enzyme activities or perform a Mito Stress Test (Seahorse Analyzer).

The data generated thus far suggested that SIRT5 ablation reduced mitochondrial function in both mice (complex II activity) and man (ATP production rate/
steady-state levels and OXPHOS activity) suggesting this may exacerbate both (i) ischaemic AKI and (ii) FA-induced CKD in vivo.

(i) Interestingly and in contrast to our predictions, Sirt5−/− mice were protected from ischaemic injury rather than showing more susceptibility. Although the animal numbers (n=3-5) were low (due to breeding issues described in Chapter 5, Section 5.4.1), the data clearly showed renoprotection by Sirt5 deletion (i.e. decreased tubulointerstitial injury, reduced mRNA levels of Kim1 and Ngal, and increased renal function; Figure 5.6-8). This finding was in line with a recent study by Chiba et al., which reported that loss of SIRT5 protected mice from IRI and proposed that the mechanism of renoprotection is a metabolic switch from mitochondrial to peroxisomal FAO.(371) However, as discussed in Chapter 5 (Section 5.5), it is unlikely that this metabolic switch alone can alleviate PT injury in Sirt5−/− IRI kidneys, as not all PT segments contain peroxisomes (7, 527-529). Therefore, it was hypothesised that in addition to the peroxisomal switch, protection may have been conveyed by a mild impairment of mitochondrial function. The protective effect of mitochondrial impairment has been suggested by a variety of studies, which have shown that inhibition of the ETC complexes I (530-532) and II (510) as well as complex V (533) alleviated ischaemic injury in the kidney, possibly by reducing ROS levels and averting ATP depletion. This, together with the observation that SIRT5 regulates the activities of these three complexes (complex I, II and V) in hepatic homogenates (336) as well as complex V in heart cardiac mitochondria (464) and complex II in renal mitochondria (Figure 5.10) suggested that reduced mitochondrial function may be an additional mechanism that alleviated IRI in Sirt5−/− kidneys. In the future, it would be key to measure complex I and V activities in WT and Sirt5−/− kidneys to assess whether the findings in Sirt5−/− livers (336) and cardiac mitochondria (464) translate to the kidneys. More importantly, given that two major downstream effectors driving IRI are oxidative stress and ATP depletion, it would be of value to determine ROS and ATP levels in CL and IRI kidneys from WT and Sirt5−/− mice. Of note, as discussed in Chapter 5 (Section 5.5), impairment of mitochondrial function is highly likely to be at the expense of the ΔΨM similar to what has been shown in SIRT5 RNAi-treated hPTECs (Figure 4.12). To confirm that this also applies to mice in vivo, it will be of interest to examine whether the ΔΨM is also reduced Sirt5−/− kidneys. This could be done by incubating WT and Sirt5−/− kidney slices with TMRM followed by measurement of fluorescence intensities using confocal microscopy. While the reduction in the ΔΨM may on one hand reduce ROS production, it is important to note that, on the other hand, it may exacerbate mitochondrial dysfunction (structure and function) in IRI. Although this may not be an issue in an acute setting, it may have repercussions in the long-term, which is why it will be central to also investigate later time-points (e.g. after 28d) in this model.
Sirt5<sup>−/−</sup> IRI kidneys showed increased mRNA expression of Sirt3 and 4 as well as Pgc1α and Tfam compared to WT IRI kidneys. While this might be the consequence of reduced injury, it cannot be excluded that germline knockout of Sirt5 may have provoked a compensatory response, especially, as Sirt5<sup>−/−</sup> CL kidneys displayed a trend to increased Sirt1, 3 and 4 as well as Pgc1α and Tfam mRNA levels, compared to WT CL kidneys (Figure 5.8 and 9). A putative compensatory response aimed at boosting mitochondrial metabolism would also be consistent with the recent findings of Chiba et al., who showed that SIRT5 ablation caused an increase in peroxisomal mass and suggested that such increase may be aimed at breaking down long chain fatty acids (by β-oxidation) and to fuel and enhance mitochondrial FAO, which is impaired in Sirt5<sup>−/−</sup> kidneys.(371) To test whether germline ablation of SIRT5 provoked a compensatory response, it would be of interest to repeat the study in mice with transient/inducible ablation of SIRT5 (i.e. by antisense oligonucleotides or inducible knockout) or the use of specific SIRT5 inhibitors (541, 542).

Of interest, bromodomain 4 (BRD4) inhibition with JQ1 (7d pre-treatment) has been shown to protect mice from IRI.(570) BRD4 is a member of the bromodomain and extraterminal (BET) proteins, which recognises and binds to acetylated histones to regulate gene expression.(571) Interestingly, BRD4 inhibition (by JQ1) resulted in downregulation of SIRT5 and 3 as well as an increase in SIRT1 mRNA levels in hPTECs (Appendix Figure 2). Both, SIRT3 and 5 boost mitochondrial enzyme activities (296, 301, 305, 326) while SIRT1 activates PGC1α to drive mitochondrial biogenesis (526). Given that the data indicated that limiting mitochondrial respiration during ischaemia combined with the fact that boosting mitochondrial biogenesis post-ischaemia (64) may be beneficial, reducing SIRT5 by BRD4 inhibition may be one promising and new pre-emptive treatment. While renoprotection by BRD4 inhibition is likely to be due to a pleiotropic effect (i.e. wider impact on gene expression) it would be of importance to further investigate its therapeutic potential in AKI. One aspect of particular interest would be to assess whether apart from pre-emptive treatment, BRD4 inhibition-post IRI is beneficial.

Taken together the findings thus far indicated that mild impairment of mitochondrial function (via SIRT5 ablation) may protect from ischaemic AKI. Yet, increasing SIRT activity and thereby, mitochondrial function with NAD<sup>+</sup> boosting agents (NAM and NMN) has equally been shown to alleviate ischaemic AKI in rats (78) and humans (80). Although this may appear contradictory, it should be emphasised that increasing NAD<sup>+</sup> is likely to exert a large number of different effects including the enhancement of overall tubular energy metabolism (boosting glycolysis and OXPHOS), attenuation of the damage response (increasing SIRT activity) and also promote
recovery (e.g. by increasing mitochondrial biogenesis via the SIRT1/PGC1α-axis) (78, 291, 572). Sirt5-deletion alone, however, may only reduce some aspects that drive ischaemic injury (e.g. ROS production and ATP shunt) with no impact on mitochondrial biogenesis. Although the present study indicated that SIRT5 ablation may be beneficial in ischaemic AKI, it will be, as discussed in the previous paragraph, important to also investigate later time-points to determine long-term repercussions. Given that Sirt5−/− kidneys show impaired mitochondrial function (371) it is possible that SIRT5 ablation exacerbates injury in the long-term. This would also be in line with our observations in the FAN model, which will be discussed in the next paragraph. Of note, SIRT5 is highly expressed in the heart where it is required for normal cardiac function (330, 337). Therefore, it is important to be aware that prolonged inhibition of SIRT5 has the potential to cause unwanted side-effects, although, acute inhibition of SIRT5 activity may not be detrimental. To establish whether targeting SIRT5 is a safe treatment option in the short- and/or long-term, further in vivo studies will have to be conducted with a major focus in cardiac effects (e.g. by measurement of cardiac output by echocardiography or by measuring ventricular wall thickness (330)).

(ii) Intriguingly, in line with our predictions and in contrast to the findings in IRI, SIRT5 ablation enhanced injury in FAN, a murine model of CKD. However, it should be emphasised that the response to FA was heterogenous resulting in highly variable injury in both WT and Sirt5−/− kidneys. Overall tubular damage 14d-post injection was very mild (Figure 5.12-18) and did not significantly reduce renal function in WT or Sirt5−/− mice (Figure 5.22). Nonetheless, despite the mild injury, the tubular injury score, Ngal and Kim1 mRNA levels indicated that SIRT5 ablation enhanced injury. With respect to mitochondrial dysfunction, mitochondrial architecture was analysed by WB for protein expression of the pro-fusion factors MFN1/2 and OPA1. While there were no changes in MFN1 or MFN2 levels, Sirt5−/− FA kidneys showed a trend to lower l-OPA1 levels compared to WT FA kidneys which may promote mitochondrial fragmentation (Figure 5.19). This finding would fit with the SIRT5 RNAi hPTEC data (Figure 4.14). Although the role of l-OPA1 in FAN has not been analysed, Xiao et al. showed that preserving l-OPA1 (by OMA1 ablation) protects from IRI in mice.(398) To determine whether Sirt5−/− FAN kidneys show enhanced l-OPA1 proteolysis (e.g. by OMA1) and examine the consequences of this change, it would be of interest to screen kidneys for OMA1 (by WB) and more importantly, determine mitochondrial structure (by super-resolution confocal microscopy (e.g. TOM20 staining by IF) and/or TEM).

Defective FAO is a major pathophysiologic factor driving FAN in mice as well as CKD in humans.(63) Considering that Sirt5−/− mice showed impaired mitochondrial FAO (371) together with the fact that this metabolic high-energy pathway is required for normal
PTEC function (33, 34, 573), it appears likely that SIRT5 ablation exacerbates chronic renal injury. The effect of SIRT5 ablation on mitochondrial function in this model was not analysed due to time limitations and therefore, would be a central focus for follow-on studies. In addition, as a single FA injection (240µg/g BW) only caused mild injury it will be key to examine the effect of increased severity (by increasing the dose and/or number of injections) and duration (e.g. 28d) of the model. Only by inducing more severe injury it will be possible to fully evaluate the effect of SIRT5 ablation in this experimental model of CKD.

Mitochondrial dysfunction is a central driver of CKD. Therefore, in the interest of finding new treatments for CKD it will be key to show that enhancing mitochondrial function reduces renal damage rather than showing that reducing mitochondrial function (by SIRT5 ablation) aggravates the disease. Although there is a strong interest in finding new therapeutics that alleviate mitochondrial dysfunction in kidney disease, the majority of studies seem to focus on AKI rather than CKD. To date, there are no curative treatments for CKD, which emphasises the unmet need to explore new therapeutic avenues including boosting mitochondrial metabolism in experimental CKD. Most of the mitochondria-targeting CKD therapies under investigation are aimed at either reducing ROS levels (399, 574) or enhancing mitochondrial biogenesis (503). One additional route, boosting NAD$^+$ levels, which has been shown to prevent AKI in humans (80), has not been widely investigated in CKD. As mentioned earlier, one study reported an improvement in renal function in patients with ESRD (566) indicating that this may be a promising new strategy. However, in this study only a small patient cohort (30 patients) (566) was analysed, which makes it difficult to draw meaningful deductions. To assess whether there is a therapeutic benefit in boosting NAD$^+$ levels in CKD patients, further detailed studies are imperative.

Taken together, these in vivo studies revealed that loss of SIRT5 on one hand alleviated ischaemic AKI and on the other enhanced nephrotoxic CKD. One underlying mechanism which may convey resistance to AKI as well as increase susceptibility to CKD might be a reduction in mitochondrial function. Further investigations will be required to confirm the present findings and to establish whether targeting SIRT5 may be a novel therapeutic approach in AKI and/or CKD.

6.2 Main conclusions and outcomes

The overall aim of this PhD project was to dissect the role of the NAD$^+$-dependent K-desuccinylase/-demalonylase SIRT5 in the kidney and assess whether it is a putative
target to treat mitochondrial dysfunction in AKI and CKD. The four major findings of this study were:

(1) SIRT5 is expressed in human kidneys, peaks in nephron segments with increased mitochondrial mass and expression is not affected by renal ageing. In addition, it was shown that SIRT5 expression is differentially regulated by acute and chronic injury in mice indicated by increased and decreased SIRT5 protein levels, respectively. It will however be important to examine SIRT5 activity rather than protein levels to determine whether the effect of age and/or injury on enzyme activity.

(2) SIRT5 knockdown (by RNAi) significantly impaired mitochondrial and glycolytic bioenergetic output, caused mitochondrial fragmentation and stimulated mitophagy in hPTECs in vitro. Moreover, SIRT5 depletion exacerbated mitochondrial dysfunction in OND as it decreased mitochondrial functional output, potentiated mitochondrial mass decline and induced swelling. Based on the premise that mitochondrial dysfunction is regarded a major pathophysiologic feature of AKI and CKD, these data indicated that SIRT5 ablation may increase injury induced by renal ischaemia (model of AKI) and FA injection (model of CKD).

(3) In contrast to our predictions, loss of SIRT5 protected mice from ischaemic AKI in vivo suggesting that slight impairment of mitochondrial function and possibly increased mitophagy alleviates acute ischaemic injury.

(4) Sirt5−/− mice showed enhanced renal damage in nephrotoxic CKD. Although FA caused only mild renal injury in both WT and Sirt5-depleted mice, Sirt5−/− kidneys showed enhanced tubular damage, immune infiltration and fibrosis suggesting that SIRT5 alleviates FAN.

In summary, this project provided evidence that SIRT5 is involved in the regulation of mitochondrial form and function in hPTECs in vitro as well as murine kidneys in vivo, however, with potential differences in the underlying mechanisms. In addition, while SIRT5 ablation per se did not impair renal function in healthy mice it had a major impact on AKI and to a lesser extent, on CKD. On one hand, loss of SIRT5 protected from ischaemic AKI suggesting that minor impairment of mitochondrial function may be beneficial in protecting PTECs from IRI. On the other hand, SIRT5 ablation enhanced nephrotoxic CKD hinting that increased mitochondrial dysfunction may exacerbate tubular injury. These findings suggest that SIRT5-depletion has differential effects on disease evolution, depending on the nature and duration of the insult and emphasise the importance of further studies to fully understand the role of SIRT5 in AKI and CKD.
6.3 Limitations of the study

Immortalised hPTECs as an in vitro system. One limitation of the present study is the use of an immortalised hPTEC cell line (HKC-8) to investigate the consequences of SIRT5 depletion on cellular metabolism. Although our data indicated that these cells primarily depend on OXPHOS for ATP generation (60/40 = OXPHOS/glycolysis; Figure 4.12) it is important to note that PTECs in vivo inherently have a very low glycolytic capacity and almost exclusively rely on OXPHOS for ATP generation. PTECs in culture, however, will utilise aerobic glycolysis over OXPHOS (Warburg effect) to maintain their proliferative phenotype and even inhibit OXPHOS when exposed to glucose (Crabtree effect). It is possible to circumvent the Crabtree effect by replacing glucose with galactose and thereby, stimulating a more OXPHOS-dependent metabolic phenotype. However, given that we were initially interested in whether SIRT5 can increase (anaerobic) glycolytic capacity in hPTECs, removing glucose would have interfered with our model, and this was not investigated further.

Rather than changing the metabolic phenotype of immortalised PTECs, attempts were made to grow primary hPTECs (from archived cryogenic stocks provided by the PKD Charity-sponsored biobank) to investigate whether findings in immortalised cells can be translated to primary hPTECs. However, these cells showed very poor viability, and it was not possible to generate sufficient cells for experimentation. A protocol has been developed for isolation of hPTECs from human kidneys in which a single cell suspension is generated by combining physical separation (via GentleMACS™ dissociator, Miltenyi Biotec) with enzymatic digestion (Liberase™ DL, Roche). hPTECs can then be isolated by MACS (Miltenyi Biotec) using CD10/13 as markers. Lack of access to non-transplantable donor kidneys in the given time frame precluded the use of this protocol and growth of primary hPTECs.

Use of the OND model as the only model of renal ischaemia. In addition to the OND model, it would also be of interest to assess whether similar findings (e.g. SIRT5 expression, autophagy/mitophagy induction) is also be seen in a combined oxygen and glucose deprivation (OGD) model, which is another model used to mimic ischaemia in vitro. This model uses glucose-deficient medium which contains key nutrients (e.g. amino acids; glucose-free DMEM) and therefore, would allow the investigation of the metabolic effects during oxygen deprivation such as accumulation of TCA cycle intermediates, which may not be possible in the same way under nutrient-deprived conditions (i.e. HBSS).
Initially, our interest was to investigate the repercussions of SIRT5 depletion during ischaemia (i.e. OND). However, given that the data suggested that SIRT5 is involved in reperfusion injury in vivo, it would be of value to also analyse hPTECs after e.g. 6h OND or OGD followed by 24h reperfusion.

**SIRT5 knockdown by RNAi.** Another limitation of this study is the use of RNAi for gene depletion experiments due to (i) potential off-target effects and (ii) residual SIRT5 expression:

(i) To minimise off-target effects, an ON-TARGETplus SMARTpool was used which contains four siRNAs allowing the use of lower concentrations of the individual siRNAs. However, effects of SIRT5 RNAi on the expression of other genes cannot be excluded, although the experiment was internally controlled using control RNAi.

(ii) SIRT5 RNAi-mediated knockdown reduced SIRT5 protein levels by >85%. To eliminate putative effects mediated by residual SIRT5 it would be of interest to remove SIRT5 completely. Deletion of **SIRT5** (by CRISPR/Cas9) was attempted using two approaches: (1) Introduction of a single point mutation; and, (2) excision of exon 5 (which codes for the active site). Although the latter approach worked very well in terms of gene deletion, it was not possible to isolate and expand individual SIRT5-/- clones. The problems encountered and potential solutions will be discussed in Section 6.4.1.

**Germline Sirt5-/- mice.** A further limitation of this study is the use of germline Sirt5-/- mice. Germline loss of a gene and lack of a phenotype may suggest compensatory mechanisms during development to circumvent the consequences of gene deletion e.g. through other SIRTs. Sirt5-/- kidneys showed a trend to increased expression of Tfam, Pgc1α, Sirt1, 3 and 4 mRNA (Figure 5.8 and 5.9). Although these data need to be confirmed at the protein level, this may hint at a mild compensatory response. To investigate whether this may have been the case and also to confirm the present findings, it would be of interest to repeat the IRI study in inducible, PT-specific Sirt5-/- mice. An additional approach would be to use antisense oligonucleotides (581), which accumulate in PTECs and therefore, may allow targeted transient ablation of SIRT5.

## 6.4 Future work

The data in Chapter 3 indicated that SIRT5 is highly expressed in human kidneys, however, expression is not affected by age. Given that SIRT5 enzymatic activity rather than total protein levels may be the crucial factor, it would be important to determine whether SIRT5 activity changes with age in human kidneys. Furthermore, the present in vitro data (Chapter 4) showed that in hPTECs SIRT5 knockdown by RNAi impaired
mitochondrial function at baseline which was exacerbated by OND indicating that SIRT5 depletion may be detrimental in AKI and possibly CKD, in vivo. Interestingly, our in vivo data (Chapter 5) revealed that germline deletion of Sirt5 protected murine kidneys from ischaemic AKI and enhanced nephrotoxic CKD suggesting a differential role for SIRT5 in the two models of kidney disease. Taken together, these findings suggest that reducing mitochondrial function (via Sirt5−/) may be beneficial in IRI but detrimental in CKD. To test these two hypotheses and determine whether SIRT5 is a putative therapeutic target in AKI/CKD the following points could be explored in vitro and in vivo:

6.4.1  Ex vivo investigations in human renal tissue

**Regulation of SIRT5 activity.** SIRT5 is an NAD⁺-dependent enzyme. As an indicator of the effect of ageing on SIRT5 activity, it would be of interest to measure NAD⁺ levels in human renal tissue. As a functional readout of SIRT5 activity WB/IHC screening for KSucc and KMal (Figure 5.1) or IP of KSucc and KMal followed by mass spectrometry could also be conducted.

6.4.2  In vitro investigations

**Regulation of SIRT5 gene expression.** Nutrient deprivation, hypoxia and OND increased SIRT5 expression in hPTECs. This, together with the ability of SIRT5 to enhance mitochondrial metabolism (335), glycolysis (288) and to protect mitochondrial from degradation (by autophagy) during nutrient starvation (338) suggested that the increase in SIRT5 levels may be a protective, stress-induced mechanism. Future experiments could explore the transcriptional circuits that regulate SIRT5 gene expression in response to (i) nutrient starvation; and (ii) oxygen deprivation:

(i) Interestingly, a preliminary screen using the Genomatrix software tool identified several binding sites of the autophagy repressor ZKSCAN3 (430), in the human SIRT5 promoter (Figure 4.10) raising the possibility that ZKSCAN3 may suppress SIRT5 expression under nutrient replete conditions and this repression is released in nutrient deficiency. One approach to assess whether SIRT5 expression is suppressed by ZKSCAN3 would be to carry out a ZKSCAN3 knockdown (by e.g. siRNA) followed by SIRT5 expression analysis.

(ii) Using the same software tool it emerged that SIRT5 expression during hypoxia may be regulated by SP1, a TF associated with stress-resistance to low-oxygen conditions in cancer cells (582). According to the software, the SIRT5 promoter contains several SP1 binding sites (data not shown), which were confirmed by chromatin
immunoprecipitation (ChIP) sequencing (ChIP-seq) analysis. In a similar way to ZKSCAN3, a siRNA-knockdown of SP1 could be conducted or mithramycin, a specific SP1 inhibitor (583), could be used to investigate the presumed SP1-SIRT5-axis.

**Analysis of primary human PTECs.** To address the potential issue of using a cell line for our in vitro investigations, it would be of interest to isolate primary PTECs from human kidneys (using MACS; CD10/13) and carry out a similar set of experiments as described in Chapter 4 (analysis of mitochondrial structure/function upon SIRT5 RNAi) to confirm our findings in HKC-8 cells. In addition, specific SIRT5 inhibitors (541, 542) could be used to determine the effects of pharmacologic inhibition of SIRT5 on mitochondrial form/function in primary hPTECs.

**SIRT5 deletion in hPTECs.** To eliminate the issue of residual SIRT5 protein levels and to determine the effects of complete SIRT5 ablation on cellular bioenergetics a complete knockout of SIRT5 (by e.g. CRISPR/Cas9) could be performed. This was attempted during this PhD project, however, problems were encountered with (i) the selection process of modified SIRT5−/− clones; and, (ii) clonal expansion of individual SIRT5−/− cells.

(i) CRISPR/Cas9 constructs were generated, which contained guide RNAs (gRNAs) and a puromycin resistance gene. Puromycin is highly toxic and most likely caused cell death in the already metabolically impaired SIRT5-deleted cells. A better, non-toxic approach would be to add a fluorescent reporter gene (e.g. GFP) into the CRISPR/Cas9/gRNA construct to allow, plasmid-containing (and most likely modified) cells to be isolated by FACS.

(ii) To successfully expand isolated SIRT5−/− clones, cells could be grown on a feeder layer (confluent layer of mitomycin A-treated PTECs) to improve cell growth.

In addition to generating SIRT5−/− hPTECs and to address possible species differences, it would be important to isolate murine PTECs from Sirt5−/− mice. Both murine and human SIRT5-deficient PTECs could be analysed with respect to mitochondrial form/function and cellular metabolism. Of note, to circumvent potential issues of germline knockout-induced compensatory responses in Sirt5−/− kidneys, murine PTECs could also be isolated from inducible, PT-specific Sirt5−/− kidneys by MACS or FACS (CD10+/CD13+). Mice could be generated by crossing loxP-flanked Sirt5 mice (generated by the Auwerx group in order to create the Sirt5−/− mice (329)) with inducible PT-specific Cre mice (e.g. tamoxifen inducible cre recombinase under a Sglt2 promoter (522)).
**Analysis of MERCs and mitochondrial Ca^{2+} levels.** TEM analysis of SIRT5 RNAi hPTECs showed that SIRT5 depletion caused mitochondrial fragmentation and enhanced OND-induced mitochondrial swelling (Figure 4.16), and suggested that this may be due to increased MERC formation. Two approaches could be used to determine whether SIRT5 depletion/deletion in hPTECs caused an increase in MERCs:

(i) For TEM analysis of MERCs, more TEM images of control and SIRT5 RNAi-treated cells (and possibly also at higher magnification) would have to be taken and MERCs quantified (439). Although this is a quantitative approach, it remains a “snapshot” and in addition, glutaraldehyde fixation may provoke changes in the organelles (artefacts).

(ii) A better, more advanced approach would be to analyse MERCs in living cells using confocal microscopy. This could be achieved by visualising mitochondrial-ER interactions using FRET-based (153) or dimerisation-dependent (477) assays.

One mechanism causing mitochondrial swelling is enhanced Ca^{2+} influx from the ER. (584) To test whether mitochondrial swelling is indeed Ca^{2+}-induced, mitochondrial Ca^{2+} levels could be quantified. Given that Ca^{2+} measurements with the calcium dye Rhod-2AM were unsuccessful (due to non-specific accumulation of the cationic dye; Figure 4.24), genetically-encoded calcium indicators (GECIs (480)) could be used (see Chapter 4, Section 4.5 for detailed discussion).

**Pharmacological interventions in experimental AKI.** The present study as well as the work of Chiba et al. indicated that SIRT5 ablation protected from IRI (371). While Chiba et al. proposed that the underlying mechanism is a switch from mitochondrial to peroxisomal FAO (353), our study indicated that impaired mitochondrial function may be an important factor contributing to the resistance to injury in ischaemic AKI. Both aspects (increased peroxisomal function and decreased mitochondrial function) are likely to add to the renoprotective effects of SIRT5 ablation. To address whether SIRT5 is a candidate therapeutic target in AKI, it would be key to determine the effects of pharmacologic interventions aimed at inhibiting SIRT5 activity and/or reducing SIRT5 levels on mitochondrial/peroxisomal function in hPTECs. While SIRT5 activity could be reduced by using specific SIRT5 inhibitors (541, 542), little is known about small molecules that can regulate SIRT5 expression. Interestingly, BRD4 inhibition (using JQ1; 500nM, 24h) reduced SIRT5 and increased SIRT1 mRNA levels in hPTECs in vitro (Appendix Figure 2) and JQ1 has been reported to ameliorate ischaemic AKI in vivo (570), although the target(s) in AKI are not known. Both decreased SIRT5 levels (371) and increased SIRT1 levels (585) have been associated with enhanced peroxisomal activity, which protected from AKI. To test whether enhancing peroxisomal function and
reducing mitochondrial respiration may be one mechanism by which BRD4 inhibition exerts renoprotection, peroxisomal/mitochondrial FAO could be determined in BRD4-treated hPTECs. This could be done by treating hPTECs with JQ1 together with either etomoxir (an inhibitor of mitochondrial FAO (586)) or thioridazine (an inhibitor of peroxisomal FAO (587)) followed by measurement of $^{14}$C-palmitate oxidation (588, 589). In addition, protein levels of peroxisomal enzymes involved in FAO such as Acyl-CoA oxidase 1 (ACOX1), could be determined by WB.

**Pharmacological interventions in experimental CKD.** As described in Chapter 5, loss of SIRT5 mildly enhanced FAN in vivo. Also SIRT5 overexpression increased the viability of cisplatin treated hPTECs (Appendix Figure 1) indicating that SIRT5 activation may be a putative therapeutic strategy in nephrotoxic CKD. Unfortunately, no specific SIRT5 activator has yet been identified. Multiple SIRTs, including SIRT1 and 3, have been shown to boost mitochondrial function (6), which suggest that renal tubular cells may benefit from a general boost of SIRT activity. Given that the SIRTs are NAD$^+$-dependent enzymes and NAD$^+$ is required for cellular bioenergetics, increasing NAD$^+$ levels may be a potential therapeutic approach. As mentioned before (Section 6.1), the Auwerx group identified a small molecule (TES-1025) which inhibits ACMSD, an enzyme which primarily localises to the kidneys and enhances NAD$^+$ biosynthesis.(79) Other compounds used to increase NAD$^+$ levels are NMN (78) and NR (590). Thus far, it is unknown whether boosting NAD$^+$ levels is beneficial in CKD. To test this hypothesis, TGFβ-stimulated hPTECs (an in vitro model of CKD (63)) could be treated with either NR (e.g. 500µM, 24h), NMN (e.g. 500µM, 24h) or TES-1025 (e.g. 100µM, 24h) followed by metabolic characterisation (e.g. Seahorse respirometry), screening for injury markers (e.g. KIM1, NGAL, IL-6, etc.) and viability/toxicity assays (e.g. CytoTox-Glow™ (Promega)).

6.4.3 **In vivo investigations**

**AKI.** Sirt5$^{-/-}$ kidneys were protected from ischaemic AKI (Chapter 5, (353)). To determine whether SIRT5 depletion/inhibition may be a novel therapeutic strategy in AKI, it would be key to reduce SIRT5 (i) levels and/or (ii) activity in AKI.

(i) SIRT5 levels could be reduced by using inducible, ideally, PT-specific Sirt5$^{-/-}$ mice or by treating WT mice with antisense oligonucleotides against Sirt5.

(ii) SIRT5 activity could be decreased by treating mice with specific SIRT5 inhibitors.(541, 542) Treatment of mice both prophylactically and therapeutically would give insight in possible treatment regimens and potential benefit to different patient
cohorts, e.g. patients at risk of developing IRI during cardiac surgery versus patients with pre-existing AKI.

To assess the effect of SIRT5 ablation/inhibition on mitochondrial function in IRI and in other forms of AKI (e.g. nephrotoxic AKI), it would be of interest to further characterise (i) mitochondrial form, (ii) function and potentially (iii) peroxisomal function in $\text{Sirt5}^{-/-}$, SIRT5-depleted (e.g. by antisense oligonucleotides) and/ or SIRT5 inhibitor-treated kidneys:

(i) Mitochondrial architecture could be determined by TEM or by super-resolution microscopy of IF-stained (e.g. TOM20) FFPE kidney sections.

(ii) Mitochondrial function (ETC and TCA cycle enzyme activities) could either be determined by Oxygraph-2k measurements (Oroboros) or by enzyme activity assays (e.g. complex I, F1/F0-ATPase, etc.). In addition, it would be of interest to determine ROS levels given that mitochondrial ROS is a major driver of tubular injury.(591)

(iii) Peroxisomal function could be measured by $^{14}\text{C}$-palmitate oxidation (588, 589) as mentioned in the in vitro paragraph above (Section 6.4).

**CKD.** FA treatment caused only mild injury in WT and $\text{Sirt5}^{-/-}$ mice. To fully evaluate the effect of SIRT5 ablation in CKD, it would be of importance to increase the severity of the injury by increasing the dose and/or extending the duration of the model e.g. to 28d. Parameters that could be changed are: FA dose (>240µg/g BW), concentration (>20mg/ml) or repeat injections. The approach which is currently being examined is to administer two doses of FA (D0: 240µg/g BW [conc. 20mg/ml]; D14: 120µg/g BW [conc. 20mg/ml]) and run the model for a total of 28d. In addition to the FA model (nephrotoxic CKD), the role of SIRT5 should be investigated in different models of CKD induced by e.g. STZ (diabetic nephropathy) or UUO (obstructive nephropathy) (592).

As shown in Chapter 4 and 5, SIRT5 depletion induced mitochondrial fragmentation and impaired mitochondrial function (Figure 4.12 and 4.13) in hPTECs, which is likely to be the reason for the aggravated damage seen in FAN in $\text{Sirt5}^{-/-}$ mice. However, to find novel treatments for CKD, it would be of importance to explore whether SIRT5 activation alleviates renal disease. There are currently no specific SIRT5 activators. Specific/exclusive activation of SIRT5 may, however, not be necessary as multiple SIRTs including SIRT1 (75, 393, 485, 585) and 3 (322, 323, 487) have been shown to exert renoprotection. As mentioned before, boosting cellular metabolism as well as SIRT activity by increasing NAD$^+$ levels, may be a new therapeutic strategy for CKD. Although some studies have indicated that increasing NAD$^+$ levels protects from AKI (78-80), to our knowledge, this has not been investigated in CKD. Therefore, it would
be of importance to test this hypothesis in multiple CKD models (e.g. FAN, UUO or STZ). The most direct approach would be to induce CKD experimentally and treat mice with TES-1025, a specific ACMSD inhibitor (15 mg/kg BW per day) (79), or NAD⁺ boosters such as NMN (500 mg/kg BW per day) (78) or NR (500 mg/kg BW per day) (593). Readouts would be assessment of: (i) renal function (e.g. proteinuria and serum creatinine), (ii) tubulointerstitial injury and fibrosis (MT and PSR staining), (iii) immune cell infiltration, (iii) mitochondrial architecture/function (TEM, super-resolution microscopy, ETC and TCA cycle enzyme activities (Oxygraph-2K)) and (iv) peroxisomal function (¹⁴C-palmitate oxidation).

**AKI-on-CKD.** Pre-existing CKD (e.g. due to diabetes, increased age or AKI) is a major risk factor for AKI. (362, 594, 595)

To determine the role of SIRT5 in AKI-on-CKD and mimic human disease more closely, it would be of interest to investigate the effects of SIRT5 ablation/inhibition on AKI in mice with mild CKD. One possible AKI-on-CKD model would be to treat mice (WT and Sirt5⁻⁻) with adenine for 28d to induce CKD (596) followed by surgical IRI.

Given that renal function declines with age, it would also be interesting to look at the response to AKI in older (e.g. 12-18 months) WT and Sirt5⁻⁻ mice. To address whether boosting SIRT activity and thereby, energy metabolism is beneficial in this model, WT mice could be treated with TES-1025 (15 mg/kg BW per day) (79), NMN (500 mg/kg BW per day) (78) or NR (500 mg/kg BW per day) (593) to increase renal NAD⁺ levels. Readouts would be the same as listed in the section above on CKD.

This PhD project generated some interesting findings on the role of SIRT5 in hPTECs *in vitro* and murine kidneys *in vivo*. In particular, the aspect of context-dependent repercussions of impaired mitochondrial function (beneficial in ischaemic AKI and detrimental in nephrotoxic CKD) may open up a number of avenues for future investigations with prospect of finding new treatments for kidney diseases.
Appendix
**Appendix Table 1: MiTF/TFE3 and ZKSCAN3 binding sites.**

<table>
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<tr>
<th>Genomatrix transcripts</th>
<th>Transcription factors</th>
<th>Position from promoter start*</th>
<th>Matrix similarity</th>
<th>Strand</th>
<th>ZKSCAN3 binding site</th>
<th>MiTF and TFE3 binding site</th>
<th>TFEB binding site (CLEAR element)</th>
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<td></td>
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*Promoter sequence: 1000 bp upstream of first transcription start site (TSS) and 100 bp downstream of last TSS. Predominant transcripts in red.
Appendix Figure 1: SIRT5 overexpression increased viability of cisplatin-treated hPTECs. (A) hPTECs were seeded in 6-well plates (6x10^5 cells/well) and treated with cisplatin (20µM) or vehicle control (saline) after 24h. Cells were harvested 24h post treatment and processed/analysed as described in Chapter 2, Section 2.3.2. Primers are listed in Chapter 4, Table 4.2. Bar graph showing mRNA levels of SIRT5 normalised to β2 macroglobulin (B2M). The data displayed are from one experiment (n=1) with n=3 replicates/group. No statistical test was carried out. (B,C) hPTECs were plated in two T25 flasks (1.5x10^6 cells/flask). After 24h, cells were transfected with a SIRT5-Flag overexpression plasmid (Addgene; Plasmid # 13816) or a pcDNA3.1 control plasmid (Thermo; Cat# V79020) (both: 5000ng plasmid/flask). Dharmafect Duo (Dharmacon; Cat# T-2010-01) was used as the transfection reagent. Medium was changed 24h-post transfection. Forty-eight hours post transfection, cells were trypsinised and replated: (B) Transfected cells were plated in 6-well plates (6x10^5 cells/well) and harvested after 24h. For the detailed protocol see Chapter 5, Section 5.3.8. WB showing protein levels of SIRT5 as well as SIRT5-Flag. Actin was used as a loading control. Antibodies are listed in Chapter 5, Table 5.4. The data displayed are from one experiment (n=1) with n=3 replicates/group (C) Transfected cells were plated in 96-well plates (1.8x10^4 cells/well) (n=5/transfection group). The following day, cells were treated with cisplatin (20µM) or vehicle control (saline). After 24h, cells were treated with WST-1 (Abcam; Cat# ab65475) according to the manufacturer’s instructions and incubated at 37°C for 2h. Absorbance was measured at 450nm (Biochrom EZ Read 400, Cambridge, UK). Dot plot showing mitochondrial dehydrogenase activity (indicator of viability) of transfected hPTECs treated with cisplatin or vehicle control. The data displayed are from one experiment (n=1) with n=5 replicates/group To determine statistical significance a two-way ANOVA was carried out followed by Sidak’s post hoc test to normalise for multiple comparisons. Data are mean ± SD. **p<0.01 and ****p<0.0001.
Appendix Figure 2: BRD4 inhibition by JQ1 affects SIRT1, 3 and 5 mRNA levels. hPTECs were seeded in 6-well plates (6x10^5 cells/ well), treated with either the BRD4 inhibitor JQ1 (500nM) or vehicle control (DMSO, 0.005% v/v) and processed/ analysed after 24h (Chapter 2, Section 2.3.2). Primers are listed in Chapter 4, Table 4.2. Bar graph showing mRNA levels of SIRT1, 3 and 5. Data were normalised to hypoxanthine phosphoribosyltransferase 1 (HPRT1). The data displayed are from one experiment (n=1) with n=3 replicates/group. No statistical test was carried out.
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