Characterisation of Pathophysiological function of NEDD4-2 in Kidney

A thesis submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

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ABSTRACT

Nedd4-2 (NEDD4L, neural precursor cell expressed, developmentally down regulated 4-like) belongs to the Nedd4 family of ubiquitin ligases. These ligases aid in maintaining cellular homeostasis by binding to, and ubiquitinating a number of membrane proteins to initiate their internalization and turnover. Previous work from our laboratory has suggested that Nedd4-2 plays an essential role in regulating ion channels, especially the epithelial sodium channel and voltage gated sodium channels. The misregulation of these channels has been implicated in multiple channelopathies, including hypertension and cystic fibrosis like disease. This study characterises a previously unknown function of Nedd4-2 in the kidney.

In order to understand this significance of Nedd4-2 in renal homeostasis, the previously generated Nedd4-2−/− (Nedd4-2 knockout) mice (Boase et al., 2011) were characterised. The initial histological examination of postnatal kidneys suggested renal cyst formation in Nedd4-2−/− animals. Further analysis revealed that Nedd4-2 loss results in renal dysplasia. Nedd4-2−/− mice showed variable renal cystic index, onset of cyst formation starting from postnatal day 2 and progressing until the Nedd4-2−/− animals die due to respiratory distress around day 19-21. To investigate the prevalence of the cystic phenotype in other tissues histological analysis was performed in pancreas, liver, spleen, colon, stomach and thymus with no significant pathological differences observed in the knockout mice.

The Nedd4-2−/− kidneys showed increased cell proliferation, with no apoptotic differences in the cells lining the cystic epithelia suggesting an imbalance between cell proliferation and apoptosis in cyst formation. The cyst formation and kidney development disorders are associated with malformation in the kidney tissue leading to extracellular matrix modification with enhanced accumulation of collagens causing increased interstitial fibrosis. The Nedd4-2−/−
kidneys showed increased interstitial fibrosis, collagen-1 accumulation and expression during progression of the disease. The renal tissue membrane is made up of polysaccharides, glycogen and mucin, the *Nedd4-2*+/− kidneys were found to have decreased accumulation of polysaccharides. The cysts in the *Nedd4-2*+/− kidneys originated from different parts within the nephron. The larger cysts originated from loop of Henle and with the smaller cysts from collecting ducts and distal convoluted tubules. The cystic progression is dependent on cAMP flux initiated by fluid secretion within the cyst. The postnatal day 19 cystic kidneys in *Nedd4-2*+/− animals showed increased cAMP levels suggesting cystic disease progression. As renal cystic disorders may arise from abnormal cilia, ciliary anomalies were found in the *Nedd4-2*+/− around the cysts suggesting importance of cilia in kidney cyst formation.

Polycystins are known to be involved in renal cyst development with polycystin-1 and polycystin-2 together known to form calcium ion channel. To investigate the role of Nedd4-2 in the regulation of these polycystins, *in vitro* and *in vivo* studies were conducted. *In vitro* studies suggested that depletion of Nedd4-2 results in increased expression of polycystin-1 on the cell membrane with a decrease in polycystin-2 levels. Further, polycystin-1 was found to be ubiquitinated by Nedd4-2 *in vitro* providing the first evidence of Nedd4-2-mediated regulation of polycystins. *In vivo* Polycystin-1 was up-regulated in the *Nedd4-2*+/− kidneys suggesting an important role of Nedd4-2 in regulation of polycystins in cyst formation.

To analyse the transcriptional signature of the phenotype seen in the knockout kidneys, postnatal day 19 kidneys from wild-type and *Nedd4-2*+/− mice were subjected to RNA sequencing highlighting 537 genes that were differentially expressed between wild-type and knockout kidneys, with 167 genes down-regulated and 370 genes significantly up-regulated in the absence of Nedd4-2. DAVID and Ingenuity pathway analyses was used to highlight the
importance of genes involved in extracellular matrix modification, cell junction formation and cell-cell communication. The work presented in this thesis thus provides new information on the pathophysiological role of Nedd4-2 in kidney and identifies polycystin-1 as a Nedd4-2 target, along with transcriptional changes which may partially explain the cystic phenotype associated with renal dysplasia.
**Declaration**

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the university of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Pranay Goel

April 2016
Publication, Awards and Conference Attendance: By Year

2012:

IPRS (International postgraduate student research scholarship 2012) from University of Adelaide / Adelaide Postgraduate Award for living allowance.

Poster presentation at Adelaide protein group meeting (2012).

Poster presentation in 2nd Adelaide ANZSCDB Cell and Developmental Biology meeting (Nov 23, 2012).

2013:

Poster presentation at Florey International Postgraduate Research Conference by University of Adelaide (September 2013).

Best student poster in 3rd Adelaide ANZSCDB Cell and Developmental Biology meeting on (November 19, 2013).

2014:

Poster presentation at the Lorne protein conference, 39th Lorne Conference on Protein Structure & Function (February 2014).

3MT thesis competition University of Adelaide (October 2014).

Howard Florey Adelaide student post graduate student conference, Adelaide (October 2014).

EMBL student symposium October 2014 abstract selected for poster presentation.


SPMSSF funding for International Lorne protein conference, Melbourne from SA pathology (1000$).

EMBL AUSTRALIA student travel grant for EMBL student symposium Heidelberg (3000$)

Publications: During PhD Candidature

1. GENE: Invited Review

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCN1</td>
<td>Hyperpolarization activated cyclic nucleotide gated</td>
</tr>
<tr>
<td>P19</td>
<td>Post natal day 19</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>ADAM23</td>
<td>ADAM metallopeptidase domain 23</td>
</tr>
<tr>
<td>ADPKD</td>
<td>Autosomal dominant polycystic kidney disease</td>
</tr>
<tr>
<td>AFF3</td>
<td>AF4/FMR2 family member 3</td>
</tr>
<tr>
<td>AGTR2</td>
<td>Angiotensin receptor 2</td>
</tr>
<tr>
<td>Akt</td>
<td>PKB- protein kinase B</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase-promoting complex</td>
</tr>
<tr>
<td>AQP2</td>
<td>Aquaporin 2</td>
</tr>
<tr>
<td>ARID5B</td>
<td>AT rich interactive domain 5B</td>
</tr>
<tr>
<td>ARPKD</td>
<td>Autosomal recessive polycystic kidney disease</td>
</tr>
<tr>
<td>Arrdc5s</td>
<td>Arrestin domain containing proteins</td>
</tr>
<tr>
<td>ATA-2</td>
<td>Amino acid transporter</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri phosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BGN</td>
<td>Biglycan</td>
</tr>
<tr>
<td>BMP-4,7</td>
<td>Bone morphogenetic protein 4, 7</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>C</td>
<td>Centigrade</td>
</tr>
<tr>
<td>C termini</td>
<td>Carboxyl termini</td>
</tr>
<tr>
<td>C2</td>
<td>Ca²⁺ phospholipid binding domain</td>
</tr>
<tr>
<td>C3AR1</td>
<td>Complement component 3a receptor 1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAKUT</td>
<td>Congential anomalies of the kidney and urinary tract</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
</tbody>
</table>
CAT  Catalase
CC   coiled–coil
CD   Collecting ducts
cDNA  complementary Deoxyribonucleic acid
CFTR Cystic fibrosis transmembrane conductance regulator
CI   Chloride
CLC-5 H⁺/Cl⁻ exchange transporter 5
ClCka/Barttin  chloride channel
cm  Centimeters
CO₂  Carbon dioxide
Collα1 Collagen I alpha 1
CSF1 Colony stimulating factor 1
DAB  3, 3’ diaminobenzidine
DAT  Dopamine transporter
DAVID Database for Annotation, Visualization and Integrated Discovery
DBA  Dolichos Biflorus Agglutinin
DCN  Decorin
DCT  Distal convoluted tubule
DCTN-5 Dynactin-5
DEPC Diethylpyrocarbonate
Dlg3 Drosophila disc large scaffolding protein
DMEM Dulbeccos modified eagle medium
DMT1 Divalent metal ion transporter
DNA Deoxy ribonucleic acid
DRG Dorsal root ganglion
DTT Dithiothreitol
DTX4 Deltex4 E3 ubiquitin ligase
DUBs Deubiquitinating enzymes
Dvl2 Dishevelled-2
E. coli Escherichia coli
E1  Ubiquitin activating enzyme
E18.5 Embryonic day 18.5 post coitum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>E2</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Ubiquitin protein ligases</td>
</tr>
<tr>
<td>EAAT1/2</td>
<td>The glial excitatory amino acid transporters</td>
</tr>
<tr>
<td>ECF</td>
<td>Enhanced chemifluorescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF2</td>
<td>EF-hand calcium binding motif</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FBLN1</td>
<td>Fibrillin</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<td>Fibrinogen alpha chain</td>
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<td>Firststrand</td>
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<td>Glomeruli</td>
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<td>GAIN</td>
<td>G protein coupled receptor auto proteolysis inducing regulatory domain</td>
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<td>GPCR</td>
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<td>G-protein coupled receptor motif</td>
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<td>H and E</td>
<td>Haematoxylin and Eosin</td>
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<td>HB-EGF</td>
<td>Heparin binding-EGF</td>
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<tr>
<td>HCO₃⁻</td>
<td>Bicarbonate</td>
</tr>
</tbody>
</table>
HECT Homologous to the E6-AP C terminus
HEK Human epithelial kidney
HepG2 Liver hepatocellular carcinoma cell line
HIF3A Hypoxia inducible factor 3 alpha
HRP Horse radish peroxidase
Hrs Hours
IB Immunoblot
ICAM1 Intercellular adhesion molecule 1
ICC Immunocytochemistry
IF Immunofluorescence
IMCD Inner medullary cortical collecting duct cells
IPA Ingenuity pathway analysis
ITGAM Integrin alpha M
iTRAQ Isobaric tags for relative and absolute quantitation
ITS Insulin/ transferrin/selenium
K Lysine
K+ Potassium
KCNQs Potassium voltage-gated channel subfamily
kDa Kilodalton
KEGG Kyoto Encyclopedia for Genes and Genomes
KIF3A Kinesin subunit 3A
KL Klotho
KO Knock out
KW Kidney weight
L Litre
LB Luria- Bertani media
LPxY Leucine-Proline-x-Tyrosine
LTL Lotus Tetragonolobus Lectin
M Molar
mA Milliamperes
mDCT       mouse Distal collecting tubule
MEF        Mouse embryonic fibroblast
MEKK1      Mitogen associated protein kinase pathway
mg/mL      Milligram/Millilitre
Min        Minutes
mL         Millilitres
mm         Millimeters
mM         Millimolar
MMP        Matrix metallo protease
mpkCCD     Mouse pyruvate kinase cortical collecting duct
n.s        Not significant
Na⁺        Sodium
Nav        Voltage-gated sodium channels
NCC        Na⁺-Cl⁻ cotransporter
NCOR2      Nuclear receptor co repressor 2
NDFIP1/2   Nedd4 family interacting protein 1/2
NDRG1      N-myc downstream regulated gene-1
Nedd       Neuronally expressed, developmentally down-regulated gene
NEM        N-ethylmaleimide
ng         Nanogram
NKCC2      Na⁺-K⁺-2Cl⁻ cotransporter
nm         Nanometers
NOX4       NADPH oxidase 4
°          Degree
OD         Optical density
Orai1      Calcium channel
PAGE       Polyacrylamide gel electrophoresis
PARP3      Polymerase family member 3
PAS        Periodic acid schiff
PAX-2  Paired box gene-2
PBS  Phosphate buffered saline
PC-1  Polycystin-1
pCNA  Proliferation cell nuclear antigen
PCR  Polymerase chain reaction
PDAC  Pancreatic ductal adenocarcinoma
PHD  Plant Homeo Domain
PIK3CD  Bisphosphate 3- kinase catalytic subunit Δ
PIK3R5  Phosphoinositide-3-kinase regulatory subunit 5
PKD  Polycystic kidney disease
PKHD1  Fibrocystin
PLAT  Polycystin-1 lipooxygenase alpha-toxin
PLCB2  Phospholipase C β2
PP/LPXY  Proline rich motifs
PVDF  Polyvinylidene fluoride
RD  Renal Dysplasia
RING  Really interesting new gene
RIPA  Radioimmunoprecipitation lysis buffer
RMA/RNF5  RING finger protein 5
RNA  Ribonucleic acid
ROMK  Renal outer medullary potassium channel
RPMI  Roswell Park Memorial Institute media
RT²  Real time / Reverse Transcriptase
RTKs  Receptor protein tyrosine kinases
RUNX1  Runt-related transcription factor 1
SALL1  SAL-like 1
SCF  Skp1- Cullin- F-box complex
SDS  Sodium dodecyl sulphate
SEM  Scanning Electron Microscope
SEM  Standard error mean
Sgk1  Serum glucocorticoid-inducible kinase  
SGLT1  Na\(^+\) glucose transporter 1  
SILAC  Stable isotope labelling of amino acid in cell culture  
siRNA  small interfering Ribonucleic acid  
Six1  Sineoculis homeobox 1  
SLC  Solute carrier family  
SLIT3  Slit homolog 3  
SMA  Smooth Muscle Actin  
SMAD2/3/7  Mothers against decapentaplegic  
SMOC2  SPARC related modular calcium binding 2  
SNPs  Single nucleotide polymorphisms  
SMOC2  SPARC related modular calcium binding 2  
SNPs  Single nucleotide polymorphisms  
SP-C  Surfactant protein C  
Src  Non-receptor protein tyrosine kinase  
STAT3  Signal transducer and activator of transcription 3  
SULF1  Sulfatase 1  
SUMO  Small Ubiquitin-like Modifier  
SVD  Singular value decomposition  
TAE  Tris acetate EDTA  
TBST  Tris-buffered saline/Tween 20  
TCA  Trichloro acetic acid  
TCF-2  Transcription factor-2  
TEM  Transmission Electron Microscope  
TGFβ  Transforming growth factor β  
TGFβR1  Transforming growth factor beta receptor 1  
THP  Tamm horsfall glycoprotein  
TINAG  Tubulointerstitial nephritis antigen  
TrkA  Neurotrophin receptor  
TRPC6  Transient receptor potential Canonical 6
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Thesis Structure

This thesis contains already published work and unpublished work in the structure described below:

**Chapter-1:** Section 1.3.2, 1.3.3 (part), 1.3.4- 1.3.5, 7 and 8 are unpublished and provide the initial review of the literature and the context of the study related to the topic of the thesis. Sections 1 (1-1.1, 1.2-1.3, 1.3.3-1.3.4, 2-7 are part of the published review providing insights on the study on Nedd4-2.


**Chapter-2:** This chapter consists of the characterisation of the Nedd4-2 knock out kidney phenotype, with introduction consisting of relevant literature on the renal disorder, materials and methods described in detail the methodology of the chapter results, the results on the kidney phenotype characterisation and discussion summarising the major findings with their relevance and limitations

**Chapter-3:** This chapter consists of the role of polycystins and their potential regulation by Nedd4-2 and its physiological relevance in context to Nedd4-2 knock out kidneys, with introduction consisting of relevant literature on the polycystin structure and function, materials and methods described in detail the methodology of the chapter results, the results describing the potential role of polycystin in context to Nedd4-2 mediated regulation and discussion summarising the major findings and limitations with their relevance to the given study.

**Chapter-4:** This chapter consists of differential gene expression analysis of Nedd4-2 knock out kidneys and their relevance in context to renal dysplasia (Nedd4-2 kidney phenotype), with introduction consisting of relevant literature on the next generation sequencing used prior to understand the disease as a model system, materials and methods described in detail the methodology of the chapter results through bioinformatics approaches, the results describing the potential role of genes and the pathways in context to Nedd4-2 mediated regulation and discussion summarising the major findings and limitations with their relevance to the given study.

**Chapter-5:** This chapter comprises of the overall summary of the major findings of the thesis and the linkage between the chapter 2, 3 and 4. This further discusses the limitations of the study and the future perspective in relevance to the given study undertaken.