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

By
Pranay Goel
Masters of Biotechnology and Bioinformatics

School of Medicine, Discipline of Medicine
Faculty of Health Sciences, University of Adelaide
Adelaide, South Australia

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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.

Attended 4th Adelaide ANZSCDB Cell and Developmental Biology meeting (Nov 19, 2014).

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1. GENE: Invited Review

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Abbreviations

HCN1	Hyperpolarization activated cyclic nucleotide gated
P19	Post natal day 19
%	Percentage
µg	Microgram
ADAM23	ADAM metallopeptidase domain 23
ADPKD	Autosomal dominant polycystic kidney disease
AFF3	AF4/FMR2 family member 3
AGTR2	Angiotensin receptor 2
Akt	PKB- protein kinase B
AP	Alkaline phosphatase
APC	Anaphase-promoting complex
AQP2	Aquaporin 2
ARID5B	AT rich interactive domain 5B
ARPKD	Autosomal recessive polycystic kidney disease
Arrdcs	Arrestin domain containing proteins
ATA-2	Amino acid transporter
ATP	Adenosine tri phosphate
BCA	Bicinchoninic acid
BGN	Biglycan
BMP-4 ,7	Bone morphogenetic protein 4, 7
bp	Base pair
BW	Body weight
C	Centigrade
C termini	Carboxyl termini
C2	Ca^{2+} phospholipid binding domain
C3AR1	Complement component 3a receptor 1
Ca^{2+}	Calcium
CAKUT	Congential anomalies of the kidney and urinary tract
cAMP	Cyclic adenosine monophosphate

CAT	Catalase
CC	coiled–coil
CD	Collecting ducts
cDNA	complementary Deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl^-	Chloride
CLC-5	$H(^+)/Cl(^-)$ exchange transporter 5
ClCka/Barttin	chloride channel
cm	Centimeters
CO_2	Carbon dioxide
Col1a1	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

E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin protein ligases
EAAT1/2	The glial excitatory amino acid transporters
ECF	Enhanced chemifluoroescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EF2	EF-hand calcium binding motif
EGF	Epidermal growth factor
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ENaC	Epithelial sodium channel
ESCRT	Endosomal sorting complex
FBLN1	Fibrillin
FBS	Foetal Bovine Serum
FGA	Fibrinogen alpha chain
fr	Firststrand
FREM1	FRAS1 related extracellular matrix 1
G	Glomeruli
GAIN	G protein coupled receptor auto proteolysis inducing regulatory domain
gDNA	Genomic DNA
GFP	Green fluoroscent protein
GO	Gene Ontology
GP78	Glycoprotein 78
GPC-3	Glypican-3
GPCR	G protein coupled receptor
GPS	G-protein coupled receptor motif
H and E	Haematoxylin and Eosin
HB-EGF	Heparin binding-EGF
HCO_3^-	Bicarbonate

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	Immunofluoroscence
IMCD	Inner medullary cortical collecting duct cells
IPA	Ingenuity pathway analysis
ITGAM	Integrin alpha M
iTRAQ	Isobaric tags for relative and absolute quantitiation
ITS	Insulin/ transferrin/selenium
K	Lysine
K^+	Potassium
KCNQs	Potassium voltage-gated channel subfamily
kDa	Kilodalton
KEGG	Kyoto Encyclopaedia 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
^{o}	Degree
OD	Optical denstity
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 Polcystin-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 lipoxygenase 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

RT^2 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 Ribonucleicacid
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

TRPM6	Transient receptor potential melastatin 6
TRPP2/PC-2	Transient receptor potential/ polycystin -2
TSHZ3	Teashirt zinc finger homeobox 3
TTYH	Tweety chloride channel
UB	Ubiquitin
UBC	Ubiquitin-conjugating domain
UBPs	Ubiquitin specific processing enzymes
UCHs	Ubiquitin carboxyl terminal hydrolases
UV	Ultra violet
V	Volts
V2R	Vasopressin receptor
VCB	Von Hippel Lindau-elongin C - elongin B- Cul2- Rbx1 complex
W	Watts
WNT4	Wingless gene 4
WT	Wild type
WT1	Wilms tumor 1
WW	Protein-Protein interaction tryptophan domains
Yeast	Saccharomyces cerevisiae
µg/ml	Microgram per millitre
µl	Micro litre
µm	Micrometer

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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.

GOEL, P., MANNING, J. A. & KUMAR, S. 2015. NEDD4-2 (NEDD4L): The ubiquitin ligase for multiple membrane proteins. *Gene,* 557**,** 1-10.

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.